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Mice lacking integrin β3 expression exhibit altered response to chronic stress

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ABSTRACT

Recent studies indicate multiple roles for integrin αvβ3 in adult neurons, including response to pharmacological agents such as cocaine and selective serotonin reuptake inhibitors. In this study, we examined the role of the integrin β3 gene (*Itgb3*) in the response to environmental stimuli by subjecting $Itgb3^{+/+}$ and $Itgb3^{-/-}$ mice to unpredictable chronic mild stressors. We found that genetic abrogation of integrin β3 expression elicits an exaggerated vulnerability to chronic unpredictable stress in the open field test. In this test, chronic stress elicited significant decreases in stereotypic behavior and horizontal locomotor activity, including increases in anxiety behaviors. Mild chronic stress led to reductions in dopamine turnover in midbrains of $Itgb3^{+/+}$, but not $Itgb3^{-/-}$ mice, suggesting a disruption of stress-dependent regulation of DA homeostasis. Chronic stress elicited altered synaptic expression of syntaxin and synaptophysin in midbrains of $Itgb3^{-/-}$ mice, when compared to $Itgb3^{+/+}$. Semi-quantitative Western blot studies revealed that the synaptic expression, but not total tissue expression, of multiple signaling proteins is correlated with integrin αv levels in the midbrain. Moreover, loss of integrin β3 expression modifies this correlation network. Together, these findings demonstrate that $Itgb3^{-/-}$ mice display a pattern of changes indicating disrupted regulation of midbrain synaptic systems involved in conferring resilience to mild stressors.

1. Introduction

Genetic influences on biological responses to stress exposure are implicated in the etiology of major depressive and anxiety disorders (Caspi and Moffitt, 2006; Conway et al., 2010; Hammen et al., 2010). Persistent exposure to unpredictable stressors results in plastic changes that involve a wide array of physiological processes in the brain including alterations in neuronal structure and synaptic plasticity (Joels et al., 2007; Yuen et al., 2012). Cell adhesion molecules, such as integrins, are ideally poised to regulate many of these processes, as many are involved in apoptosis, dendritic reorganization, the regulation of synaptic connectivity, and receptor localization (Kerrisk and Koleske, 2013; Scheiffele et al., 2000). Integrins are particularly enriched in synaptic regions (Bahr et al., 1997; Kramar et al., 2002; Mazalouskas et al., 2015; Nishimura et al., 1998), where they participate in synaptic development, maintenance and the cytoskeletal rearrangements that accompany synaptic activity (Bahr, 2000; Chavis and Westbrook, 2001; Hama et al., 2004; Karanian et al., 2005; Nikonenko et al., 2003). Integrin expression and downstream signaling are modulated by antidepressant exposure, implying that integrins are involved in mood regulation (Malki et al., 2012; Oved et al., 2013). However, to our knowledge, the role of integrins in vulnerability to stress has yet to be examined.

The vitronectin receptor, integrin αvβ3, bi-directionally connects the extracellular matrix (ECM) with intracellular signaling pathways (Hynes, 2002). Many integrin subunits, including αvβ3,

Abbreviations: 5-HIAA, 5-Hydroxyindoleacetic acid; 5-HT, 5-hydroxytryptamine; AMPA, α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; ANOVA, analysis of variance; A.U., arbitrary units; BSA, bovine serum albumin; DOPAC, 3,4-Dihydroxyphenylacetic acid; ECL, electrochemiluminescence; ECM, extracellular matrix; EPM, elevated plus maze; ERK, extracellular signal-related kinase; FAK, focal adhesion kinase; FST, forced swim test; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high pressure liquid chromatography; HVA, homovanillic acid; LCL, lymphoblastoid cell lines; NMDA, N-Methyl-D-aspartic acid; OFT, open field test; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; PP2a, protein phosphatase 2a; PSD-95, post-synaptic density 95 protein; PVDF, polyvinylidene fluoride; TBS, tris-buffered saline; SEM, standard error of the mean; Src, c-Src tyrosine kinase; UCMS, unpredictable chronic mild stress; WT, wild-type.

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are expressed at hippocampal, midbrain, and cortical synapses in the brain (Cingolani and Goda, 2008; Cingolani et al., 2008; Mazalouskas et al., 2015). Integrin αvβ3 modulates both serotonergic and glutamatergic neurotransmission in the central nervous system by modifying pre-and post-synaptic protein function (Bisaz and Sandi, 2012; Cingolani and Goda, 2008; Whyte et al., 2014). These effects may influence both structure and function of synapses, as mice lacking the expression of functional integrin αvβ3 (*Itgb3*$^{-/-}$) exhibit reductions in hippocampal, cortical, and dorsal raphe nucleus volumes, with concomitant increases in amygdala volume, a pattern of changes analogous to structural changes observed after prolonged chronic stress exposure (Christoffel et al., 2011; Ellegood et al., 2012; McEuen et al., 2008; Vyas et al., 2002). Behavioral repercussions of integrin αvβ3 loss of function include diminished anxiety-like behaviors in the open field test and elevated plus maze, a lack of preference for social novelty, and elevated novelty-induced grooming behaviors (Carter et al., 2011; McGeachie et al., 2012).

Given the behavioral significance of integrin αvβ3 expression, its genetic and functional interactions with serotonergic and glutamatergic systems, and substantial evidence linking serotonin, glutamate, and stress responses, we sought to delineate the role of integrin αvβ3 in several facets of the response to environmental stressors. Accordingly, we evaluated the role of integrin αvβ3 in the neurochemical and behavioral responses to acute and chronic stress by subjecting *Itgb3*$^{+/+}$ and *Itgb3*$^{-/-}$ mice to an unpredictable chronic stress paradigm. Taken together, our studies indicate a role for integrin αvβ3 in both stress reactivity and resilience mechanisms resulting, in part, from differential expression of synaptic proteins in the midbrain.

2. Materials and methods

2.1. Animals and housing

Three cohorts of adult male C57BL/6 *Itgb3*$^{+/+}$ and *Itgb3*$^{-/-}$ mice (Hodivala-Dilke et al., 1999) were generated by *Itgb3*$^{+/-}$ x *Itgb3*$^{+/-}$ crosses. All three *Itgb3* cohorts were subjected to chronic unpredictable stress. At the beginning of stress exposure, mice ranged from 8 to 13 weeks of age. Control mice were group housed (except mice subjected to chronic stress) in temperature and humidity controlled conditions under a 12 h light–dark cycle with food and water available *ad libitum* in the Vanderbilt Murine Neurobehavioral Core. All experimental procedures were approved by the Vanderbilt Institutional Animal Care and Use Committee under the protocol M/12/167.

2.2. Unpredictable chronic mild stress (UCMS)

UCMS mice were individually housed and subjected to a randomized stress protocol modified from the procedure described by Nollet and colleagues (Nollet et al., 2013; Strekalova et al., 2004). Stressors were applied *once* daily at randomized times for 7 weeks. The following stressors were utilized: 1. changing of bedding: mice were placed permanently onto a novel cage containing clean bedding; 2. Exposure to another male's cage: mice were placed permanently onto a cage which used to house another male mouse, thus containing soiled bedding and a formed nest; 3. cage shaking: cages were shaken three times for 30 s (total 1:30 min); 4. Swim stress: mice were placed in a clear cylinder with 23 °C water approximately 20 cm deep for 5 min; 5. Nestlet destruction: after measuring nestlet shredding, the nestlet of the stressed mice were destroyed and spread throughout the cage. Mice were not exposed to stressors for 12 h before testing, and behavioral testing was conducted during the seventh week of chronic stress.

2.3. Behavioral tests

Animals were tested during the light phase and acclimated to testing room conditions for 30 min. All apparatuses were cleaned with chlorine dioxide disinfectant (Vimoba, Quip Labs, Wilmington, DE) prior to the first testing session and between sessions. Mice were tested in a randomized order for each test. Test order was designed to minimize carryover anxiety on subsequent assays. Individual tests were conducted with a minimum of 24 h between each test.

2.3.1. Nestlet shredding

Nestlet shredding behavior was analyzed weekly in stressed animals and during the first and last weeks of the experiment in non-stressed animals as previously described (Deacon, 2006). Nestlet shredding marked the beginning of the stress paradigm. All mice were separated from their littermates and placed in a clean cage. A pre-weighed cotton nestlet (approximately 5 cm × 5 cm x .3 cm, 2.5 g, Ancare, Ancare Bellmore, NY, USA) was placed in the middle of each cage approximately one hour prior to the beginning of the dark phase. The following morning, all unshredded material 0.1 g or heavier was weighed and recorded. During the seventh week of stressor application, nestlet shredding behavior was also assessed in the non-stressed group by placing a nestlet in the home cage. Data is shown as percentage of initial weight shredded for the first day after isolation of mice and the last day of stress (weeks 1 and 7, respectively).

2.3.2. Open field test (OFT)

The OFT was used to examine locomotor activity and anxiety-related behavior. The apparatus, purchased from Med Associates (Med Associates Inc., St. Albans, Vermont, USA), consisted of a square box 27.3 cm × 27.3 cm. The apparatus was placed in a sound-attenuating chamber purchased from Med Associates. Horizontal and vertical arrays of 16 infrared beams tracked horizontal and vertical movements. The arena was brightly lit throughout the test. Animals were placed in the center of the arena and allowed to explore the chamber for 10 min. Med Associates Open Field Activity software was used to track and analyze animals' movements. Stereotypy counts were defined as the number of beam breaks that occur during a period of stereotypic activity. If the animal breaks the same beam (or set of beams) repeatedly then the software considers that the animal is exhibiting stereotypy. Thigmotaxis was analyzed by defining a center zone consisting of the area more than five centimeters from the walls.

2.4. Neurochemistry

Within one week of behavioral testing, mice were euthanized by decapitation. Tissue samples were dissected from the cerebral cortex and midbrain. Midbrain was dissected by peeling off the cortex and cerebellum to expose the third ventricle and the acqueduct and making two coronal sections at the beginning of the superior colliculus at Bregma −3.52 and another at the end of the inferior colliculus at Bregman −5.20. One hemisphere, randomly assigned per mouse, was dissected and immediately frozen in dry ice, and the other hemisphere was dissected and stored in 0.32 M sucrose for preparation of synaptoneurosomes. Samples were analyzed for serotonin (5-HT), 5-hydroxyindoleacetic acid (5-HIAA), dopamine, homovanillic acid (HVA), norepinephrine, and 3,4-dihydroxyphenylacetic acid (DOPAC). Monoamine levels were determined by high-pressure liquid chromatography (HPLC) using an Antec Decade II electrochemical detector (oxidation, 0.5) operated at 33 °C in the Vanderbilt Brain Institute Neurochemistry Core. Supernatant samples (2-μl) from trichloroacetic acid tissue extracts

were injected via a Water 717 + autosampler onto a Phenomenex Nucleosil C^{18}HPLC column (5u, 100 A; 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. Results are expressed as ng/mg protein or as a percent of *Itgb3*$^{+/+}$ control levels. Frozen protein pellets were saved for biochemical analysis.

2.5. Biochemistry

Synaptoneurosomes were prepared from midbrains dissected onto 0.32 M sucrose in HEPES containing 0.1 mM $CaCl_2$ and 1.0 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 were separated by centrifugation at 1000 × g. Supernatants were collected and spun at 10,000 × g for isolation of crude synaptoneurosomes. Frozen trichloroacetic acid pellets from midbrain were resuspended in 1% sodium dodecyl sulfate in phosphate buffered saline pH 7.4. Immediately after preparation, total protein in synaptoneurosomal and frozen tissue pellets were measured using a modified Lowry protocol with bicinchoninic acid (BCA Protein Assay Kit, Pierce Chemical Company, Rockford, IL). Approximately 50 μg was used immediately for Western blot studies, as previously described (Phillips et al., 2001). Briefly, equivalent amounts of protein in lithium dodecyl sulfate (LDS) sample buffer were loaded into 4–20% Tris-HEPES gels (Thermo Scientific, Waltham, MA, USA) and transferred to methanol-activated polyvinylidene fluoride membrane. Membranes were blocked in 5% nonfat dry milk/tris-buffered saline (TBS), pH 7.4 and incubated in primary antibodies (1.0 μg/mL) at 4 °C overnight (Supplemental Table 1). Following incubation with secondary horseradish peroxidase-coupled antibodies, electrochemiluminescence was used to detect immunocomplexes (Western Blotting Prime, GE Healthcare). Films were scanned and bands quantified using ImageJ. Expression values were normalized to Na^+/K^+ ATPase expression as a protein loading control.

2.6. Statistical analyses

Data were analyzed using Prism 6 (for Mac OS X, GraphPad Software). Two-way ANOVA with stress and genotype as factors with Bonferroni-corrected post-tests were used for multiple comparisons (alpha = 0.0125). Pearson correlation analyses with linear regression models were used to correlate integrin αv expression and other measures. Correlation matrices for all measures can be found in the Supplemental Files. Data are presented as mean ± SEM.

3. Results

3.1. Loss of integrin αvβ3 expression influences the neurochemical and behavioral responses to chronic stress

To examine the role of integrin αvβ3 in the neurochemical and behavioral responses to chronic stress, we utilized a modified version of unpredictable chronic mild stress procedure (UCMS) (Nollet et al., 2013). After exposure to the UCMS, *Itgb3*$^{+/+}$ mice exhibited increases in nestlet shredding, while *Itgb3*$^{-/-}$ mice exhibited no stress-dependent responses in this behavior (Fig. 1a. 2-way ANOVA Gene x time effect: $F_{(3,78)} = 3.175$, P = 0.028. Post-tests with Bonferroni's corrections: *Itgb3*$^{+/+}$ Stress: Week 1 vs. Week 7 P = 0.0001). We then exposed mice to the open field to determine genotype- and stress-induced alterations in locomotor activity and anxiety behaviors. UCMS induced a significant reduction in ambulatory distance only in *Itgb3*$^{-/-}$ mice (Fig. 1b. Stress: $F_{(1,68)} = 7.80$; P = 0.007. *Itgb3*$^{-/-}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.015). We also observed reductions in stereotypy in response to UCMS only in *Itgb3*$^{-/-}$ mice, in both number of stereotypic counts (Fig. 1c. Interaction: $F_{(1,68)} = 4.911$; p = 0.030. *Itgb3*$^{-/-}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.001), or time spent engaging in stereotypic behaviors (Fig. 1d. Stress: $F_{(1,68)} = 2.88$; P = 0.004. *Itgb3*$^{-/-}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.008). Thigmotaxis analysis revealed a significant gene × stress interaction on the time spent in the center of the open field chamber (Fig. 1e. Interaction: $F_{(1,67)} = 4.900$; P = 0.030) and in the number of entries in the center of the open field (Fig. 1f. Stress effect: $F_{(1,68)} = 5.15$; P = 0.026. *Itgb3*$^{-/-}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.032).

Neurochemical analysis of brain tissue samples harvested from the cerebral cortex and midbrain was conducted to investigate whether perturbed monoamine homeostasis is differentially associated with chronic stress in *Itgb3*$^{-/-}$ mice (Table 1). Midbrain monoamines 5-HT and DA, and the 5-HT metabolite 5-HIAA, were significantly elevated in control *Itgb3*$^{-/-}$ mice, when compared to *Itgb3*$^{+/+}$. We observed a gene × stress interaction in midbrain DA turnover ratio, as calculated by the ratio of DOPAC to DA (Interaction: $F_{(1,66)} = 5.99$; P = 0.017. *Itgb3*$^{+/+}{}_{\text{Control}}$ vs. *Itgb3*$^{+/+}{}_{\text{UCMS}}$, P = 0.019, *Itgb3*$^{+/+}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{Control}}$, P = 0.012.). Mice lacking integrin αvβ3 expression have reduced DOPAC/DA ratios, levels comparable to those observed in UCMS-exposed *Itgb3*$^{+/+}$ mice (*Itgb3*$^{+/+}{}_{\text{Control}}$ = 0.535 ± 0.025, *Itgb3*$^{-/-}{}_{\text{Control}}$ = 0.433 ± 0.018, *Itgb3*$^{+/+}{}_{\text{UCMS}}$ = 0.451 ± 0.026, *Itgb3*$^{-/-}{}_{\text{UCMS}}$ = 0.475 ± 0.017). Taken together, these data reveal that loss of integrin αvβ3 expression results in differential responses to unpredictable chronic mild stressors in anxiety behaviors in the open field, as well as changes in and DA metabolism in the midbrain.

3.2. Chronic stress and Itgb3$^{-/-}$ differentially influence protein expression at the synapse

We used Western blotting of whole-tissue and synaptoneurosomal preparations from midbrains to identify potential molecular determinants of stress-induced phenotypes influenced by integrin αvβ3 (Fig. 2a). We focused on two major protein groups: proteins involved in canonical integrin signaling (talin, FAK, Src, PP2A, and ERK) and synaptic proteins involved in synapse formation and plasticity (synaptophysin, syntaxin, PSD-95, GluR2, and the NR1 subunit of the NMDA receptor). No significant changes in total tissue protein levels were observed. We then isolated midbrain synaptoneurosomes to identify changes in trafficking and/or synaptic translation events. Synaptic expression of integrin αv, FAK, Src, ERK, PP2A, GluR2, and NMDAR were unaltered by chronic stress or *Itgb3* genotype. We observed no changes in post-synaptic PSD-95 levels (Fig. 2b), but observed significant gene × stress interactions in the synaptic levels of syntaxin (Fig. 2c. Interaction: $F_{(1,25)} = 10.43$; P = 0.003. *Itgb3*$^{-/-}{}_{\text{Control}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.022. *Itgb3*$^{+/+}{}_{\text{UCMS}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.029) and synaptophysin (Fig. 2d. Interaction: $F_{(1,11)} = 15.47$; P = 0.002. *Itgb3*$^{+/+}{}_{\text{UCMS}}$ vs. *Itgb3*$^{-/-}{}_{\text{UCMS}}$, P = 0.009). Therefore, loss of *Itgb3* expression also confers significant reductions in presynaptic protein expression in the context of chronic unpredictable stress.

3.3. Synaptic midbrain integrin αv levels are correlated with expression of midbrain synaptic structural and signaling proteins

As integrin αvβ3 is one of the many integrin receptors expressed in neurons, other receptors may compensate for the loss of integrin β3 expression and modify synapse function. One example is the integrin αvβ1 receptor, also found to modulate gluatamatergic signaling in the hippocampus (Babayan et al.,

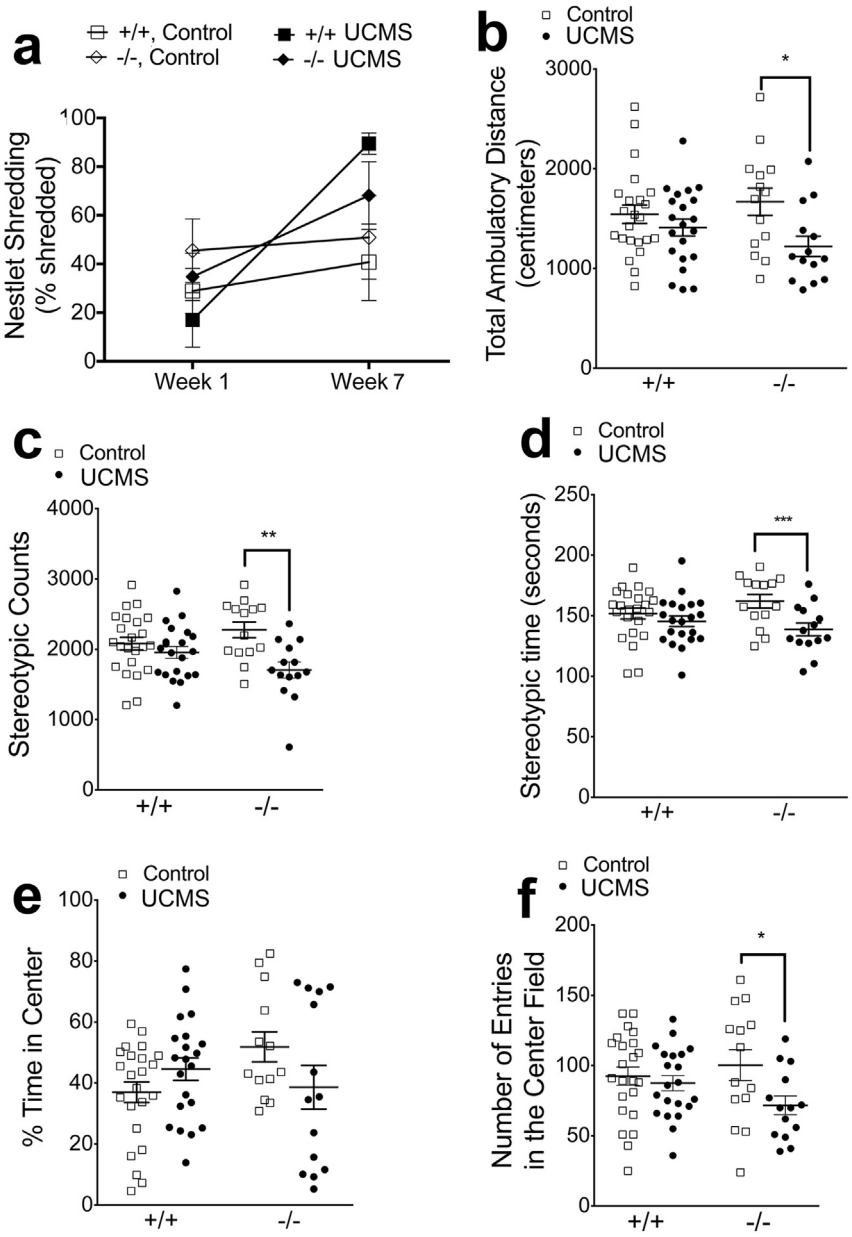

Fig. 1. Exposure to unpredictable chronic mild stress reveals altered behavioral responses in *Itgb3*$^{-/-}$ mice. a, Shredding of a cotton nestlet was measured in mice exposed to UCMS and controls. The amount shredded is shown as percentage of total nestlet weight. Number of animals: Week 1: *Itgb3*$^{+/+}{}_{\text{Control}}$ N = 15; *Itgb3*$^{+/+}{}_{\text{UCMS}}$ N = 12; *Itgb3*$^{-/-}{}_{\text{Control}}$ N = 11; *Itgb3*$^{-/-}{}_{\text{UCMS}}$ N = 13. Week 8: *Itgb3*$^{+/+}{}_{\text{Control}}$ N = 10; *Itgb3*$^{+/+}{}_{\text{UCMS}}$ N = 10; *Itgb3*$^{-/-}{}_{\text{Control}}$ N = 7; *Itgb3*$^{-/-}{}_{\text{UCMS}}$ N = 8. b–f, Mice were exposed to the open field without habituation and activity measured for the first 10 min b, Open field total ambulatory distance. c–d, Stereotypic counts (c) and time spent on grooming and other stereotypic behaviors (d) were altered by UCMS. e–f, Thigmotaxis analysis of % of time spent in the center of the open field (e) and number of times the mouse explored the center of the filed (f). b–f Number of animals: *Itgb3*$^{+/+}{}_{\text{Control}}$ N = 22; *Itgb3*$^{+/+}{}_{\text{UCMS}}$ N = 20; *Itgb3*$^{-/-}{}_{\text{Control}}$ N = 13; *Itgb3*$^{-/-}{}_{\text{UCMS}}$ N = 12. *P < 0.05, **P < 0.01, and ***P < 0.001 for control vs. UCMS post-tests, and $^{\#}$P < 0.05 for genotype comparisons within treatment group. All post-tests P values are Bonferonni corrected.

2012). To identify phenotypes modified by integrin αv expression, independently of the β3 subunit, we performed correlations between behavioral, neurochemical, and biochemical phenotypes and synaptic integrin αv expression (Table 2). Tissue αv levels were correlated with FAK and GluR2 expression in both genotype groups. Positive correlations were observed between tissue αv and PSD-95, syntaxin and synaptophysin in *Itgb3*$^{+/+}$, but not *Itgb3*$^{-/-}$ samples. Synaptic αv was correlated with synaptic Src, ERK, syntaxin and GluR2, in both genotypes, suggesting a coordinated targeting of these proteins to synapses by yet unknown mechanisms. Synaptic FAK, PSD-95, and NMDAR were correlated with synaptic αv in *Itgb3*$^{+/+}$ samples only, suggesting a coordinated targeting of these proteins and the αvβ3 receptor. Two neurochemical and behavioral phenotypes were correlated with tissue αv expression in *Itgb3*$^{-/-}$ samples. Of those, total ambulatory distance in the OFT was also specifically altered by UCMS in *Itgb3*$^{-/-}$ mice. Vertical time in the OFT was the only phenotype significantly correlated with both αv and β1 subunits in *Itgb3*$^{-/-}$ samples, indicating a potential role for this receptor in vertical activity (correlation with synaptic β1: Pearson r = −0.905, P = 0.002). The strong correlations with both pre- and post-synaptic proteins may indicate a role of integrin αv in synapse formation in the midbrain, which could influence the neurochemical and behavior phenotypes.

Table 1
HPLC analysis of tissue levels of monoamines in mice exposed to UCMS.

	Itgb3$^{+/+}$		*Itgb3*$^{-/-}$		Two-way ANOVA
	Control n = 23	Stress n = 21	Control n = 13	Stress n = 13	
	ng/mg protein SEM	ng/mg protein SEM	ng/mg protein SEM	ng/mg protein SEM	
Midbrain					
5-HT	14.382 ± 0.425	14.982 ± 0.681	16.762 ± 0.671*	15.637 ± 0.432	Genotype: $F_{(1,66)} = 6.440$, P = 0.0135
5-HIAA	5.883 ± 0.276	5.635 ± 0.471	7.132 ± 0.605	6.932 ± 0.728	Genotype: $F_{(1,66)} = 6.430$, P = 0.0136
Dopamine	1.473 ± 0.097	1.599 ± 0.109	1.883 ± 0.096*	1.738 ± 0.094	Genotype: $F_{(1,65)} = 6.388$, P = 0.0139
DOPAC	0.763 ± 0.048	0.792 ± 0.077	0.815 ± 0.048	0.812 ± 0.026	
HVA	1.213 ± 0.057	1.339 ± 0.145	1.422 ± 0.072	1.354 ± 0.058	
Norepinephrine	8.767 ± 0.205	8.697 ± 0.423	9.326 ± 0.327	8.992 ± 0.376	
Cerebral Cortex					
5-HT	9.113 ± 0.619	9.562 ± 0.519	9.929 ± 0.593	9.791 ± 0.654	
5-HIAA	2.441 ± 0.203	2.684 ± 0.211	3.068 ± 0.211	2.936 ± 0.134	Genotype: $F_{(1,66)} = 4.384$, P = 0.0401
Dopamine	2.110 ± 0.686	2.675 ± 1.093	4.653 ± 2.635	2.430 ± 1.209	
DOPAC	0.443 ± 0.070	0.485 ± 0.119	0.513 ± 0.189	0.506 ± 0.191	
HVA	0.936 ± 0.112	0.907 ± 0.172	1.115 ± 0.320	1.028 ± 0.240	
Norepinephrine	5.548 ± 0.164	5.809 ± 0.256	5.641 ± 0.194	5.613 ± 0.213	

Genotype comparisons: Bonferroni-corrected post-tests with a *P < 0.05.

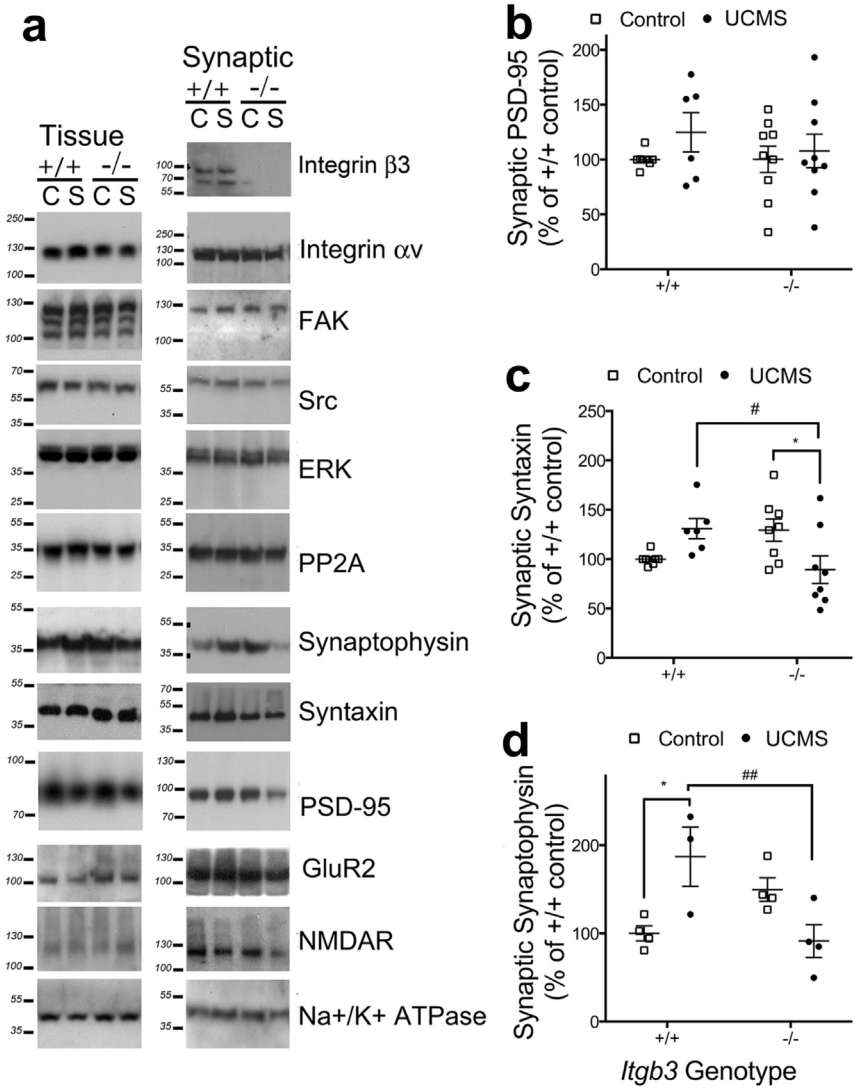

Fig. 2. Western blot analysis of synaptic expression of several signaling, structural, and glutamate receptor subunits in the midbrain. Expression levels for each protein are normalized to both Na^+/K^+ ATPase and *Itgb3*$^{+/+}{}_{Control}$. a, Representative Western blots from whole tissue (left column) and synaptosome fractions (right column). b, Synaptic PSD-95 expression is not modified by chronic stress in *Itgb3*$^{-/-}$ mice. *Itgb3*$^{+/+}{}_{Control}$ N = 9, *Itgb3*$^{+/+}{}_{UCMS}$ N = 6, *Itgb3*$^{-/-}{}_{Control}$ N = 9, *Itgb3*$^{-/-}{}_{CS}$ N = 9. c, Synaptic syntaxin expression. *Itgb3*$^{+/+}{}_{Control}$ N = 7, *Itgb3*$^{+/+}{}_{UCMS}$ N = 6, *Itgb3*$^{-/-}{}_{Control}$ N = 8, *Itgb3*$^{-/-}{}_{UCMS}$ N = 8. c, Synaptic synaptophysin expression. *Itgb3*$^{+/+}{}_{Control}$ N = 4, *Itgb3*$^{+/+}{}_{UCMS}$ N = 3, *Itgb3*$^{-/-}{}_{Control}$ N = 4, *Itgb3*$^{-/-}{}_{UCMS}$ N = 4). *P < 0.05 for control vs. UCMS post-tests, and $^{\#}$P < 0.05 for genotype comparisons within treatment group. All post-tests P values are Bonferonni corrected.

Table 2
Correlation analyses between tissue and synaptic integrin αv expression levels and biochemical, neurochemical and behavioral phenotypes.

	Total Tissue Integrin av				*Synaptic Integrin av*			
	Itgb3$^{+/+}$		*Itgb3*$^{-/-}$		*Itgb3*$^{+/+}$		*Itgb3*$^{-/-}$	
	Pearson r	P Value	Pearson r	P Value	Pearson r	P Value	Pearson r	P Value
Protein Levels								
Whole Tissue								
Talin	0.037	0.873	−0.039	0.854	−0.316	0.489	0.341	0.409
FAK	***0.718***	***2.47E-04***	***0.435***	***0.030***	−0.503	0.250	0.428	0.290
SRC	0.359	0.101	***0.439***	***0.028***	−0.014	0.976	0.637	0.089
ERK	0.217	0.331	0.268	0.196	−0.495	0.259	0.303	0.466
PP2A	0.136	0.657	0.351	0.199				
PSD95	***0.581***	***0.005***	0.203	0.331	−0.474	0.282	0.525	0.181
Synaptophysin	***0.658***	***0.003***	0.303	0.170	0.246	0.691	0.663	0.151
Syntaxin	***0.714***	***0.031***	0.145	0.689	−0.370	0.415	0.680	0.064
mGluR1	0.630	0.069	0.239	0.506	−0.341	0.454	0.672	0.068
GluR2 (AMPAR)	***0.776***	***0.024***	***0.685***	***0.042***	0.212	0.648	0.600	0.116
NMDAR	0.493	0.123	0.029	0.924				
Synaptoneurosomes								
Talin	−0.345	0.503	−0.429	0.396	0.531	0.279	−0.227	0.665
FAK	−0.277	0.548	−0.207	0.624	***0.924***	***0.003***	0.699	0.054
SRC	−0.088	0.851	0.246	0.594	***0.811***	***0.027***	***0.918***	***0.004***
ERK	−0.376	0.406	0.333	0.420	***0.956***	***0.001***	***0.910***	***0.002***
PP2A	−0.352	0.561	***0.913***	***0.004***	0.563	0.323	0.570	0.181
PSD95	−0.627	0.132	0.122	0.774	***0.871***	***0.011***	0.629	0.095
Synaptophysin	−0.436	0.328	0.330	0.425	0.376	0.406	0.470	0.240
Syntaxin	−0.182	0.696	0.360	0.381	***0.865***	***0.012***	***0.928***	***0.001***
mGluR1	−0.345	0.449	−0.084	0.843	0.684	0.090	0.330	0.424
GluR2 (AMPAR)	−0.051	0.924	0.120	0.798	***0.948***	***0.004***	***0.871***	***0.011***
NMDAR	−0.559	0.192	0.148	0.726	***0.913***	***0.004***	0.599	0.117
Neurochemistry − HPLC								
Midbrain								
Noradrenaline	0.035	0.876	0.241	0.246	−0.156	0.739	0.235	0.575
DOPAC	0.142	0.527	***−0.637***	***0.001***	0.332	0.468	−0.690	0.058
Dopamine	0.119	0.599	***−0.469***	***0.018***	0.238	0.607	−0.371	0.366
5-HIAA	0.029	0.897	−0.019	0.930	0.123	0.792	−0.454	0.258
HVA	0.114	0.615	***−0.407***	***0.044***	0.156	0.738	***−0.770***	***0.025***
5-HT	−0.107	0.634	−0.065	0.756	−0.009	0.985	−0.353	0.390
5-HIAA/5HT	0.097	0.667	−0.012	0.954	0.289	0.529	−0.153	0.718
DOPAC/Dopamine	−0.128	0.569	−0.247	0.233	−0.065	0.890	0.163	0.700
HVA/Dopamine	−0.169	0.453	0.071	0.736	−0.290	0.528	−0.033	0.939
Cortex								
Noradrenaline	0.086	0.704	0.366	0.072	−0.002	0.997	0.334	0.419
DOPAC	0.275	0.215	−0.092	0.662	−0.387	0.391	−0.156	0.712
Dopamine	0.209	0.350	0.031	0.883	−0.352	0.439	0.149	0.725
5-HIAA	−0.008	0.972	−0.182	0.383	−0.199	0.669	−0.244	0.560
HVA	0.197	0.380	−0.003	0.988	−0.304	0.507	0.098	0.817
5-HT	−0.122	0.588	−0.087	0.680	−0.064	0.891	0.129	0.762
5HIAA/5HT	0.273	0.220	−0.165	0.431	−0.621	0.137	−0.555	0.154
DOPAC/Dopamine	0.030	0.895	***0.753***	***1.42E-05***	0.526	0.225	−0.316	0.445
HVA/Dopamine	0.058	0.799	***0.727***	***3.90E-05***	0.551	0.200	−0.124	0.770
Open Field								
Total Ambulatory Time	−0.071	0.755	***0.439***	***0.028***	−0.583	0.170	0.253	0.546
Total Ambulatory Distance	−0.018	0.936	***0.435***	***0.030***	−0.723	0.067	0.288	0.489
Total Vertical Time	−0.047	0.835	0.088	0.676	−0.032	0.946	***−0.790***	***0.020***
Total Stereotypic Time	0.098	0.665	0.268	0.195	−0.203	0.663	0.291	0.485
% Center Time	0.111	0.623	0.049	0.817	−0.371	0.413	0.297	0.475

4. Discussion

The present study provides evidence of the influence of integrin αvβ3 on vulnerability to stress. In the context of unpredictable chronic mild stress, we observed significant genotype × stress interactions where *Itgb3*$^{-/-}$ mice presented increased anxiety and reduced stereotypy in the open field. We also identified significant reductions in the synaptic expression of syntaxin and synaptophysin in *Itgb3*$^{-/-}$ mice exposed to UCMS, suggesting a role for integrin αvβ3 in the molecular and behavioral responses to chronic unpredictable stressors. To our knowledge, these findings are the first to assess behavioral and physiological responses to adverse environmental events in mice lacking integrin β3 expression.

We utilized a modified chronic stress procedure in order to identify exaggerated responses to chronic stressors in mice lacking integrin β3 expression. Integrin β3 influences synaptic plasticity via AMPA and NMDA receptor trafficking mechanisms (Bahr, 2000; Bernard-Trifilo et al., 2005; Cingolani et al., 2008; Juhasz et al., 2008; Lin et al., 2003; Pozo et al., 2012), which are involved in the functional and structural alterations observed after exposure to chronic stress (Christian et al., 2011; Kallarackal et al., 2013). Integrin β3 also influences midbrain high-affinity 5-HT reuptake via the serotonin transporter (SERT) (Mazalouskas et al., 2015; Whyte et al., 2014), and mice lacking SERTs display enhanced sensitivity

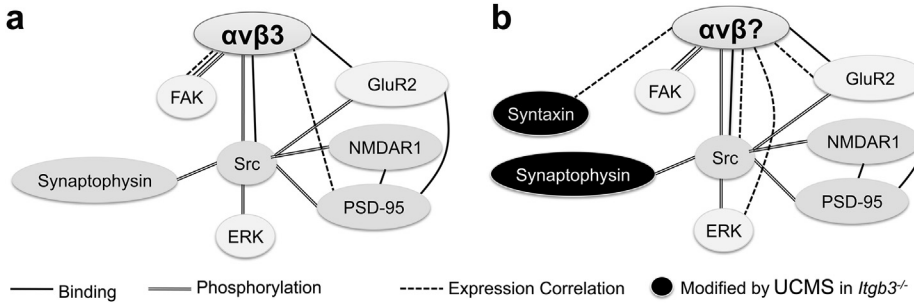

Fig. 3. Network diagram of proteins modulated by integrin αvβ3 expression levels during chronic stress response. We examined proteins that are either known to modulate stress vulnerability or response to antidepressants, or proteins that are regulated by integrin αvβ3 signaling. Of the 12 proteins examined, only 2 proteins have stress-induced reductions in expression in the context of *Itgb3* loss-of-function (shown in black:syntaxin and synaptophysin), and 6 proteins have their synaptic expression correlated with midbrain synaptic αv (syntaxin, FAK, Src, ERK, PSD-95, NMDAR and GluR2). Expression correlations (indicated by a dashed line) are shown in wild-type mice (a), and in the context of integrin β3 loss of expression (b). Several of those proteins either form physical complexes or are phosphorylated by either FAK or Src, indicated by solid or double lines, respectively.

to repeated stress, which are associated with altered dendritic morphology of pyramidal neurons in the prefrontal cortex (Wellman et al., 2007). Our neurochemical studies revealed increased midbrain levels of 5-HT and its metabolite, 5-HIAA, in mice lacking β3 expression, which were not influenced by UCMS. Instead, midbrain DA turnover, as defined by DOPAC/DA ratios, was significantly reduced by UCMS exposure in $Itgb3^{+/+}$, but not $Itgb3^{-/-}$ mice. In fact, these neurochemical alterations paralleled changes in behavior, as we observed positive correlations between midbrain DA turnover and total ambulatory distance (Pearson's $r = 0.504$, $P = 0.009$) and stereotypic time (Pearson's $r = 0.513$, $P = 0.015$) in $Itgb3^{+/+}$ mice (Supplemental Tables). Alterations of DA systems in response to chronic stress have been established, although only recently the ventral tegmental area (VTA) of the midbrain, where dopaminergic cell bodies are located, have been studied (Friedman et al., 2014). Importantly, selective modulation of VTA neurons revealed DA circuits that promote resiliency (VTA to the nucleus accumbens) and susceptibility (VTA to medial prefrontal cortex) to social stress (Chaudhury et al., 2013). It is tempting to speculate that modulation of DA metabolism may be an important facet in the adaptive response to UCMS that is somehow impaired in $Itgb3^{-/-}$ mice.

$Itgb3^{-/-}$ mice display behavioral phenotypes indicative of an exaggerated response to persistent environmental stressors that are not recapitulated in $Itgb3^{+/+}$ mice. This pattern was observed across several behavioral modalities assayed in the OFT, including locomotor activity, stereotypy, and thigmotaxis. Our results confirm the previously observed lack of basal differences in locomotor activity in $Itgb3^{-/-}$ mice relative to wild-type controls (Carter et al., 2011; McGeachie et al., 2012). While chronic stress-driven alterations in thigmotaxis seen in $Itgb3^{-/-}$ mice cannot be definitively extricated from potentially confounding parallel changes in locomotor activity, the lack of basal genotype effects suggests that these changes result from altered adaptation to chronic stressors in $Itgb3^{-/-}$ mice rather than a generalized reduction in activity levels, per se. However, no stress-induced changes were observed in the elevated zero maze (data not shown), suggesting that the OF changes do not result from a generalized sensitivity to anxiogenic environments.

Biochemical studies revealed correlation between midbrain synaptic expression levels of αv with signaling kinases, especially in the context of $Itgb3^{-/-}$. The αv-containing receptors may participate in a molecular network modulating pre- and post-synaptic plasticity during the adaptive response to chronic stress (Fig. 3). In the absence of integrin β3 expression, αv correlates with synaptic expression of multiple proteins that modulate synaptic plasticity (GluR2, NMDAR and PSD-95), perhaps via compensatory expression of other integrin subunits. Of those, only GluR2 levels are correlated with synaptic integrin β1 levels (Pearson's $r = 0.929$, $P = 0.003$), indicating that other β subunits may also participate in this network. A possible interpretation of correlation results is that *Itgb3* modifies a common factor influencing all proteins within this network. We also observed significant decreases in midbrain synaptic expression of syntaxin in $Itgb3^{-/-}$ mice, effects that were also correlated with total vertical activity time (Pearson's $r = -0.923$, $P = 0.001$), and synaptic levels of integrin β1 (Pearson's $r = 0.871$, $P = 0.005$). Thus, loss of integrin β3 expression may allow for the coordinated targeting of integrin αvβ1 and syantaxin to synapses. However, synaptoneurosomal preparation precludes the identification of specific neuronal subtypes in which these changes may be occurring, and our findings may arise from small changes in multiple systems, or in large alterations in specific neurotransmitter pathways. Future studies with conditional mutant lines should reveal the pathways that are directly influenced by *Itgb3*. Taken together, our data indicates that loss of integrin β3 expression significantly alters the coupling of integrins to monoamine metabolism and trafficking of presynaptic proteins to synapses, thus influencing the response to environmental stimuli.

In conclusion, our results provide the first description of an interaction between *Itgb3* and stress exposure, as well as identification of potential monoaminergic and synaptic mechanisms by which this interaction may exert its effects. The evidence presented here suggests that the αvβ3 integrin receptor may exist as a central member of pre- and post-synaptic midbrain protein networks that influence the behavioral and neurochemical responses to chronic stressors.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ynstr.2015.05.002.

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