NEUROSCIENCE

GENETIC AND FUNCTIONAL INTERACTIONS BETWEEN *Itgb3* AND *Slc6a4* IN MOUSE BRAIN

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Thesis under direction of Assistant Professor Ana Carneiro

ABSTRACT

In the brain, serotonin (5-hydroxtryptamine, 5-HT) is synthesized in the raphe nucleus. Raphe serotonergic projections modulate neurotransmissions throughout the brain influencing mood and behavior. The serotonin transporter (SERT; *SLC6A4*) clears 5-HT from the synapse for degradation or reuse, thus regulating levels of 5-HT and limiting its actions on 5-HT receptors. Dysfunction in 5-HT modulation of neurotransmission is associated with mood and developmental disorders including anxiety, depression, and autism and there is genetic evidence for increased risk for depression in individuals possessing polymorphisms in *SLC6A4* as well as genes which interact with *SLC6A4*. *ITGB3* encodes integrin β3, a cell adhesion molecule which has been implicated as a modulator of serotonergic systems via genetic and functional interactions with *SLC6A4*, as well as in regulation of synaptic plasticity and maturation. In the brain, integrin β3 couples to integrin αv to form a functional receptor, making integrin αvβ3 an interesting target for regulation of neural 5-HT systems. Immunohistochemical experiments revealed integrin β3 localization in serotonergic neurons, colocalized with SERT. Examination of genetic interactions utilizing an *Itgb3*$^{-/+}$ x *Slc6a4*$^{-/+}$ mouse model revealed reduced SERT expression, and an anxiety- and depression-like phenotype compared to wildtype littermates. Further experimentation of the functional interaction between integrin αvβ3 and SERT via pharmacological targeting of integrin αvβ3 revealed integrin αvβ3 regulation of SERT uptake activity. These studies highlight integrin β3 as a potential modulator of brain 5-HT systems and subsequently 5-HT mediated behavioral phenotypes.

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By

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Thesis

Submitted to the Faculty of the

Graduate School of Vanderbilt University

in partial fulfillment of the requirements

for the degree of

MASTER OF SCIENCE

in

Neuroscience

May, 2013

Nashville, Tennessee

Approved:

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CHAPTER I

INTRODUCTION

The serotonin (5-hydroxytryptamine, 5-HT) signaling pathway modulates neurons and their function, and dysregulation of the 5-HT system has been implicated in anxiety, depression, and autism (Akimova *et al*, 2009; Arango *et al*, 2001; Cook *et al*, 1990; Fink and Gothert, 2007; Gadow *et al*, 2013; Hoehn-Saric *et al*, 2000; Leventhal *et al*, 1990). 5-HT is cleared from the extracellular milieu by the Na^+/Cl^- dependent 5-HT transporter (SERT) encoded by the *SLC6A4* gene (Blakely *et al*, 1991; Ramamoorthy *et al*, 1993). SERT is the principal target of selective 5-HT reuptake inhibitors (SSRIs), a class of therapeutics used extensively in the treatment of anxiety, depression, and autism (Anderson, 2004; Serretti and Artioli, 2004). It is probable that SSRIs work by preventing reuptake of 5-HT thereby prolonging its actions on 5-HT receptors. A diagram of the synaptic serotonergic system is depicted in Figure 1.

In addition to its documented involvement in the treatment of psychiatric conditions, polymorphisms in *SLC6A4* have been associated with the etiology of anxiety, depression and autism (Caspi *et al*, 2003; Cook *et al*, 1990; Kaufman, 2005; Lesch *et al*, 1996; Monti, 2011; Roiser *et al*, 2006). Many of these studies focus on persons who have experienced stressful life events and report a gene x environment interaction. Also implicated in 5-HT related mental illnesses are genes which may interact with *SLC6A4* such as *ITGB3* (Weiss *et al*, 2005) which encodes the integrin β3 subunit.

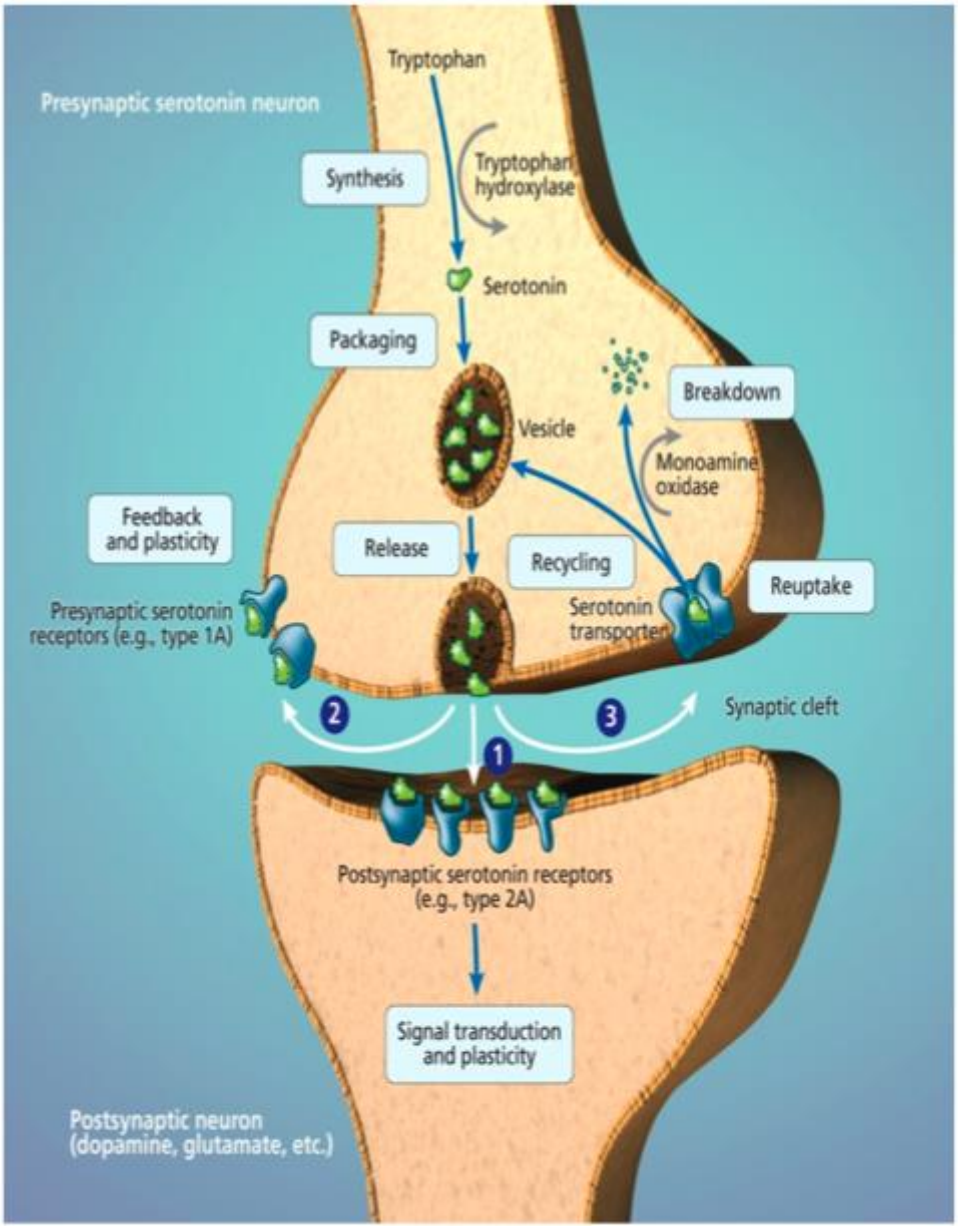

Figure 1. Model of 5-HT synaptic actions (aan het Rot *et al*, 2009). 5-HT is synthesized in the presynaptic neuron where it is packaged into vesicles for release from the presynaptic terminal. Once released into the synaptic cleft 5-HT can act upon postsynaptic 5-HT receptors (or presynaptic 5-HT autoreceptors), which then initiate intracellular signaling cascades. The actions of 5-HT receptors allow 5-HT to regulate feeding behavior, sleep, locomotor activity, learning and memory, and mood (Arango *et al*, 2001; Monti, 2011; Murphy *et al*, 1999). SERT is located perisynaptically where it can transport extracellular 5-HT back into the presynaptic neuron for degradation or reuse, thus limiting 5-HT actions.

Integrins are heterodimeric, bidirectional allosteric signaling receptors formed of an α and β subunit (Hynes, 2002b). The integrin β3 subunit is expressed in both platelets and in neurons. In the brain, integrin β3 is enriched at glutamatergic and glycinergic synapses where it has been ascribed various roles including the developmental regulation of glutamatergic synapses (Chavis and Westbrook, 2001), synaptic strength (Cingolani *et al*, 2008; Pozo *et al*, 2012) and glycine receptor localization (Charrier *et al*, 2010). Recent genetic analyses have provided evidence that *ITGB3* may be involved in serotonergic function (Carneiro *et al*, 2008; Coutinho *et al*, 2007; Cross *et al*, 2008; Napolioni *et al*, 2011; Weiss *et al*, 2005; Weiss *et al*, 2006a; Weiss *et al*, 2006b). Expression levels of *ITGB3* and *SLC6A4* are correlated in both mice and humans (Weiss *et al*, 2006b) and several independent studies have revealed *ITGB3* as a quantitative trait locus for whole blood 5-HT levels (Coutinho *et al*, 2007; Napolioni *et al*, 2011; Weiss *et al*, 2005; Weiss *et al*, 2006a). Carneiro, et al previously reported that integrin αIIβ3 directly interacts with SERT and activation of integrin αIIβ3 results in enhanced SERT uptake activity and elevated SERT plasma membrane expression in platelets (Carneiro *et al*, 2008). Thus, integrin β3 has become an interesting candidate for regulation of 5-HT systems.

The focus of this thesis is the genetic and functional interaction between *Itgb3* and *Slc6a4*. The results provide evidence that a genetic interaction between *Itgb3* and *Slc6a4* modifies, SERT expression, transport activity, and anxiety- and depression-like behaviors. Pharmacological analysis revealed that synaptic SERT uptake can be modulated by integrin αvβ3 targeted compounds indicating a functional integrin αvβ3 x SERT interaction. These data highlight *Itgb3* in brain 5-HT system regulation.

CHAPTER II

IMMUNOHISTOCHEMICAL ASSESSMENT

Localization of Integrin β3 in Serotonergic Neurons

5-HT pathways have been well characterized in rodents as they have become a model system for studying pharmacotherapies and 5-HT dysregulation. In the brain, 5-HT is synthesized in the raphe nucleus specifically, the dorsal raphe (DR) which contains approximately 50% of the brains serotonergic neurons (Jacobs and Azmitia, 1992). Fibers originating from the dorsal raphe nuclei near ubiquitously innervates the brain including the cortex and hippocampus (Vertes, 1991). To determine if integrin β3 was present in serotonergic neurons, immunohistochemistry experiments were performed utilizing wildtype (WT) and *Itgb3*$^{-/-}$ mice. Confocal imaging revealed integrin β3 expression in DR neurons (identified by NeuN) in WT (Figure 2B) but not *Itgb3*$^{-/-}$ mice (Figure 2A). Consistent with a recent finding using magnetic resonance imaging in mice (Ellegood *et al*, 2012) preliminary evidence was found for reduced neuron number in the DR of *Itgb3*$^{-/-}$ mice (Figure 2A). Sections probed for integrin β3 and the 5-HT synthesizing enzyme tryptophan hydroxylase 2 (TPH2) revealed robust colocalization in DR serotonergic neurons (Figure 2C). Integrin β3 x TPH2 colocalization was also found in the cortex and hippocampus (Figure 3) areas where the actions of 5-HT are known to mediate neuronal function and subsequent behavior (Akimova *et al*, 2009; Alexandre *et al*, 2006; Andrade, 2011; Schmidt *et al*, 2012). Last, confirmation of integrin β3 x SERT colocalization was found in DR neurons (Figure 4). These preliminary results indicate

integrin β3 localization in serotonergic neurons where it can affect 5-HT signaling. Furthermore, consistent with previous findings in platelets (Carneiro *et al*, 2008) the integrin β3 x SERT colocalization in the DR may be a direct protein-protein interaction, however this theory remains to be evidenced in the DR.

Methods and Materials

Immunohistochemistry

Mice were perfused with 30 mL of 4% paraformaldehyde (Sigma). Following rapid decapitation mouse brains were removed and stored in 30% sucrose for 48 hrs at 4^{o} C. Brains were then sectioned on a frozen stage microtome (Leica) at 20 μM and stored in a cryoprotectant solution containing; 30 mL ethylene glycol, 30 mL H_20, 10mL PBS, and 30 mL glycerol. Sections collected between ~4 mm and ~5 mm from bregma were defined as DR sections. Immunohistochemistry was performed using specific antibodies against integrin β3 (1:250, AbCam), SERT (1:2000, Frontier), TPH2 (1:250, Millipore), and NeuN (1:250, Millipore). Fluorescent secondary antibodies were applied at a 1:500 concentration. Images were captured using an LSM 510 Meta confocal.

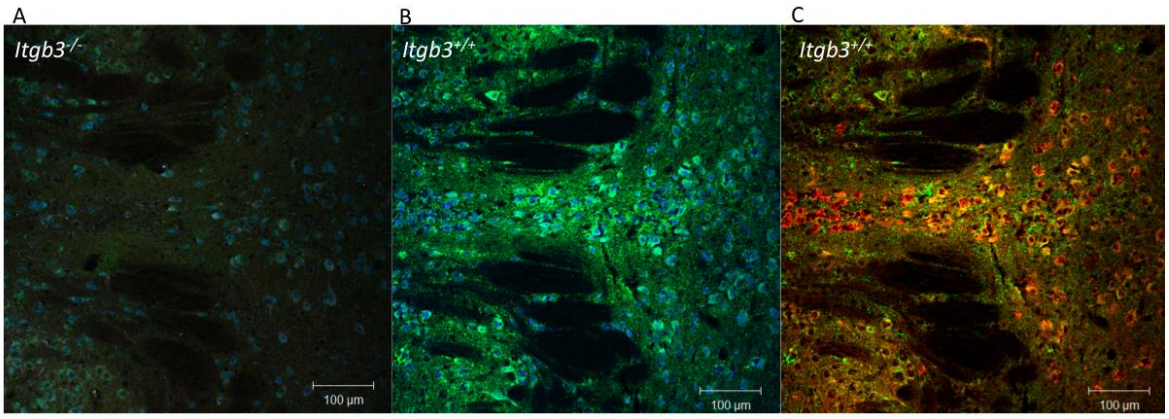

Figure 2. Confocal image of integrin β3 (green) and NeuN (blue) immunohistochemical staining in the dorsal raphe nucleus of A) *Itgb3*$^{-/-}$ and B) *Itgb3*$^{+/+}$ mice. C) Immunohistochemical probe for TPH2 (red) indicates integrin β3 localization in serotonergic neurons.

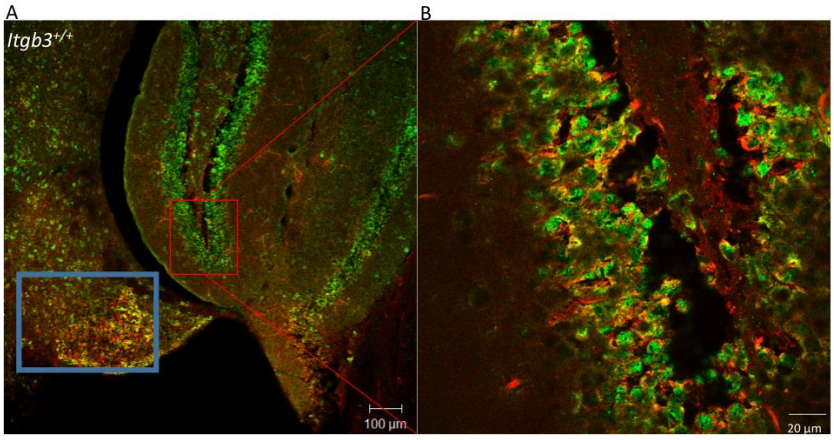

Figure 3. A) Integrin β3 (green) and TPH2 (red) co-localization was also found the hippocampus (red box) and cortex (blue box). B) Increased magnification of the dentate gyrus.

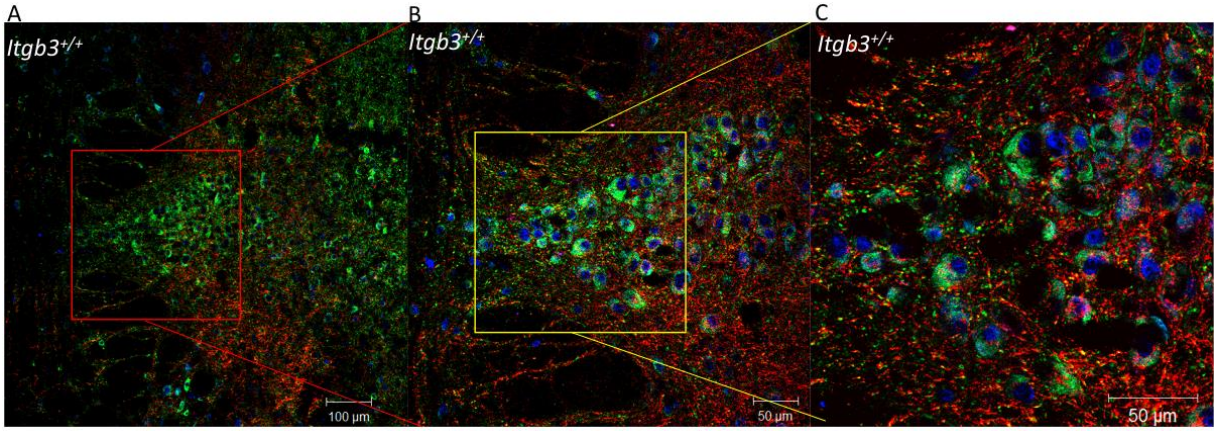

Figure 4 A) Colocalization of integrin β3 (green), SERT (red) and NeuN (blue) immunohistochemical staining in the DR. B-C) Magnification of the region increases from left to right in the panel.

CHAPTER II

GENETIC ASSESSMENT: *Itgb3*$^{-/+}$ x *Slc6a4*$^{-/+}$ MOUSE MODEL

Mouse Model

To examine the influence of *Itgb3* heterozygosity on SERT expression and uptake activity, 5-HT levels, and SERT-related behavioral phenotypes, mice heterozygous for *Itgb3* (*Itgb3*$^{-/+}$; I) and heterozygous for *Itgb3 and Slc6a4* (*Itgb3*$^{-/+}$ x *Slc6a4*$^{-/+}$; IS) were generated. These mice were bred by crossing C57BL/6 males with a silencing mutation in the *Itgb3* promoter region, *Itgb3*$^{-/-}$, (Hodivala-Dilke *et al*; McHugh *et al*) with C57BL/6 females in which the *Slc6a4* gene contains a silencing mutation in exon 14 which encodes the C-terminus, *Slc6a4*$^{-/-}$ (Zhao *et al*). Mice derived from this crossing were not used for experiments to avoid rearing effects caused by *Slc6a4*$^{-/-}$ dam phenotypes (Holmes *et al*; Kalueff *et al*, 2007a). Instead, the IS male offspring were paired with wildtype C57BL/6J females producing offspring of four genotypes: wildtype (WT), *Itgb3*$^{-/+}$ (I), *Slc6a4*$^{-/+}$ (S), *Itgb3*$^{-/+}$ x *Slc6a4*$^{-/+}$ (IS). Littermate males and females were utilized for all biochemical, neurochemical and behavioral assays.

Analysis of SERT Expression and Function

Slc6a4$^{-/+}$ mice have been previously reported to express ~50% of SERT compared to WT (Bengel *et al*, 1998) and expression of *Itgb3* and *Slc6a4* are known to correlate (Weiss *et al*, 2006b). To determine effects of *Itgb3* x *Slc6a4* heterozygosity on midbrain 5-HT levels, SERT expression, and SERT transport function, were examined

in both tissue and synaptoneurosome preparations. Analysis of tissue levels of 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) with were performed with high performance liquid chromatography (HPLC). No individual differences were found between genotypes, despite a *Slc6a4* significant contribution to elevated midbrain 5-HT levels (WT: 11.2 ± .46 ng/mg, *n* = 14; I: 10.63 ± .36 ng/mg, *n* = 18; S: 12.03 ± .34 ng/mg, *n* = 18; IS: 11.49 ± .55 ng/mg, *n* = 13; two-way ANOVA: *Slc6a4* $p < .05$; Figure 5A). No significant differences were found in 5-HT turnover as measured by 5-HIAA/5-HT in the midbrain (WT: .75 ± .06, *n* = 14; I: .79 ± .04, *n* = 18; S: .68 ± .04, *n* = 18; IS: .73 ± .06 ng/mg, *n* = 13; Figure 5B), however *Slc6a4* significantly contributed to reduced turnover in the cortex (WT: .43 ± .02, *n* = 14; I: .45 ± .02, *n* = 18; S: .37 ± .01, *n* = 18; IS: .39 ± .02, *n* = 13; two-way ANOVA: *Slc6a4* $p < .05$; Figure 5C), and hippocampus (WT: .61 ± .06, *n* = 13; I: .67 ± .05, *n* = 18; S: .54 ± .04, *n* = 18; IS: .52 ± .03 ng/mg, *n* = 12; two-way ANOVA: *Slc6a4* $p < .05$; Figure 5D). Western blot analysis of midbrain tissue revealed reduced expression of SERT in IS mice, (WT: 100 ± 0%, *n* = 7; I: 98.42 ± 14.14%, *n* = 8; S: 51.9 ± 9.33%, *n* = 7; IS: 34.81 ± 7.98%, *n* = 6; Kruskal-Wallis one-way ANOVA: $p < .005$, Dunn's *post-hoc* WT vs. IS $p < .01$, I vs. IS $p < .05$; Figure 5E).

Next, synaptic SERT expression and function were analyzed in synaptoneurosome preparations which contain isolated pre- and post-synaptic components and attachments (Phillips *et al*, 2001). Again Western blot analysis revealed reduced SERT expression in midbrain samples (WT: 100 ± 0%, *n* = 9; I: 89.88 ± 19.4%, *n* = 12; S: 66.26 ± 18.4%, *n* = 10; IS: 33.68 ± 9.79%, *n* = 8; Kruskal-Wallis one-way ANOVA: $p < .05$, Dunn's *post-hoc* WT vs. IS $p < .05$; Figure 6A), and a significant contribution of *Slc6a4* to reduced [^{3}H] citalopram binding in midbrain

synaptoneurosomes (WT: 143.8 ± 14.46 fmols/min/mg, *n* = 12; I: 115.6 ± 19.47 fmols/min/mg, *n* = 12; S: 98.26 ± 13.06 fmols/min/mg, *n* = 12; IS: 97.18 ± 8.43 fmols/min/mg, *n* = 12; two-way ANOVA: *Slc6a4* $p < .05$; Figure 6B). To determine if reductions in SERT midbrain expression affected 5-HT uptake, synaptoneurosomes were exposed to increasing concentrations of [^{3}H] 5-HT. Although I mice displayed significant reductions in midbrain V_{max} compared to WT, IS mice exhibited normal 5-HT uptake (WT: 144.4 ± 4.58 fmols/min/mg, *n* = 4; I: 91.19 ± 3.33 fmols/min/mg, *n* = 4; S: 142.1 ± 5.3 fmols/min/mg, *n* = 4; IS: 156.7 ± 8.45 fmols/min/mg, *n* = 4; one-way ANOVA: $p < .0001$; Bonferroni *post-hoc* WT vs. I $p < .001$, S vs. I $p < .001$. IS vs. I $p < .0001$; Figure 6B). No significant differences were detected in [^{3}H] citalopram binding in synaptoneurosomes in the cortex (WT: 94.73 ± 9.28 fmols/min/mg, *n* = 12; I: 75.13 ± 13.57 fmols/min/mg, *n* = 12; S: 107.4 ± 12.47 fmols/min/mg, *n* = 12; IS: 91.69 ± 15.74 fmols/min/mg, *n* = 12; Figure 6C) although similar V_{max} reductions were found in I mice compared to WT, S, and IS mice (WT: 84.37 ± 5.79 fmols/min/mg, *n* = 4; I: 53 ± 2 fmols/min/mg, *n* = 4; S: 73.76 ± 3.1 fmols/min/mg, *n* = 4; IS: 73.76 ± 3.02 fmols/min/mg, *n* = 4; one-way ANOVA: $p < .0001$; Bonferroni *post-hoc* WT vs. I $p < .001$, S vs. I $p < .05$, IS vs. I $p < .05$; Figure 6C). In the hippocampus, again no significant differences were found in [^{3}H] citalopram binding (WT: 222 ± 25.54 fmols/min/mg, *n* = 12; I: 233.6 ± 20.7 fmols/min/mg, *n* = 12; S: 208.5 ± 16.19 fmols/min/mg, *n* = 12; IS: 231.2 ± 28.19 fmols/min/mg, *n* = 12; Figure 6D), despite significant V_{max} reductions in I mice compared to WT (WT: 160 ± 12 fmols/min/mg, *n* = 4; I: 119.1 ± 4.55 fmols/min/mg, *n* = 4; S: 150 ± 4.99 fmols/min/mg, *n* = 4; IS: 148.5 ± 3.5 fmols/min/mg, *n* = 4; one-way ANOVA: $p < .01$; Bonferroni *post-hoc* WT vs. I $p < .01$; Figure 6D).

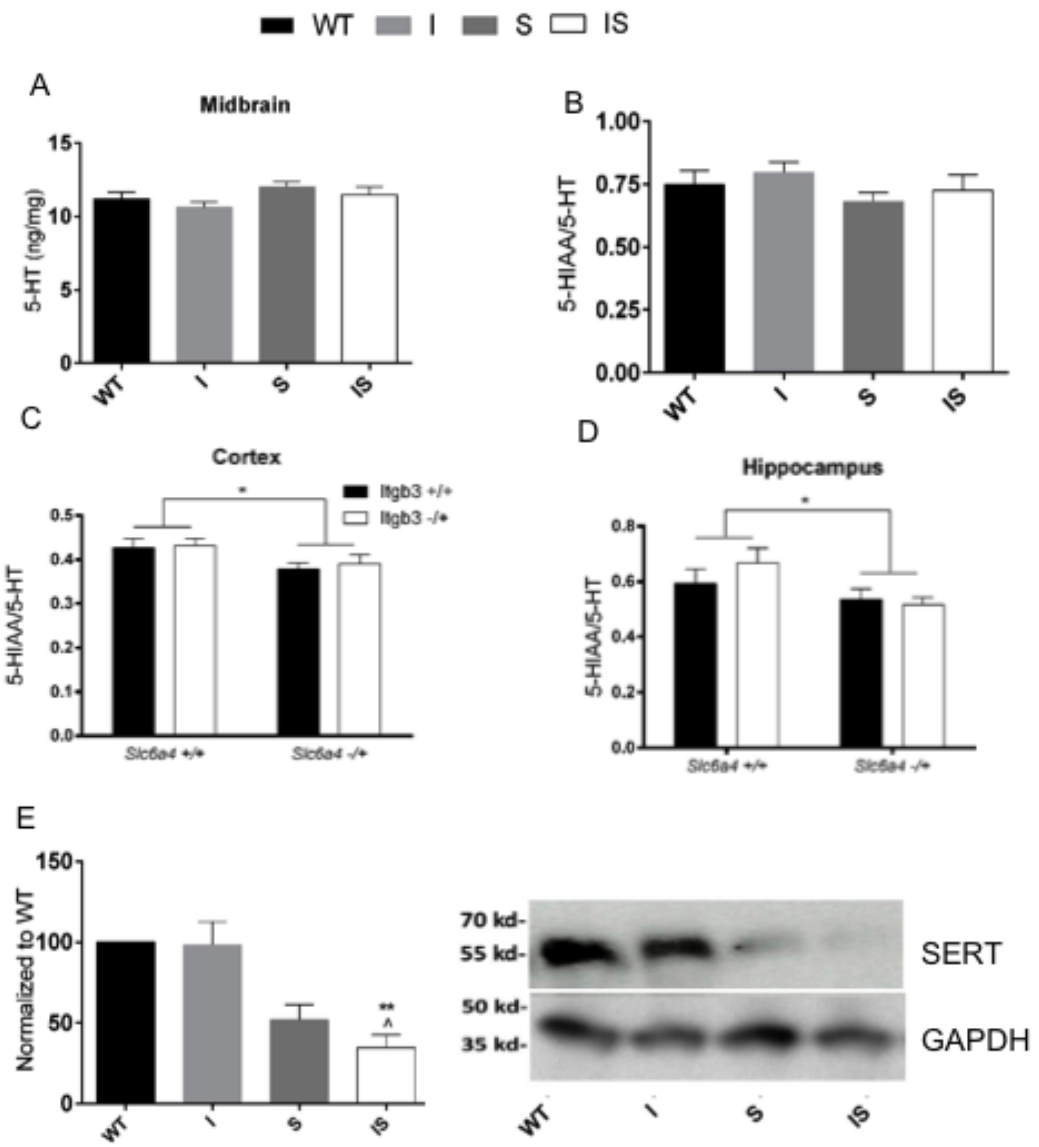

Figure 5. *Slc6a4* contributes to brain 5-HT neurochemical measures. (A) HPLC analysis of midbrain tissue 5-HT revealed a significant contribution from *Slc6a4* to elevated 5-HT levels (two-way ANOVA main effect for *Slc6a4*: $F(1,59) = 4.04$, $p < .05$, $n = 13\text{-}18$) although no significant differences were found between genotypes (one-way ANOVA, $p = .09$). (B) Two- way ANOVA analysis also revealed *Slc6a4* significant contributions to 5-HIAA/5-HT in (C) cortex (main effect for *Slc6a4*: $F(1,58) = 6.28$, $p < .05$, $n = 13\text{-}18$) and (D) hippocampus (main effect for *Slc6a4*: $F(1,58) = 5.05$, $p < .05$, $n = 12\text{-}18$) however Bonferroni *post-hoc* test could not detect individual genotype differences in either region. (E) Western blot analysis of midbrain tissue reveals significant reductions in IS mice compared to WT and I mice (Kruskal-Wallis one-way ANOVA, $p < .005$; Dunn's *post-hoc* analysis revealed significant differences between IS mice and both WT ($p < .01$), and I ($p < .05$) mice n=6-7). All error bars = SEM, significance indicators as follows: * compared to WT, ^ compared to I.

Although IS mice displayed normal 5-HT uptake kinetics, I mice exhibited significant reductions in V_{max}. The molecular alterations required for IS mice to exhibit normal 5-HT uptake may have be due to effects on the serotonergic system which may have gone undetected in the analysis. Previous reports of *Slc6a4* double heterozygous or double knockout mouse models report only deficits due to the gene x gene interactions (Hagino *et al*, 2011; Page *et al*, 2009; Ren-Patterson *et al*, 2005), although gender differences in BDNF x SERT double knockout phenotypes have been reported (Ren-Patterson *et al*, 2006). The finding of rescued 5-HT uptake in IS mice may indicate a genetic compensation that is protective against *Itgb3* heterozygosity and is engaged in the context of concurrent *Itgb3* and *Slc6a4* heterozygosity.

In regards to brain 5-HT levels, HPLC tissue analysis of 5-HT levels may be insensitive to differences in extracellular and intracellular 5-HT. The zero-net flux method has been evidenced to be more sensitive than dialysate measurements and has routinely reported elevated extracellular 5-HT levels in mice with reduced SERT expression (Guiard *et al*, 2008; Mathews *et al*, 2004). It is likely that use of this method can find significant increases in extracellular 5-HT in S and IS mice compared to WT.

Methods and Materials

HPLC Assessment of Brain Amine Levels

Mice were euthanized by rapid decapitation. The midbrain was dissected by making a coronal cut straight down through the brain just anterior to the superior colliculus (approximate bregma, −3.28). The cerebellum was removed and a second coronal cut was made just posterior to the inferior colliculus (approximate bregma,

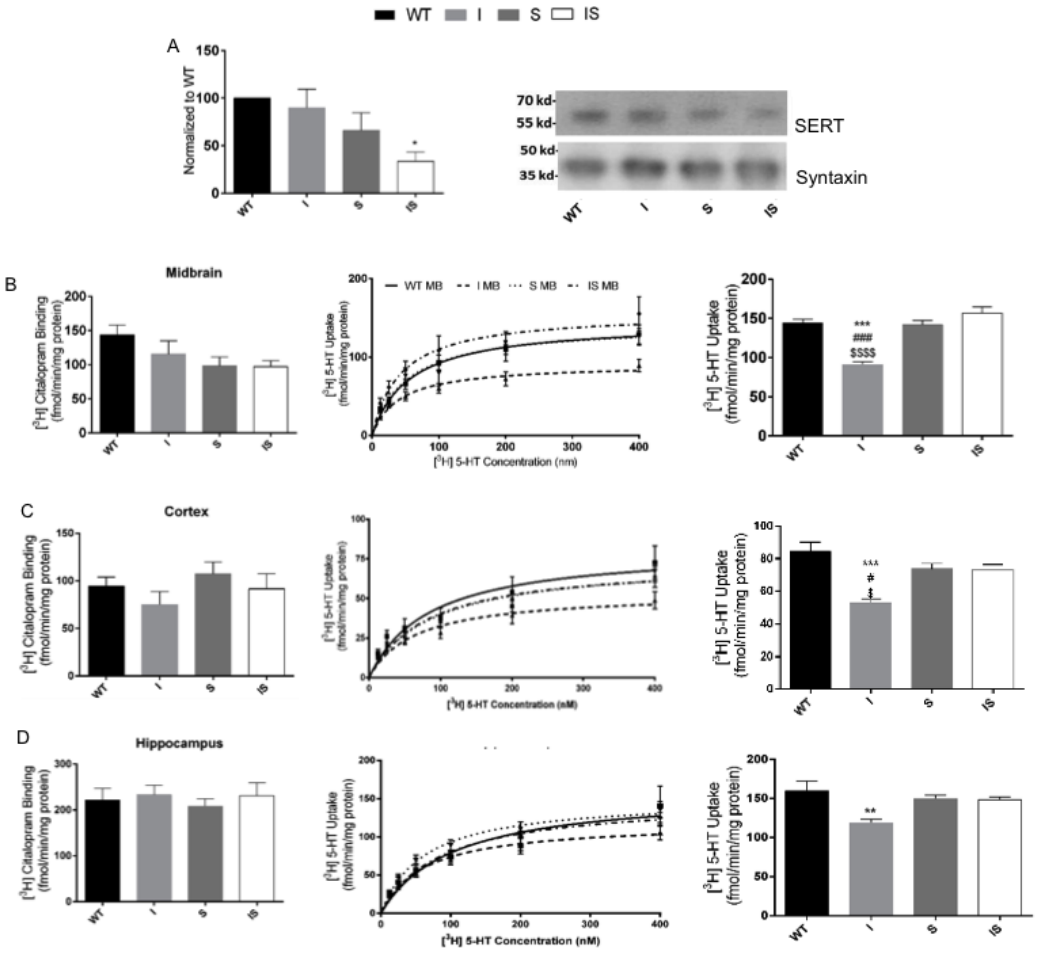

Figure 6. Despite reduced SERT Expression, IS mice have normal 5-HT uptake (A) Western blot analysis of midbrain synaptoneurosomes reveals synapse specific reductions in SERT expression in IS mice compared to WT (Kruskal-Wallis one-way ANOVA, $p < .05$, n = 8-12; Dunn's *post-hoc* analysis revealed significant differences between IS mice and WT, $p < .05$). (B) [^{3}H] citalopram binding reveals *Slc6a4* dependent reductions in SERT expression in midbrain synaptoneurosomes (two-way ANOVA main effect for *Slc6a4*: $F(1,44) = 4.92$, $p < .05$, $n = 12$), however *post-hoc* analysis could not reveal individual genotype differences, saturation analysis revealed normal uptake in IS mice despite significant reductions uptake capacity in I mice (two-way ANOVA main effect for genotype: $F(3,162) = 13.46$, $p < .0001$, and 5-HT concentration $F(5,162) = 41.45$, $p < .0001$, $n = 4$), and V_{max} measure shows I mice have significantly less uptake capacity than WT ($p < .001$), S ($p < .001$), and IS mice ($p < .0001$) due to a significant *Itgb3* x *Slc6a4* interaction (two-way ANOVA main effect for genotype: $F(1, 8) = 8.73$, $p < .05$, $n = 4$).Similar experiments were performed in on cortex and hippocampus synaptoneurosomes. (C) Analysis of cortical synaptoneurosomes revealed no significant differences in [^{3}H] citalopram binding ($p = .38$), however saturation analysis again revealed normal uptake in IS mice despite significant reductions uptake capacity in I mice (two-way ANOVA main effect for genotype: $F(3, 119) = 5.79$, $p = .0001$, and 5-HT concentration $F(5,119) = 59.41$, $p < .0001$, $n = 4$), and V_{max} measure shows I mice have significantly less uptake capacity than WT ($p < .005$), S ($p < .05$), and IS mice ($p < .05$). (D) The hippocampus also displayed no significant differences in [^{3}H] citalopram binding ($p = .24$), however saturation analysis again revealed normal uptake in IS mice despite significant reductions uptake capacity in I mice (two-way ANOVA main effect for genotype: $F(3, 168) = 3.66$, $p < .05$, and 5-HT concentration $F(5,168) = 75.52$, $p < .0001$, $n = 4$), and V_{max} measure shows I mice have significantly less uptake capacity than WT mice ($p < .01$). All error bars = SEM, significance indicators as follows: * compared to WT, # compared to S, and $ compared to IS.

−5.80). 5-HT, dopamine, and norepinephrine levels in tissue extracts were determined by HPLC by using an Antec Decade II electrochemical detector (oxidation, 0.5) operated at 33 °C in the Vanderbilt Center for Molecular Neuroscience Neurochemistry Core. Twenty microliter samples of the supernatant from trichloroacetic acid tissue extracts were injected via a Water 717+ autosampler onto a Phenomenex Nucleosil C18HPLC column (5u, 100A; 150 × 4.60 mm). Amines were eluted with a mobile phase consisting of 89.5% 0.1 M trichloroacetic acid, 10−2 M sodium acetate, 10−4 M EDTA, and 10.5% methanol (pH 3.8). Solvent was delivered at 0.6 ml/min by using a Waters 515 HPLC pump.

Synaptoneurosome Preparation

Synaptoneurosomes were obtained as previously described (Veenstra-VanderWeele *et al*, 2012). Briefly, mice were rapidly decapitated and brain regions were dissected and stored at 4 °C. Samples were homogenized in 10 mL of .32 M sucrose and centrifuged to isolate synaptoneurosomes.

Western Blotting

Midbrain synaptoneurosomes or trichloroacetic acid pellets retrieved from HPLC were resuspended in 1% sodium dodecyl sulfate in phosphate buffered saline pH 7.4 and protein was measured by bicinchoninic acid kit (BCA Protein Assay Kit, Pierce Chemical Company, Rockford, IL). Concentrations of 20-50 µg of protein were loaded onto 17-well Pierce Protein Gels (Thermo Scientific). Gel electrophoresis was performed at 100 v for 3 hours then proteins were transferred overnight at 4 °C onto

PVDF membranes (Immoblin). 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. Multiple exposures were taken to address linearity of the data. Films were scanned and proteins quantified by densitometry using Image J. Samples that showed significant background or degradation were excluded from analysis. Antibodies included: mouse anti- GAPDH (Ambion), mouse anti-syntaxin (Millipore); and guinea pig anti-serotonin transporter (Frontier Science Co., LTD).

[^{3}H] Citalopram Binding

Each tube contained 100 µg of midbrain synaptoneurosomes 50 µl binding buffer, 50 µl of 1 mM 5-HT or 250 µM fluoxetine, and 50 µl of 5 nM [^{3}H] citalopram. Tubes were incubated on ice for 20 min then harvested via Brandel onto GF/B Whatman filters. Filters were dissolved overnight in scintillation fluid then radioactivity was quantified in a Packard counter by QuantaSmart 4.0 software.

[^{3}H] 5-HT Saturation Assays

Each tube contained 100 µl of midbrain synaptoneurosomes (at 1 µg/µl) and 50 µl assay buffer (containing 100 µM ascorbic acid and 100 µM paraglycine in KRH buffer). Parallel tubes were incubated with 10 µM citalopram to determine SERT specific uptake. Tubes were incubated for 10 min at 37 °C then 50 µl of vehicle, 500 nM, 1 mM, 1.5 mM or 2 mM of [^{3}H] 5-HT were added to duplicate tubes. Samples were incubated for 10 min at 37° C then harvested via Brandel onto GF/B Whatman filters. Filters were

dissolved overnight in scintillation fluid then radioactivity was quantified in a Packard counter by QuantaSmart 4.0 software.

Autism-like Phenotype Analysis

Itgb3 has been implicated in whole blood 5-HT levels (Weiss *et al*, 2004), and elevated whole blood 5-HT is a known biomarker of autism (Cook and Leventhal, 1996). HPLC analysis of whole blood 5-HT revealed no significant difference in IS mice compared to WT, however there was a *Slc6a4* significant contribution to reduced blood 5-HT (WT: 3597 $\pm$ 246.7 ng/mg, n = 9; I: 3698 $\pm$ 428.8 ng/mg, n = 8; S: 2675 $\pm$ 354 ng/mg, n = 7; IS: 3097 $\pm$ 269.2 ng/mg, n = 9; two-way ANOVA: *Slc6a4* $p < .05$; Figure 7A). Repetitive and stereotypic behaviors are core symptoms of autism disorder and were examined in the marble burying and open field assays. No significant differences were found between genotypes in number of marbles buried (WT: 3.39 $\pm$ 1, n = 13; I: 2.6 $\pm$ 1.02, n = 14; S: 4.14 $\pm$ .84, n = 14; IS: 2.43 $\pm$ 2.93, n = 14; one-way ANOVA: p = .57; Figure 7B). Although *Slc6a4* significantly contributed to reduced stereotypic counts in the open field chamber, no genotype differences were found (WT: 5135 $\pm$ 243.2, n = 20; I: 4947 $\pm$ 268.4, n = 24; S: 4437 $\pm$ 258.8, n = 22; IS: 4404 $\pm$ 276.4, n = 17; two-way ANOVA: *Slc6a4* $p < .05$; Figure 7C). During a 10 min 3 chamber test of social behavior, *Itgb3*$^{-/-}$ mice displayed reduced interest in social interaction (Carter *et al*, 2011) another common symptom of autism disorder. WT and IS mice assayed in the same paradigm displayed a significant preference for the social stimulus ($p < .001$) while there were no significant differences between genotypes (WT: 200.83 s, $\pm$ 22.36, n =12; IS: 184.77 s, $\pm$ 28.86, n =13; two-way ANOVA: stimulus $p < .005$; Figure 7D). In the novel interaction

condition, a significant preference was found for the novel mouse compared to the familiar mouse ($p < .05$), however no significant differences were found between genotypes (WT: 200.83 s, ± 22.36, n =12; IS: 184.77 s, ± 28.86, n =13; two-way ANOVA: stimulus $p < .005$; Figure 7E). No significant difference was found in entries into the left or right chamber (Figure 7F) indicating a side preference did not skew the data. The normal whole blood 5-HT levels, and stereotypic and social behavior, suggests that IS mice are *not* an autistic mouse model.

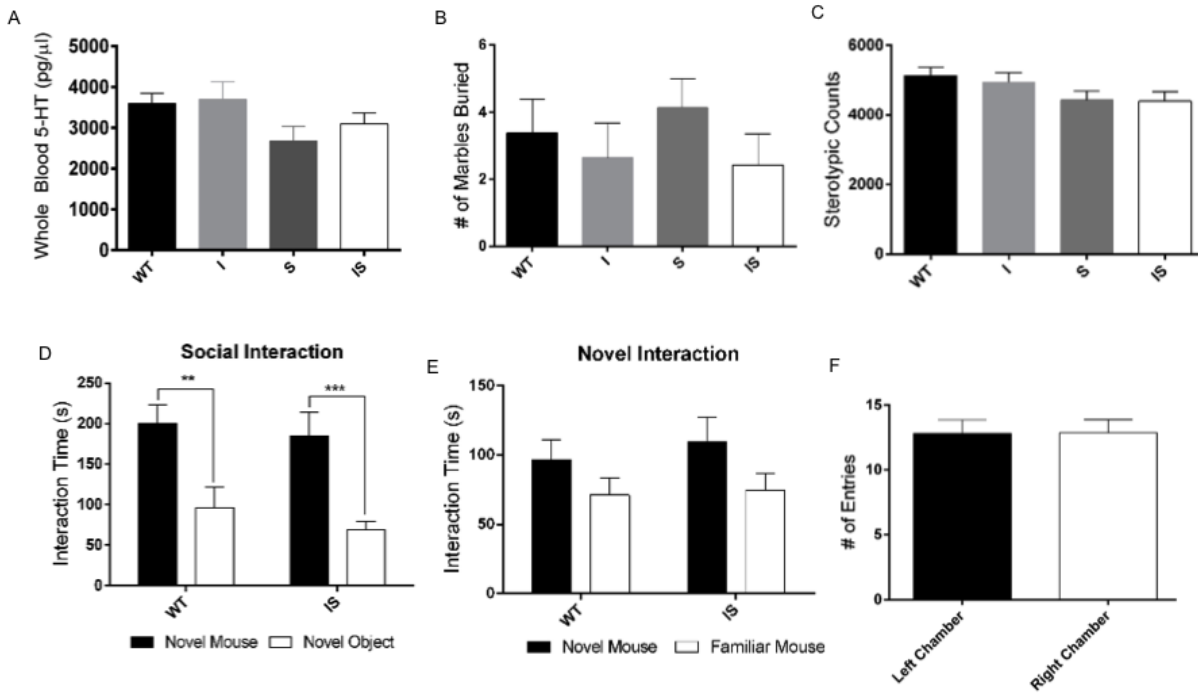

Figure 7. IS mice exhibit normal whole blood 5-HT, stereotypic, and social behaviors (A) Whole blood 5-HT in IS mice is normal compared to WT despite significant contribution from *Slc6a4* to reduced 5-HT (two-way ANOVA main effect for *Slc6a4*: $F(1,27) = 6.38$, $p < .05$, n = 7-9). (B) IS mice do not exhibit increases in marble burying behavior (one-way ANOVA, $p = .57$, n = 13-14). (C) IS mice do not exhibit increases in stereotypic behaviors during a 30 min OF (one-way ANOVA, $p = .14$, n = 18-24). (D) IS mice show no deficit in social preference (two-way repeated measures ANOVA: main effect for stimulus $F(1, 23) = 31.5$, $p < .0001$ Bonferroni *post-hoc* analysis revealed a significant preference for the social stimulus in WT ($p < .01$, n = 12) and IS ($p < .005$, n = 13) mice. (E) IS mice did not display significant differences in the amount of time spent with a novel mouse compared to a familiar mouse in (two-way repeated measures ANOVA: main effect for novelty $F(1,23) = 6.4$, $p < .05$). Analysis of side preference revealed the random counterbalanced stimulus presentation was effective as mice did not exhibit preference for entering either side, unpaired t-test, t (.041, 98), $p = .98$, n = 13-14. All error bars = SEM.

Methods and Materials

Open field

After at least 1 hr acclimation under red light, mice were placed in light- and air controlled open field activity chambers (Med Associates, 27.9 x 27.9 x 20.5 cm) for 30 min. Locomotor patterns were reported by 16 photocells in each horizontal direction. Data was extrapolated using the Activity Monitor software (Med Associates). The inner zone was defined as greater than 2 cm from the chamber wall.

Marble burying

Each mouse was placed in a novel cage with a 5 cm deep layer of bedding and allowed 30 min to acclimate. After 30 min the mice were removed from the cage. 20 clean, transparent glass marbles (1.5 cm diameter) were placed on top of bedding in five rows of four marbles each, equally spaced apart. The mouse was then replaced in the cage for 20 min. After 20 min the mouse was removed from the cage and the number of marbles buried (at least 2/3 covered in bedding) was recorded.

3-chamber social interaction test

Social behavior was evaluated in a three chamber polycarbonate apparatus with 4−inch sliding gates separating the 7 × 9−inch chambers. After at least 1 hr acclimation under red light, the subject mouse was initially allowed to explore all three chambers for 10 min to acclimate to the apparatus. A stimulus mouse (social stimulus) was then introduced inside an inverted wire pencil cup (Spectrum Diversified Designs) in one side

of the chamber with a clean empty pencil cup (inanimate stimulus) introduced in the opposite side chamber. The stimulus mouse was an adult male WT mouse, previously habituated to the pencil cup. Videos were scored by trained observers blinded to genotype.

Anxiety- and Depression-like Phenotype Analysis

To determine contributions of *Itgb3* heterozygosity to SERT-mediated anxiety- and depression-like phenotypes, further behavioral tests were analyzed. In a 30 minute open field test, *Slc6a4* heterozygosity significantly contributed to reduced locomotor activity (WT: 3427 $\pm$ 295.8 cm, n = 20; I: 3334 $\pm$ 245.5 cm, n = 24; S: 2622 $\pm$ 214.5 cm, n = 22; IS: 2744 $\pm$ 317.2 cm, n = 17; two-way ANOVA: *Slc6a4* p = .01; Figure 8A), however *post-hoc* analysis did not detect individual genotype differences. Further analysis revealed vertical exploratory behavior was significantly reduced in IS mice (WT: 156.1 $\pm$ 19.16, n = 20; I: 135 $\pm$ 19.48, n = 24; S: 126 $\pm$ 25.38, n = 22; IS: 76 $\pm$ 9.55, n = 17; Kruskal-Wallis one-way ANOVA: $p < .05$; Dunn's *post-hoc* WT vs. IS $p < .05$; Figure 8B) specifically in the inner zone of the chamber (WT: 22.49 $\pm$ 2.94, n = 20; I: 15.99 $\pm$ 2.36, n = 24; S: 22.8 $\pm$ 3.9, n = 22; IS: 7.3 $\pm$ 1.21, n = 17; Kruskal-Wallis one-way ANOVA: $p < .005$; Dunn's *post-hoc* WT vs. IS $p < .01$, S vs. IS $p < .05$; Figure 8C). Additionally, IS mice spent significantly less time in the inner zone than WT mice (WT: 20.5 $\pm$ 2.59%, n = 20; I: 18.11 $\pm$ 2.4%, n = 24; S: 14.04 $\pm$ 2.5%, n = 22; IS: 10.45 $\pm$ 1.82%, n = 17; one-way ANOVA: $p < .05$; Bonferroni *post-hoc* WT vs. IS $p < .05$; Figure 8D). During a 5 min elevated zero maze assay no individual genotype differences were found however there was a significant contribution from *Slc6a4* heterozygosity to reduced percent time in the open arm (WT: 47.41 $\pm$ 2.43%, n = 20; I: 47.08 $\pm$ 2.65%, n

= 21; S: 41.4 ± 2.28%, *n* = 23; IS: 43.5 ± 2.26%, *n* = 20; one-way ANOVA: *p* = .05; Figure 8F). Results from these exploratory and anxiety measures are consistent with previous reports of *Slc6a4* heterozygosity effects on exploratory and anxiety-like behaviors (Holmes *et al*, 2003; Kalueff *et al*, 2007a; Kalueff *et al*, 2007b).

As SERT deficiency is also implicated in depression-like behaviors (Ansorge *et al*, 2008; Lira *et al*, 2003; Zhao *et al*, 2006), behavioral tests of SERT related depression-like behaviors were performed (Bodnoff *et al*, 1988; Lucki *et al*, 2001). During a six minute forced swim test (FST), significant differences were found in immobility time during the first 2 min of the test (WT: 52.83 ± 7.81 s, *n* = 14; I: 36.33 ± 4.53 s, *n* = 15; S: 46.57 ± 10.4 s, *n* = 21; IS: 82.59 ± 13.68 s, *n* = 16; Kruskal-Wallis one-way ANOVA: *p* < .05; Figure 8G), however *post-hoc* tests did not detect genotype differences. IS mice also exhibited significantly increased immobility during the last 4 min of the FST (WT: 85.18 ± 9.18 s, *n* = 27; I: 119.6 ± 9.76 s, *n* = 27; S: 138 ± 11.24 s, *n* = 21; IS: 143.3 ± 11.35 s, *n* = 22; one-way ANOVA: *p* < .001; Bonferroni *post-hoc* WT vs. IS *p* < .005, WT vs. S *p* < .01; Figure 8H). As a further indicator to SERT regulation of these behaviors, correlation analysis revealed a significant correlation between SERT expression and immobility time in minute 4 of the FST (*r* = -.52, *p* < .005; Figure 8I).

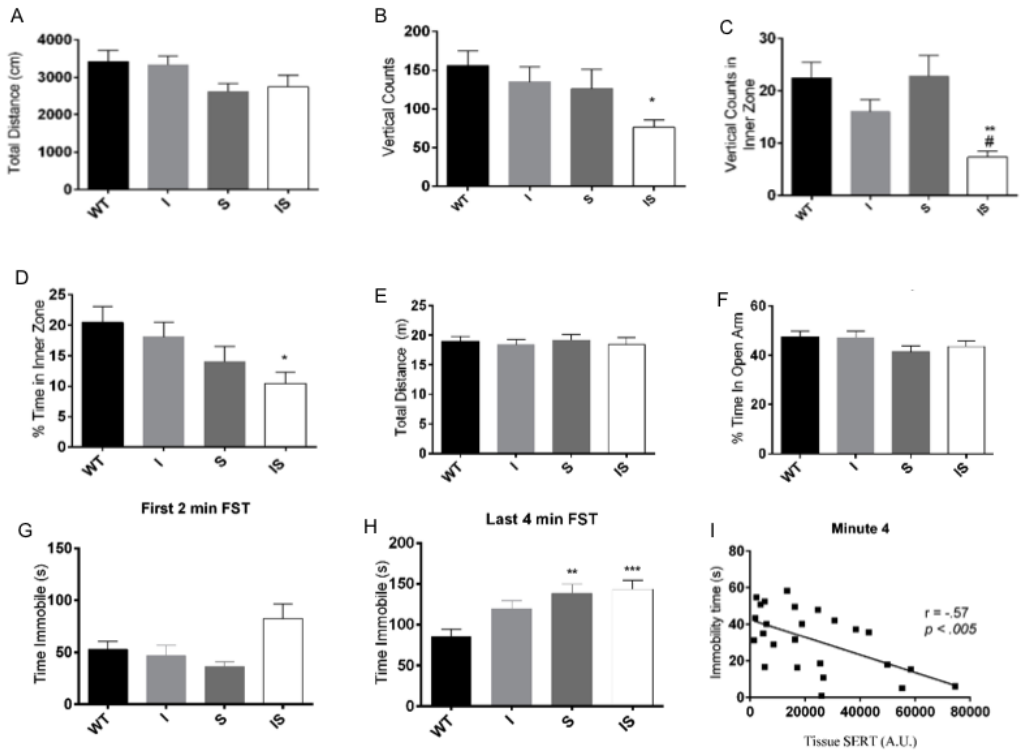

Figure 8. IS mice exhibit a hypoexploratory, anxiety-, and depression-like phenotype. (A) *Slc6a4* significantly contributes to a hypolocomotor phenotype in the open field test (two-way ANOVA main effect for *Slc6a4*: F(1, 79) = 6.8, *p*=.01, *n* =17-24. IS mice show reduced vertical exploration behavior in the open field apparatus (Kruskal-Wallis one-way ANOVA; *p* < .05, *n* = 17-24; Dunn's *post-hoc* analysis reveals a significant reduction in IS mice compared to WT, *p* < .05) and, (C) in the center zone (Kruskal-Wallis one-way ANOVA, *p* <.05, *n* = 16-23; Dunn's *post-hoc* analysis reveals a significant reduction in IS mice compared to WT mice, *p* < .05, I mice, *p* < .05, and S mice *p* < .05). (D) IS mice spend significantly less time in the center zone of the OF (two-way ANOVA main effect for *Slc6a4*: F(1, 79) = 8.4, *p* <.005, *n* = 17-24; Bonferroni *post-hoc* analysis reveals a significant difference in IS mice compared to WT, *p* < .05). (E) Although no significant differences were found in total distance traveled in the EZM (one-way ANOVA; *p* = .91, *n* = 20-23), (F) *Scl6a4* significantly contributed to time spent in the open arm of the maze (two-way ANOVA main effect for *Slc6a4*: F(1, 80) = 39.5, *p* =.05, *n* = 20-23). (G) A significant difference was detected in immobility time during the first two minutes of the FST (Kruskal-Wallis one-way ANOVA *p* < .05, *n* = 14-21), however *post-hoc* analysis did not reveal individual genotype differences. (H) During the last four min of the FST *Slc6a4* heterozygosity contributed to greater immobility time (two-way ANOVA main effect for *Slc6a4*: F(1, 93) = 13.65, *p* = .004; main effect for *Itgb3*: F(1, 93) = 3.67, *p* = .058; WT, *n* = 27; I, *n* = 27; S, *n* = 22; IS, *n* = 16; Bonferroni *post-hoc* reveals a significant difference in both IS and S mice compared to WT (*p* < .005 and *p* < .01 respectively). (I) Correlation analysis revealed a significant correlation between tissue SERT expression and time immobile in minute 4 of the FST (Pearson *r*(25) = -.57, *p* < .005). All error bars = SEM. Significance indicators as follows: * compared to WT, # compared to I.

Methods and Materials

Elevated Zero Maze

The apparatus is 40 cm x 50 cm, and has four equidistance 5 cm wide arms. The two closed arms face opposite each other and have 15 cm walls. Each mouse was placed gently into the open arm of the maze and allowed to explore freely for 5 min. Mouse behavior was video-tracked and analyzed via ANY-maze software (Stoelting).

Forced Swim Test

The Porsolt forced swim test was used to measure depression-related behaviors (Cryan *et al*, 2005; Porsolt *et al*, 1977). Experimentation and analysis was conducted with the experimenter blinded to animal genotypes. The testing apparatus consisted of a clear Plexiglas cylinder with water approximately 20 cm deep and 23° C. After at least 1 hr acclimation under red light, mice were tested for 6 min. After 6 min, the number of fecal boli produced during the test was counted and the animals were removed and placed in clean, heated cages for 15 min to recover. All tests were recorded by video camera and scored by an observer blinded to the genotypes. Following each test, the testing cylinders were drained, cleaned, and refilled with clean water. Immobility was defined as minimal movement necessary for the animal to keep its head above water. Immobile behavior was recorded in one-minute bins. The primary dependent variable was immobility time in the last 4 min of the test, which has been shown to be sensitive to anti-depressant effects. Additional dependent variables included latency to first immobile period and immobility time in the first 2 min.

CHAPTER IV

PHARMACOLOGICAL ASSESSMENT: INTEGRIN αvβ3 X SERT FUNCTIONAL INTERACTION

Integrin αvβ3 Regulation of SERT Uptake Activity

Integrin antagonists have traditionally utilized the high affinity Arginine-Glycine-Aspartic acid (RGD) binding domain (Hynes, 1992), and integrin effects on synaptic functions in response to cyclo-RGD (cRGD) peptide mimetics have been reported (Watson *et al*, 2007). RGD ligands are known to regulate the integrin αIIβ3 x SERT interaction in platelets (Carneiro *et al*, 2008). Based on these findings, integrin αvβ3 targeted cRGD analogs IDT 494 and IDT 500 were synthesized to explore integrin αvβ3 x SERT functional interactions. Carneiro, et al., demonstrated that activation of platelet integrin αIIβ3 significantly increases SERT [^{3}H] 5-HT uptake (Carneiro *et al*, 2008). Similarly, in WT midbrain synaptoneurosomes [^{3}H] 5-HT uptake assays revealed effects on SERT uptake activity in response to integrin αvβ3 targeted compounds. Specifically, at concentrations of 1-100 nm IDT 494 increased SERT uptake ($p < .05$, $n = 4$; Figure 9B), while .1 nM IDT 500 decreased SERT uptake ($p < .05$, $n = 3$; Figure 9C), indicating that integrin αvβ3 can differentially modulate synaptic SERTs. Also assayed were other compounds which contain RGD sites or otherwise bind to integrin αvβ3. 10 nM RGD significantly increased SERT uptake ($p < .05$, $n = 4$; Figure 9A). Resveratrol is a natural polyphenol found in grapes and other fruits. It has come under investigation as an anti-cancer agent and has been found to act through its RGD sequence binding integrin

αvβ3 (Hsieh *et al*, 2011; Lin *et al*, 2006). At concentrations of 50-100 μm resveratrol significantly reduced SERT uptake ($p < .005$, $n = 3$; Figure 9D). Echistatin is a naturally occurring disintegrin which also contains the high affinity RGD sequence (Kumar *et al*, 1997). Echistatin had no effect on SERT uptake at comparable concentrations ($p = .71$, $n = 3$; Figure 9E). Lastly, $MnCl_2$, which is known to bind to metal ion-dependent adhesion sites as opposed to the RGD binding-site (Hynes, 2002a), also had no effect on SERT uptake ($p = .75$, $n = 4$; Figure 9F).

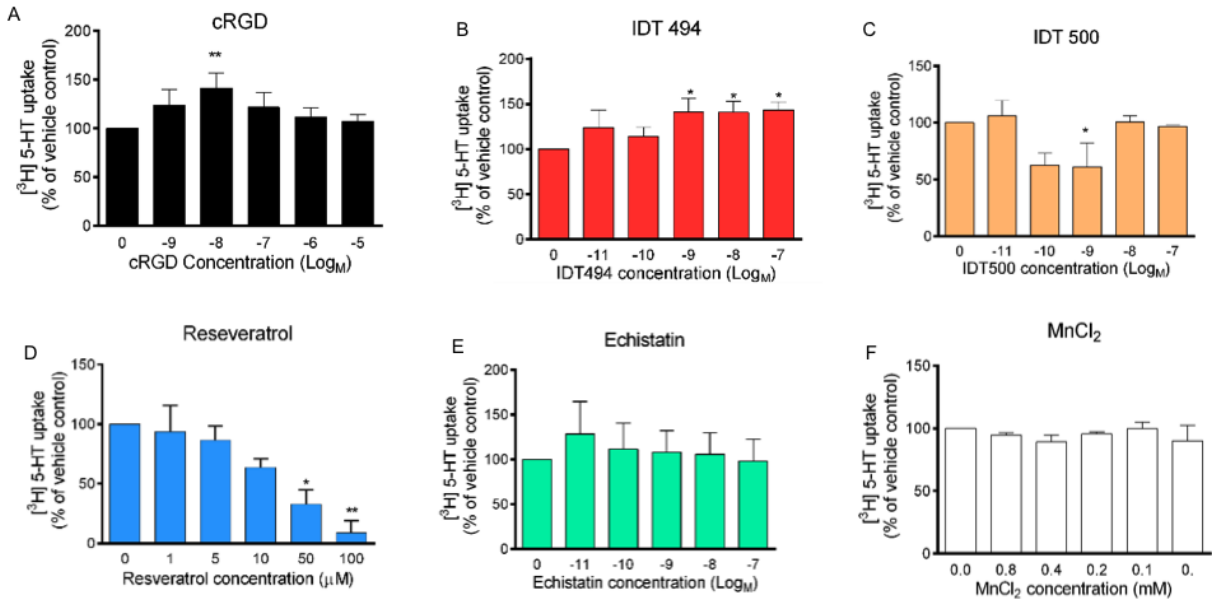

Figure 9. Pharmacological targeting of the integrin αvβ3 RGD binding site regulates SERT uptake activity. A) 10 nM cRGD significantly increased uptake ($p < .01$). B) IDT 494 significantly increased uptake at concentrations of 1-100 nM ($p < .05$). C) IDT 500 decreased uptake at .1 nM ($p < .05$). D) Resveratrol reduced uptake at concentrations of 50-100 μM ($p < .05$), $p < .01$, respectively). (E) Echistatin had no effect of SERT uptake at comparable concentrations. (F) $MnCl_2$ which binds to an alternate site in integrin αvβ3 also had no effect on uptake activity. Repeated measures ANOVA with Dunnett's *post-hoc* analysis, $n = 3$-6 for each experiment. All error bars = SEM.

As IDT 500 and resveratrol both exhibited SSRI-like effects on SERT uptake, they were further probed to determine if they were specific for integrin αvβ3. Since integrin β3 only forms dimers with integrin αv and αIIb (Hynes, 2002a), and integrin αIIb is not expressed in brain (Wu and Reddy, 2012), *Itgb3*$^{-/-}$ mice experiments allowed for determination of specificity for integrin αvβ3 as opposed to the multitude of integrin α- and β- subunit combinations that form RGD receptors and are expressed in the brain (Wu *et al*, 2012). [^{3}H] 5-HT uptake assays in *Itgb3*$^{-/-}$ mice proved specificity of IDT 500 for integrin αvβ3 uptake ($p < .005$, $n = 5$; Figure 10a), since the compound had no effect on [^{3}H] 5-HT uptake in *Itgb3*$^{-/-}$ midbrain synaptoneurosomes ($p = .94$). Resveratrol proved to be non-specific for integrin αvβ3 as it significantly reduced uptake in both WT and *Itgb3*$^{-/-}$ synaptoneurosomes ($p < .005$, $n = 5$; and $p < .05$, $n = 4$; respectively; Figure 10B).

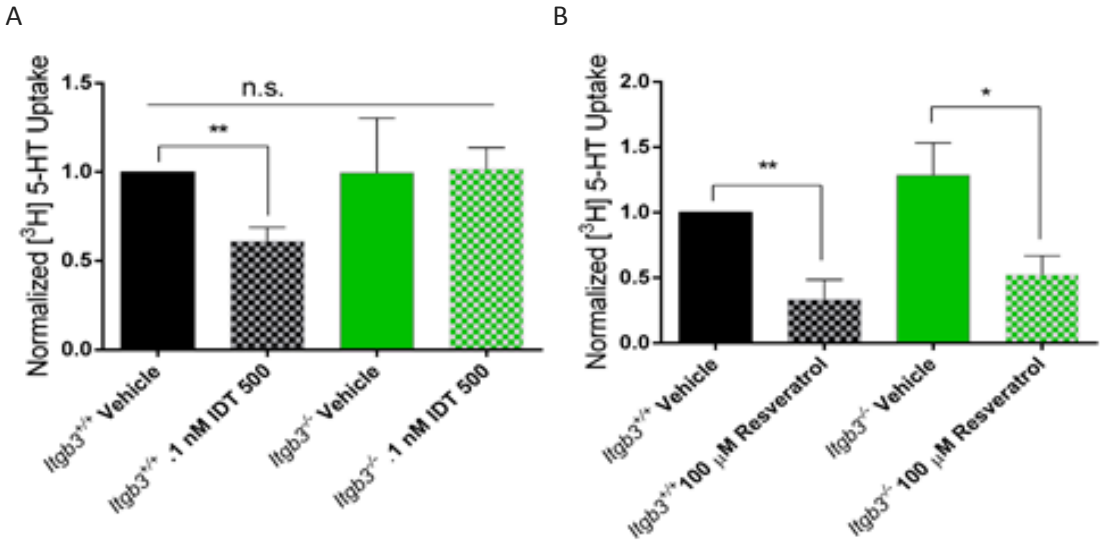

Figure 10. A) IDT 500 reduces uptake in WT [t (4.96, 8), $p < .005$, $n = 5$], and has no effect on uptake in *Itgb3*$^{-/-}$ [t (.07, 8), $p = .95$, $n = 5$] midbrain synaptoneurosomes. B) Resveratrol significantly reduces 5-HT uptake in both WT [t (4.37, 6), $p < .005$, $n = 4$] and *Itgb3*$^{-/-}$ [t (2.64, 6), $p < .05$, $n = 4$] midbrain synaptoneurosomes. Two-tailed unpaired t-tests. All error bars = SEM.

Results from these experiments suggests that RGD targeting of integrin αvβ3 can regulate midbrain SERT uptake activity leading to the working model in Figure 11.

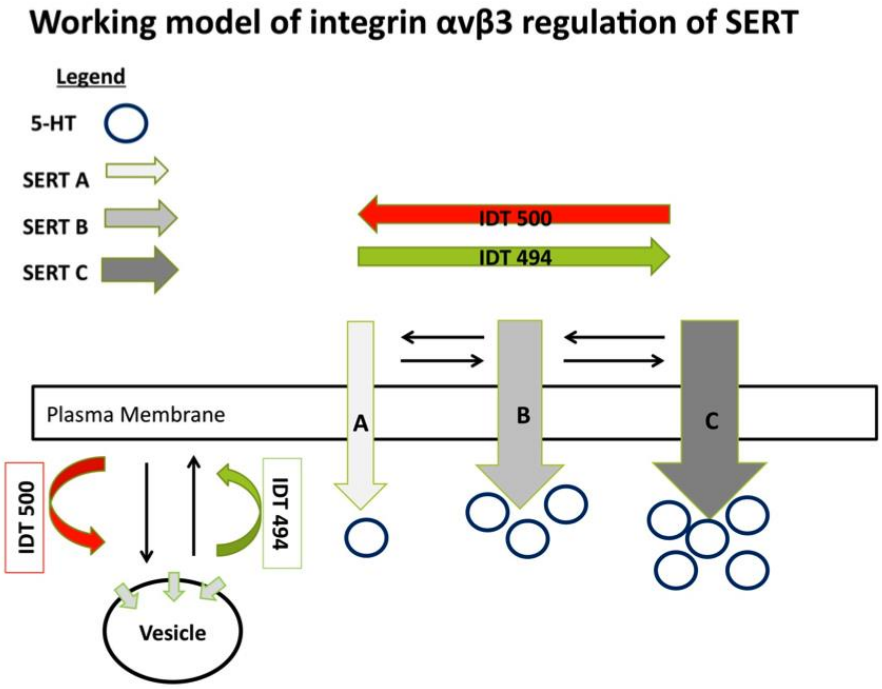

Figure 11. Working model of IDT 494 and IDT 500 regulation of SERT activity via integrin αvβ3. IDT 494 may regulate SERT uptake activity via increasing SERT expression at the plasma membrane, or increasing SERT catalytic activity, while IDT 500 may regulate SERT uptake by decreasing SERT expression or decreasing SERT catalytic activity.

Methods and Materials

[^{3}H] 5-HT uptake assays

Midbrain synaptoneurosomes were obtained and normalized to a concentration of 1 µg/ µl. 100 µl of synaptoneurosomes were then incubated for 10 min at 37^o C in test tubes containing 100 µl of assay buffer, and 50 µl of drug. Next, samples were incubated with [^{3}H] 5-HT for 10 min at 37^o C. An identical set of tubes contained 50 µl of 10 nM citalopram to define SERT specific uptake. Next samples were harvested via Brandel onto GF/B Whatman filters. Filters were dissolved overnight in scintillation fluid then radioactivity was quantified in a Packard counter by QuantaSmart 4.0 software. Echistatin, resveratrol, and cRGD were purchased from Tocris Biosciences. IDT 494 and IDT 500 were synthesized at Vanderbilt University by Ian Tomlinson, Ph.D.

Analysis of Kinase Activity

Since integrins are enzymatically inactive, they must rely on adaptor molecules to confer signaling and regulate cell functions. Integrin αIIβ3 signaling through p38 MAPK is a known regulator of SERT plasma membrane expression and catalytic activity in platelets (Carneiro *et al*, 2008). Recently, the tyrosine kinase Src was found to regulate synaptosome plasma membrane expression and uptake activity of SERT via phosphorylation of Tyr47 and Tyr 142 (Annamalai *et al*, 2012). Src is known to directly associate with the c-terminus of integrin β3 (Arias-Salgado *et al*, 2003), and activation of integrin αvβ3 by RGD peptides leads to Src phosphorylation and activation (Alghisi *et al*, 2009).

To determine if kinase signaling mediated the integrin αvβ3 x SERT uptake effect, midbrain synaptoneurosomes were incubated in vehicle, 10 nM cRGD, 10 nM IDT 494, 100 μM resveratrol, or 1 mM $MnCl_2$. Synaptoneurosomes were then probed for kinase activity via Western blot. No significant differences were found for any of the tested RGD compounds in Src activity, however 1 mM $MnCl_2$ significantly reduced Src activity ($p < .05$, $n = 7$; Figure 11A). None of the tested compounds significantly affected ERK activity ($p = .3$, $n = 7$; Figure 11B).

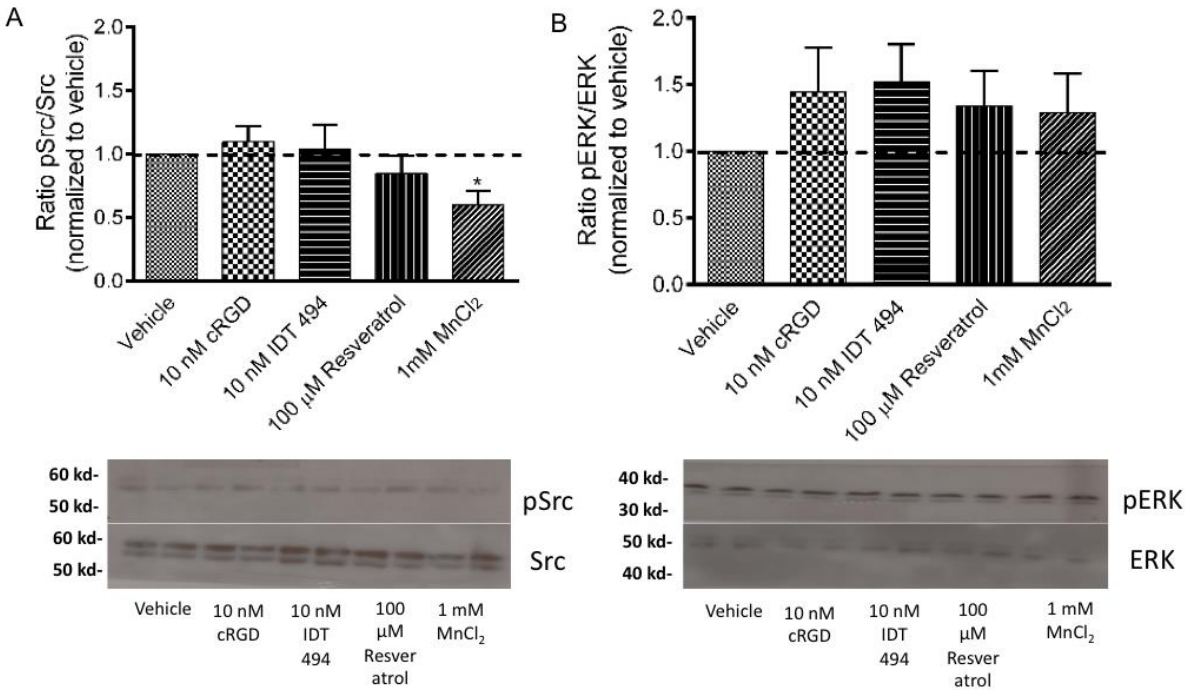

Figure 12. Western blot analysis of midbrain synaptoneurosomes did not reveal changes in kinase activity in response to RGD compounds. However, Src activity was significantly reduced by $MnCl_2$ (Repeated measures one-way ANOVA, Dunnett's *post-hoc* analysis, $p < .05$, $n = 7$). (B) ERK activity was not significantly altered by any of the compounds (Repeated measures one-way ANOVA, $p = .30$, $n = 7$). All error bars = SEM.

It is possible that kinase signaling is not responsible for integrin αvβ3 mediated changes in SERT activity. The cytoplasmic tail of integrin β3 is known to regulate expression and function of GluA2 AMPA (Pozo *et al*, 2012) and VEGF type 2 (West *et al*, 2012) receptors via direct interactions with their cytoplasmic tails. Binding of integrin αvβ3 specific compounds could result in conformation changes in the integrin β3 cytoplasmic tail which can then directly regulate SERT.

Methods and Materials

Microcentrifuge tubes contained 50 µl of vehicle/drug and to 450 µl of midbrain synaptoneurosomes (at 1 µg/µl). Samples were incubated for 20 minutes at 37^{o} C. Samples were re-pelleted by centrifugation. The supernatant was removed and the pellet was resuspended in 100µl of 1x-Tris (pH-7.4), 1% SDS, and 25µl lammeli buffer (containing β-mercaptoethanol). Western blotting was performed as previously described in chapter III. Primary antibodies included: Total and phosphorylated Src (Cell Signaling, 1:1000), and total and phosphorylated ERK (Cell Signaling, 1:1000). Protein expression was quantified as described in chapter III.

CHAPTER V

SYNOPSIS AND CONCLUSION

Immunohistochemistry experiments provided preliminary evidence for integrin β3 localization in serotonergic neurons (Figure 2C & 3), in close proximity to SERT (Figure 4). Additionally, it appeared that knockout of *Itgb3* led to a reduction in the number of neurons in the DR, consistent with recent imaging results (Ellegood *et al*, 2012). The presence of integrin β3 in DR serotonergic neurons, previous evidence of a functional integrin αIIbβ3 x SERT interaction in platelet function (Carneiro *et al*, 2008), a genetic interaction in human and mouse brain (Weiss *et al*, 2006b), and an *Itgb3* x *Slc6a4* contribution to increased autism risk (Ma *et al*, 2010; Napolioni *et al*, 2011; Weiss *et al*, 2006a) provided the evidence needed to further investigate the integrin αvβ3 x SERT interaction in brain.

Despite previous reports implicating *ITGB3* in whole blood 5-HT levels (Weiss *et al*, 2005; Weiss *et al*, 2004) and autism risk (Ma *et al*, 2010; Napolioni *et al*, 2011; Weiss *et al*, 2006a), and *Itgb3*$^{-/-}$ mice exhibiting reduced social preference (Carter *et al*, 2011), experiments reported here did not reveal *Itgb3* heterozygosity influences on whole blood (Figure 7A) or midbrain tissue 5-HT levels (Figure 5A). Neither did *Itgb3* heterozygosity influence stereotypic and repetitive, or social behavior (Figure 7B-D). In humans, the gain-of function Leu33Pro *ITGB3* polymorphism is associated with whole blood levels (Weiss *et al*, 2004), while the Leu33 allele is associated with autism risk (Weiss *et al*, 2006a). Additionally, there is confusion regarding involvement of *SLC6A4*

polymorphisms in autism risk. Polymorphisms in the extensively studied *SLC6A4* promoter region (5-HTTLPR) have implicated increased risk for autism for both the *s* allele (Cook *et al*, 1997), associated with reduced transporter expression and function (Lesch *et al*, 1996), and the nominal *l* allele (Klauck *et al*, 1997). There is also evidence that each 5-HTTLPR polymorphism is linked to a particular symptomology of the disorder (Brune *et al*, 2006). Recently, the gain-of-function Gly56Ala polymorphism has been shown to cause hyperserotonemia, social impairments, and increased repetitive behavior in a transgenic mouse model (Veenstra-VanderWeele *et al*, 2012), while *Pten*$^{-/+}$ x *Slc6a4*$^{-/+}$ mice exhibit reductions in social behavior (Page *et al*, 2009). These mixed result suggest that it is likely that no autism-like phenotypes were detected in the IS mouse model because different combinations of genetic and molecular interactions result in different phenotypes.

Additional results from the IS mouse model experiments indicate that while *Itgb3* heterozygosity can significantly reduce 5-HT uptake (Figure 6B-D), and exaggerate biochemical and behavioral phenotypes such as reduced *Slc6a4* expression (Figure 5E and Figure 6A), reduced exploratory behavior (Figure 8B-C), increased anxiety-like behavior (Figure 8D), and increased depression-like behavior (Figure 8G-H), the phenotypes are largely driven by *Slc6a4* heterozygosity. Taken together with IS mice being the sole genotype to have significant reductions in midbrain tissue and midbrain synaptoneurosome SERT expression, it is likely that dose-dependent reductions in SERT expression underlie these behavioral phenotypes, with IS mice demonstrating phenotype characteristics between S and *Slc6a4*$^{-/-}$ mice (Holmes *et al*, 2002; Kalueff *et al*, 2007b; Lira *et al*, 2003). Thus it would appear that integrin β3 acts on the

serotonergic system either directly through SERT or in a similar fashion as SERT to modulate brain function and subsequent behavioral phenotypes.

The pharmacological assessment provided preliminary insight into how integrin αvβ3 can modulate the serotonergic system since targeting of integrin αvβ3 could increase or decrease SERT-mediated 5-HT uptake (Figure 9A-D). That integrin αvβ3 targeting compounds differentially regulate SERT uptake suggests that *in vivo* 5-HT actions can be enhanced or limited via endogenous integrin αvβ3 ligands. Furthermore, these experiments provide evidence for integrin αvβ3 as a potential therapeutic target for serotonergic systems in human brain, and highlight 5-HT phenotypes such as anxiety and depression, as possible side effects for persons prescribed integrin αvβ3 targeted therapeutics. The exact mechanism underlying the integrin αvβ3 X SERT interaction remained elusive as experiments into the prime candidate (kinase signaling) did not yield results (Figure 12). However, it is possible that Western blot is not a sensitive enough technique to detect kinase activity alterations in as complex a system as the brain, where multitudes of kinase signaling pathways are regulating numerous synaptic functions.

These findings highlight integrin β3 as a modulator of brain serotonergic systems. It remains to be elucidated if this occurs through a direct interaction or integrin β3 kinase signaling. The results suggest that persons possessing polymorphisms which result in an *Itgb3*$^{-/+}$ x *Slc6a4*$^{-/+}$ genotype and persons prescribed integrin αvβ3 compounds may be at increased risk for developing 5-HT related disorders, such as anxiety. Further experimentation is required to confirm these findings, determine the mechanism of action, and provide insight into *in vivo* relevance of the integrin αvβ3 x SERT interaction.

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