Neurochemistry International xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/nci

Serotonin transporter and integrin beta 3 genes interact to modulate serotonin uptake in mouse brain

Alonzo Whyte [a], Tammy Jessen [b], Seth Varney [b], Ana M.D. Carneiro [b,*]

[a] Neuroscience Graduate Program, Vanderbilt Brain Institute, U1205 Medical Research Building III, 465 21st Avenue South, Nashville, TN 37232, United States
[b] Department of Pharmacology, Vanderbilt University School of Medicine, 215 Light Hall, Nashville, TN 37232, United States

ARTICLE INFO

Article history:
Available online xxxx

Keywords:
Serotonin transporter
Integrin
Genetic interaction
Neuropsychiatric disorders
Autism

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 changes 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.

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. Materials and methods

2.1. Animals

Mouse studies were performed in accordance with humane guidelines established by the Vanderbilt Institutional Animal Care

* Corresponding author. Address: 461 Preston Research Building, Vanderbilt University Medical Center, 23rd Ave. South at Pierce, Nashville, TN 37232-6600, United States. Tel.: +1 615 875 5635.
E-mail address: ana.carneiro@vanderbilt.edu (A.M.D. Carneiro).

0197-0186/$ - see front matter © 2013 Published by Elsevier Ltd.
http://dx.doi.org/10.1016/j.neuint.2013.09.014

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-h 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 0.1 mM $CaCl_2$ and 1 mM $MgCl_2$ 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 1000*g*. Supernatants were collected and spun at 10,000*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 1 mg 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 (130 mM NaCl, 1.3 mM KCl, 2.2 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1.8 g/L glucose, 10 mM HEPES, pH 7.4 containing 100 μM ascorbic acid and 100 μM pargyline). Synaptoneurosomes (100 μg for midbrain and 200 μg for hippocampus and cortex) were incubated for 10 min at 37 °C in test tubes containing 100 μl of KRH buffer, and 50 μl [^{3}H] 5-HT (Concentrations ranging from 12.5–400 nM. Hydroxytryptamine Creatinine Sulfate, 5-[1,2-3H(N)]-(Serotonin). Perkin Elmer, Walthman, MA). An identical set of tubes contained 50 μl of 1 μM 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 (100 μg for midbrain and 250 μg 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 1 μM 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. 50 μg of protein were loaded onto 17-well Pierce Protein Gels (Thermo Scientific). Gel electrophoresis was performed at 100 v for 3 h 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 min 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 wildtype (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

Please cite this article in press as: Whyte, A., et al. Serotonin transporter and integrin beta 3 genes interact to modulate serotonin uptake in mouse brain. Neurochem. Int. (2013), http://dx.doi.org/10.1016/j.neuint.2013.09.014