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SEROTONIN TRANSPORTER AND INTEGRIN BETA 3 GENES INTERACT TO MODULATE SEROTONIN UPTAKE IN MOUSE BRAIN

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Abstract

Dysfunctions in serotonin (5-hydroxytryptamine, 5-HT) systems have been associated with several psychiatric illnesses, including anxiety, depression, obsessive-compulsive disorders and autism spectrum disorders. Convergent evidence from genetic analyses of human subjects has implicated the integrin β3 subunit gene (*ITGB3*) as a modulator of serotonergic systems via genetic interactions with the 5-HT transporter gene (*SLC6A4*, SERT). While genetic interactions may result from contributions of each gene at several levels, we hypothesize that *ITGB3* modulates the 5-HT system at the level of the synapse, through the actions of integrin αvβ3. Here we utilized a genetic approach in mouse models to examine *Itgb3* contributions to SERT function both in the context of normal and reduced SERT expression. As integrin αvβ3 is expressed in postsynaptic membranes, we isolated synaptoneurosomes, which maintain intact pre- and post-synaptic associations. Citalopram binding revealed significant *Slc6a4*-driven reductions in SERT expression in midbrain synapses, whereas no significant changes were observed in hippocampal or cortical projections. Expecting corresponding changes to SERT function, we also measured 5-HT uptake activity in synaptoneurosomal preparations. *Itgb3* single heterozygous mice displayed significant reductions in 5-HT $V_{max,}$ with no chages in K_m, in midbrain preparations. However, in the presence of both *Itgb3* and *Slc6a4* heterozygozity, 5-HT uptake was similar to wild-type levels, revealing a significant *Slc6a4* by *Itgb3* genetic interaction in the midbrain. Similar findings were observed in cortical preparations, whereas in the hippocampus, most V_{max} changes were driven solely by *Slc6a4*. Our findings provide evidence that integrin αvβ3 is involved in the regulation of serotonergic systems in some, but not all 5-HT synapses, revealing novel contributions to synaptic specificity within the central nervous system.

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Keywords

serotonin transporter; integrin; genetic interaction; neuropsychiatric disorders; autism

1. Introduction

Dysfunction in serotonin (5-hydroxytryptamine, 5-HT) neurotransmission has been implicated in the etiology of mood and developmental disorders including anxiety, depression, and autism-spectrum disorders (ASD). Several genetic variants in the 5-HT transporter gene (SERT, *SLC6A4*) have been associated with behavioral phenotypes manifested in these disorders, especially in the context of genetic interactions or under specific environmental conditions (Caspi et al. 2003, Sutcliffe et al. 2005, Murphy and Moya 2011). Variations in whole blood 5-HT levels, found in several neuropsychiatric disorders, including autism, bipolar disorder and seasonal affective disorder (Velayudhan et al. 1999, Willeit et al. 2008), are associated with non-coding variation in *ITGB3* (Weiss et al. 2004). Genetic interaction of *ITGB3*, which encodes the integrin β3 subunit (forming the integrin αIIbβ3 in platelets and integrin αvβ3 in brain), and *SLC6A4*, either in mRNA expression or autism susceptibility, further reinforces the suggestion that these two genes may interact to modify 5-HT homeostasis (Weiss et al. 2004). Whereas genetic interactions do not typically translate into functional or biochemical interactions, we have reported a physical and functional association between integrin αIIbβ3 and SERT in platelets (Carneiro et al. 2008). Thus we hypothesize that *ITGB3* and *SLC6A4* interact to modulate SERT expression and function in the brain. Here we utilized a genetic approach to document unique and interactive contributions of these genes to transporter expression and function in the mouse synaptic preparations.

2. Material and Methods

2.1. Animals

Mouse studies were performed in accordance with humane guidelines established by the Vanderbilt Institutional Animal Care and Use Committee under approved protocol (M/09/198). Both *Itgb3*$^{-/-}$ (Hodivala-Dilke et al. 1999) and *Slc6a4*$^{-/-}$ (Bengel et al. 1998) mouse lines were previously backcrossed onto C57BL/6 for more than 20 generations. *Slc6a4*$^{+/-}$, *Itgb3*$^{+/-}$ mice were generated by crossing C57BL/6 *Itgb3*$^{-/-}$ males and C57BL/6 *Slc6a4*$^{-/-}$ females. Mice derived from this crossing were not used for experiments to avoid rearing effects caused by *Slc6a4*$^{-/-}$ dam phenotypes. Instead, the *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ male offspring were paired with wildtype C57BL/6J females producing offspring of four genotypes: *Itgb3*$^{+/+}$, *Slc6a4*$^{+/+}$ (WT)*; Itgb3*$^{+/-}$, *Slc6a4*$^{+/+}$ (*Itgb3*$^{+/-}$*); Itgb3*$^{+/+}$, *Slc6a4*$^{+/-}$ (*Slc6a4*$^{+/-}$); and *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$. Male and female offspring were housed by sex with mixed-genotype littermates in groups of 2–5 per cage. Mice were maintained on a 12-hour light-dark cycle, and provided with food and water *ad libitum*. Littermate males and females were utilized for all biochemical and neurochemical assays.

2.2. Synaptoneurosome Preparation

Synaptoneurosomes were obtained as previously described (Phillips et al. 2001). Briefly, mice were rapidly decapitated and brain regions were dissected onto 0.32 M sucrose in HEPES containing 0.1mM $CaCl_2$ and 1mM $MgCl_2$ at 4°C. Samples were homogenized in a piston-type Teflon® pestle with stainless steel shaft and replaceable grinding vessel and cell debris/nuclei separated by centrifugation at 1,000 x g. Supernatants were collected and spun at 10,000 x g for isolation of crude synaptoneurosomes. Immediately after preparation, synaptoneurosomal protein was measured using a modified Lowry protocol with bicinchoninic acid (BCA Protein Assay Kit, Pierce Chemical Company, Rockford, IL). Approximately 1mg was used immediately for 5-HT saturation kinetic studies of 5-HT uptake, and the remaining was frozen for citalopram binding and western blot studies.

2.3. Saturation Kinetic Studies of [^{3}H] 5-HT Uptake

Synaptoneurosome pellets were resuspended in Krebs-Ringer's HEPES (KRH) buffer (130mM NaCl, 1.3mM KCl, 2.2mM $CaCl_2$, 1.2mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8g/L glucose, 10mM HEPES, pH 7.4 containing 100 μM ascorbic acid and 100 μM pargyline). Synaptoneurosomes (100μg for midbrain and 200μg for hippocampus and cortex) were incubated for 10 min at 37° C in test tubes containing 100μl of KRH buffer, and 50 μl [^{3}H] 5-HT (Concentrations ranging from 12.5–400nM. Hydroxytryptamine Creatinine Sulfate, 5-[1, 2-3H(N)]-(Serotonin). Perkin Elmer, Walthman, MA). An identical set of tubes contained 50 μl of 1μM paroxetine (Sigma Aldrich, Saint Louis, MO) to define SERT-specific uptake. Next, samples were harvested via Brandel tissue harvester and filtered onto GF/B Whatman filters (Brandel, Gaithersburg, MD). Filters were dissolved overnight in scintillation fluid (Econo-Safe™, Research Products International Corp. Mount Prospect, IL) then radioactivity was quantified in a Packard counter by QuantaSmart 4.0 software.

2.4. [^{3}H]-Citalopram Binding

Synaptoneurosomes (100μg for midbrain and 250μg for hippocampus and cortex) were incubated with 5 nM [^{3}H]-citalopram (Racemic citalopram,[N-Methyl-^{3}H]. Perkin-Elmer, Walthman, MA) on ice for 20 min then harvested using a Brandel tissue harvester onto GF/B Whatman filters. An identical set of tubes contained 1μM paroxetine (Sigma Aldrich, Saint Louis, MO) to define SERT-specific binding. Filters were dissolved overnight in scintillation fluid then radioactivity was quantified in a Packard counter by QuantaSmart 4.0 software.

2.5. Western Blotting

Midbrain synaptoneurosomes pellets were resuspended in 1% sodium dodecyl sulfate in phosphate buffered saline pH 7.4 and protein was measured using a modified Lowry protocol with bicinchoninic acid (BCA Protein Assay Kit, Pierce Chemical Company, Rockford, IL). No hippocampal or cortical samples were available for western blots. 50μg of protein were loaded onto 17-well Pierce Protein Gels (Thermo Scientific). Gel electrophoresis was performed at 100v for 3 hours then proteins were transferred overnight at 4°C onto PVDF membranes (Immobilon, Millipore, Billerica, MA). After transfer, membranes were blocked with 5% milk in 1x tris-buffered saline pH 7.4 and incubated with

antibodies at 1:250 or 1:1000 dilutions overnight at 4 °C. Secondary antibodies were added at 1:2500 dilution and proteins detected with chemiluminescence. Amersham Hyperfilm ECL films were exposed at 1,5,10, and 30 minutes to address linearity of the data (GE Healthcare, Pittsburgh, PA). Films were scanned in tagged image file format (.tiff) and bands quantified by densitometry using Image J. Antibodies included: rabbit anti-integrin αv and rabbit anti-integrin β3 (Cell Signaling Technology, Denvers, MA), mouse anti-syntaxin (Millipore, Billerica, MA), and guinea pig anti-5-HT transporter (Frontier Science Co., LTD, Hokkaido, Japan).

2.6. Data Analysis

All data was analyzed in Prism 4.0c (Graphpad Software, Inc., LaJolla, CA). Two-way ANOVA was used with *Slc6a4* and *Itgb3* as variables to identify contributions of each gene. Dunnett's multiple comparison tests were used to compare each genotype to wild-type (WT). Kruskal-Wallis test was used to analyze western blot samples as each group of samples was run in a different day and normalized to each individual control (WT =100%). In this particular case we used Dunn's post-tests to identify statistical significant genotype differences. Saturation data was fit to a one-site non-linear regression model. Scatchard plots were fit by linear regression for calculation of V_{max} and K_m. A *P* value of less than 0.05 was considered statistically significant. All data are shown as mean ± standard error of the mean (SEM, represented by error bars).

3. Results and Discussion

3.1. Synaptic SERT expression is reduced in the midbrains of double heterozygous mice

To examine the influence of *Itgb3* heterozygosity on SERT expression and uptake activity, we studied *Itgb3*$^{+/-}$ and *Slc6a4*$^{+/-}$, *Itgb3*$^{+/-}$ mice. Whereas SERT expression patterns in midbrain neurons and in projection areas have been extensively studied (Bengel et al. 1997, Tao-Cheng and Zhou 1999), we have little information on the expression of integrin αvβ3 in the intact brain. Few studies have identified post-synaptic expression of integrin αvβ3 in hippocampal synapses (Cingolani et al. 2008); moreover, it is possible that extracellular-matrix proteins, which bind integrins, maintain synaptic structure and thus pre- and post-synaptic interactions may be essential for proper synaptic function (Wang et al. 2008). Therefore, to examine the influence of *Slac6a4* and *Itgb3* heterozygozity in synaptic SERT expression and uptake activity, we isolated synaptoneurosomes in the presence of $CaCl_2$ and $MgCl_2$, maintaining N-cadherin, NCAM, and integrin-mediated interactions (Phillips et al. 2001).

We prepared synaptoneurosomes from midbrain, hippocampus, and cortices dissected from WT, *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$; and *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ littermates and assessed [^{3}H]-citalopram binding. The data revealed a significant a significant reduction in [^{3}H]-citalopram binding in the context of *Slc6a4* heterozygosity in midbrain synaptoneurosomes (Figure 1a). We used western blot analysis to determine whether these changes may correspond to reductions in SERT expression in terminals. Our data indicates that *Slc6a4* modifies SERT expression in midbrain terminals (Figure 1b, c). Similar findings were found in previous studies of the *Slc6a4*$^{+/-}$ mice (Bengel et al. 1998). As synapse number/size may be influenced by 5-HT

signaling (Udo et al. 2005) or integrin function (Cingolani et al. 2008), we assessed syntaxin expression as a control for pre-synaptic terminal expression. No significant changes were found in integrin αv, integrin β3 or syntaxin expression (Figure 1b). We found no significant alterations in [^{3}H]-citalopram binding in synaptic preparations from two terminal fields: hippocampus and cortex (Figure 1d and 1e, respectively). These findings indicate that, although SERT tissue expression may be influenced by genotype, neither *Slc6a4* nor *Itgb3* modified synaptic SERT expression in the two terminal fields examined. The discrepancy between midbrain and cortical and hippocampal SERT synaptic expression may be due to differences in the localization of SERT in these brain regions. While SERT is strictly localized to axonal/pre-synaptic terminals in cortex and hippocampus, both at the perisynaptic plasma membrane and in intracellular vesicles, midbrain SERTs localize to both axonal/pre-synaptic and dendritic/post-synaptic terminals (Tao-Cheng and Zhou 1999). It is possible that axonal SERT localization is tightly regulated by trafficking mechanisms, independent of the total protein expressed in the cell body, whereas dendritic expression, predominantly intracellular, may be directly correlated with mRNA/protein expression at the cell body. To determine whether these changes in expression are correlated with changes in SERT function, we performed 5-HT reuptake studies.

3.2. *Itgb3* and *Slc6a4* interact to modulate SERT uptake

Saturation kinetic analysis of SERT 5-HT uptake from midbrains synaptoneurosomes revealed a significant interaction between *Slc6a4* and *Itgb3* genes (Figure 2a–c). *Itgb3*$^{+/-}$ mice exhibited significant reductions in midbrain V_{max} (Figure 2b) compared to WT, while *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ mice exhibited normal 5-HT uptake (Figure 2b). No significant changes were observed in K_m (Figure 2c). This result supports the hypothesis that axonal/pre-synaptic plasma membrane SERT expression is not reduced in *Slc6a4*$^{+/-}$ or *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ mice and replicates the initial functional studies performed in *Slc6a4*$^{+/-}$ mice (Bengel et al. 1998). Our results reveal that heterozygosity in the *Itgb3*$^{+/-}$ line is sufficient to reduce SERT activity, by a mechanism yet unidentified, as no changes in integrin αvβ3 were observed in the same preparations. It is possible that pre-synaptic levels of integrin αvβ3 are modified, but remain undetected in synaptoneurosomal preparations. Importantly, no 5-HT uptake changes were observed in *Itgb3*$^{+/-}$ platelets (Carneiro et al. 2008), suggesting a specific neuronal mechanism for integrin αvβ3 modulation of SERT activity in the midbrain.

5-HT uptake was differentially modulated in the two terminal fields studied. In the hippocampus (Figure 2d–f), we found significant V_{max} increases driven by *Slc6a4* (Figure 2e). No changes in K_m were observed (Figure 2f). In cortical preparations, V_{max} reductions were found in *Itgb3*$^{+/-}$ mice compared to WT (Figure 2h). These changes in hippocampal uptake are not replicated in other studies; most laboratories report reductions in hippocampal V_{max} in *Slc6a4*$^{+/-}$ mice (Murphy and Moya 2011). These differences may result from different fractionation methods used to isolate 5-HT synapses i.e. synaptosomes vs. synaptoneurosomes. Thus, we have detected changes in SERT transport activity, increases driven by genetic alterations in *Slc6a4* in the hippocampus, and decreases driven by *Itgb3*. Together, in *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ preparations, we observe no significant changes in SERT uptake activity.

How can we interpret alterations in SERT uptake vs. expression levels in *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ mice? One possibility is the presence of two distinct SERT populations at the plasma membrane. One population has high capacity for 5-HT uptake, which is preferentially expressed at the plasma membrane of *Slc6a4*$^{+/-}$ mice. The second population, perhaps with a low-capacity uptake, is absent in *Slc6a4*$^{+/-}$ mice. Therefore 100% of SERTs at the plasma membrane of *Slc6a4*$^{+/-}$ mice have high capacity uptake, independently of *Itgb3*. As integrin αvβ3 may modulate SERT levels at the plasma membrane (Carneiro et al. 2008), *Itgb3*$^{+/-}$ mice may have reduced plasma membrane SERT expression in both low- and high-capacity uptake populations, and thus the resulting 5-HT reuptake observed is significantly reduced. While highly speculative, this interpretation is supported by the data observed in hippocampal preparations, where *Itgb3* heterozygozity has no effect in SERT uptake and *Slc6a4*$^{+/-}$ mice show elevated SERT activity. Interestingly, these data suggest a protective effect of *Slc6a4* heterozygozity in the context of *Itgb3* heterozygozity. As both genes are highly polymorphic in humans, it is possible that this genetic interaction may play an important role in conferring risk for neuropsychiatric disorders.

4. Conclusions

Our studies reveal a surprising genetic interaction between *Slc6a4* and *Itgb3* in the modulation of 5-HT uptake in midbrain and cortical synapses. In accordance to previous studies, deletion or loss of function in *Itgb3* reduces SERT uptake capacity in some brain areas, whereas *Slc6a4* heterozygozity seems to increase or has no effect in SERT uptake. These effects suggest an important role of integrin αvβ3 in the midbrain. Future studies will reveal whether these alterations are sufficient to modify 5-HT signaling.

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Highlights

- Epistatic interaction between *Slc6a4* and *Itgb3* in the modulation of the serotonin system.
- *Slc6a4* heterozygosity dictates SERT expression in midbrain synapses.
- Midbrain and cortical serotonin uptake was significantly reduced in *Itgb3*$^{-/+}$ mice.
- A significant *Slc6a4* x *Itgb3* genetic interaction drives SERT V_{max} in the midbrain.
- Integrin αvβ3 is involved in the regulation of SERT in cortex and midbrain.

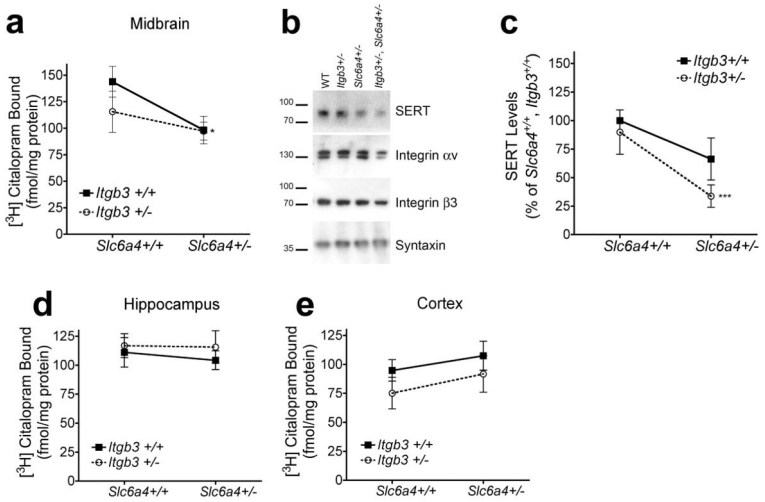

Figure 1.

SERT expression levels are reduced in midbrain synapses of *Itgb3*$^{+/+}$, *Slc6a4*$^{+/-}$ and *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ mice. (**a**) Two-way ANOVA reveals significant contributions of *Slc6a4* to midbrain synaptoneurosomal [^{3}H]-citalopram binding. WT: 143.8 ± 14.46 fmol/mg, *n* = 12; *Itgb3*$^{+/-}$: 115.6 ± 19.47 fmol/mg, *n* = 12; *Slc6a4*$^{+/-}$: 98.26 ± 13.06 fmol/mg, *n* = 12; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: 97.18 ± 8.43 fmol/mg, *n* = 12; two-way ANOVA: *Slc6a4 P* <0.05. (**b**) Representative western blot showing SERT, integrin αv, integrin β3 and syntaxin (as a presynaptic control) expression in midbrain synaptoneurosomes. (**c**) Densitometry analysis of western blots for SERT expression levels in midbrain synaptoneurosomes. *WT:* 100 ± 0%, *n* = 9; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/+}$: 89.88 ± 19.4%, *n* = 12; *Itgb3*$^{+/+}$, *Slc6a4*$^{+/-}$: 66.26 ± 18.4%, *n* = 10; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: 33.68 ± 9.79%, *n* = 8; Kruskal-Wallis one-way ANOVA: *P* < 0.05, Dunn's *post-hoc* WT vs. *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: **P*<0.05. Hippocampus (**d**) and cortex (**e**) synaptoneurosomal [^{3}H]-citalopram binding is not significantly different across terminal fields examined. Data is shown as means ± SEM.

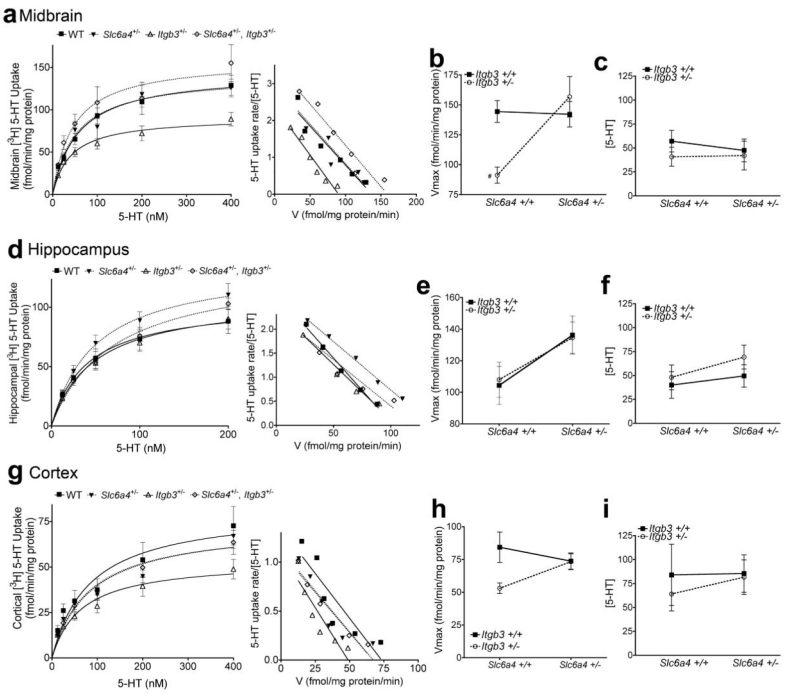

Figure 2.

^{3}[H] 5-HT uptake in synaptoneurosomes reveals differential changes in midbrain and cortices of *Itgb3*$^{+/-}$ mice. (**a**) Saturation kinetic studies and Scatchard plot for midbrain synaptoneurosomes. (**b**) Two-way ANOVA of V_{max} for 5-HT uptake in the midbrain reveals a significant statistical interaction between *Slc6a4* and *Itgb3*. WT: 144.4 ± 4.58 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$: 91.19 ± 3.33 fmol/min/mg protein, *n* = 4; *Slc6a4*$^{+/-}$: 142.1 ± 5.3 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: 156.7 ± 8.45 fmol/min/mg protein, *n* = 4; Two-way ANOVA: Interaction *P* = 0.0183; Dunnett's *post-hoc* WT vs. *Itgb3*$^{+/-}$: $^{\#}P < 0.05$. (**c**) No changes in K_m were observed in the midbrain. (**d**) Saturation kinetics studies and Scatchard plots for SERT-mediated 5-HT uptake from hippocampal synaptoneurosomes. (**e**) Two-way ANOVA analysis of V_{max} values obtained in **d** reveal significant contributions of *Slc6a4* to 5-HT V_{max} in the hippocampus. WT: 160 ± 12 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$: 119.1 ± 4.55 fmol/min/mg protein, *n* = 4; *Slc6a4*$^{+/-}$: 150 ± 4.99 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: 148.5 ± 3.5 fmol/min/mg protein, *n* = 4; two-way ANOVA: *Slc6a4: P* = 0.0330. (**f**) No significant changes were observed in K_m. (**g**) Saturation kinetics studies and Scatchard plots for SERT-mediated 5-HT uptake in cortical synaptoneurosomes. (**h**) Two-way ANOVA analysis of cortical SERT V_{max} reveals significant reductions in *Itgb3*$^{+/-}$ samples. WT: 84.37 ± 5.79 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$: 53 ± 2 fmol/min/mg protein, *n* = 4; *Slc6a4*$^{+/-}$: 73.76 ± 3.1 fmol/min/mg protein, *n* = 4; *Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$: 73.76 ± 3.02 fmol/min/mg protein, *n* = 4; two-way ANOVA: Interaction *P* = 0.0731; Dunnett's *post-hoc Itgb3*$^{+/-}$, *Slc6a4*$^{+/-}$ vs. *Itgb3*$^{+/-}$: $^{\#}P < 0.05$. (**i**) No significant changes were observed in SERT K_m in cortical preparations. Data is shown as means ± SEM.