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Absence of Preference for Social Novelty and Increased Grooming in Integrin $\beta 3$ Knockout Mice: Initial Studies and Future Directions

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Scientific Abstract

Elevated whole blood serotonin (5HT), or hyperserotonemia, is a common biomarker in autism spectrum disorder (ASD). The integrin $\beta 3$ receptor subunit gene (*ITGB3*) is a quantitative trait locus for whole blood 5-HT levels. Recent work shows that integrin $\beta 3$ interacts with the serotonin transporter (SERT) in both platelets and in the midbrain. Furthermore, multiple studies have now reported gene-gene interaction between the integrin $\beta 3$ and SERT genes in association with ASD. Given the lack of previous data on the impact of integrin $\beta 3$ on brain or behavioral phenotypes, we sought to compare mice with decreased or absent expression of the integrin $\beta 3$ receptor subunit (*Itgb3* +/- and -/-) with wildtype littermate controls in behavioral tasks relevant to ASD. These mice did not show deficits in activity level in the Open Field or anxiety-like behavior on the Elevated Plus Maze, two potential confounds in the evaluation of mouse social behavior. In the three-chamber social test, mice lacking integrin $\beta 3$ were shown to have normal sociability but did not show a preference for social novelty. Importantly, the absence of integrin $\beta 3$ did not impair olfaction or the ability to recall familiar social odors. Additionally, mice lacking integrin $\beta 3$ showed increased grooming behavior in novel environments. These preliminary studies reveal altered social and repetitive behavior in these mice, which suggests that the integrin $\beta 3$ subunit may be involved in brain systems relevant to ASD. Further work is needed to fully characterize these behavioral changes and the underlying brain mechanisms.

Keywords

Autism; Genetic; Integrin; Cell Adhesion; Serotonin; Social Memory; Grooming; Obsessive Compulsive Disorder

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Introduction

Autism spectrum disorder (ASD) is characterized by deficits in communication and social behaviors coupled with the presence of restricted and repetitive behaviors (American Psychiatric Association, 2000). As a behaviorally defined syndrome without clear boundaries, ASD presents a diagnostic challenge to both clinicians and researchers (Constantino & Todd, 2003; Risi, et al., 2006). Nevertheless, twin studies suggest a strong genetic component to autism susceptibility (Bailey, et al., 1995; Folstein & Rutter, 1977). Linkage and association studies have provided some evidence of common susceptibility alleles in ASD, pointing primarily to genes encoding synaptic proteins, but this evidence has been somewhat inconsistent across studies (Buxbaum, 2009; Geschwind, 2009; O'Roak & State, 2008). This lack of consistency is not surprising given the striking heterogeneity of cognitive and behavioral profiles in autism (Munson, et al., 2008; Richler, et al.). Multiple uncommon copy number and *de novo* variants have also been identified in ASD (Berkel, et al., 2010; Glessner, et al., 2009; Jamain, et al., 2003; Kim, et al., 2008; Laumonier, et al., 2004; Marshall, et al., 2008; Pinto, et al., 2010; Weiss, et al., 2008), including some that disrupt synaptic adhesion molecules and scaffolding proteins in individuals.

In the presence of abundant clinical heterogeneity, heritable biomarkers may allow the identification of susceptibility genes specific to a subgroup of individuals with ASD. One such biomarker is elevated whole blood serotonin (5-HT), or hyperserotonemia, which occurs in approximately 30% of individuals with ASD (Cook & Leventhal, 1996; Mulder, et al., 2004; Schain & Freedman, 1961). In the blood, 5-HT is contained almost exclusively in platelets, which take up 5-HT as they pass through the enteric circulation (Anderson, et al., 1987). Elevated whole blood 5-HT levels are also seen in first-degree relatives of probands with ASD (Abramson, et al., 1989; Cross, et al., 2008; Cuccaro, et al., 1993; Leboyer, et al., 1999; Leventhal, et al., 1990). In the Hutterites, a large founder population, whole blood 5-HT levels approach a heritability of 1.0 (Abney, et al., 2001). The integrin $\beta 3$ gene (*ITGB3*) and the vitamin D receptor gene (*VDR*) in an initial genome-wide association study to map whole blood 5-HT quantitative trait loci in the Hutterites (Weiss, et al., 2004). Given that males are much more likely to be affected with ASD (Chakrabarti & Fombonne, 2001), a follow-up study searched for sex-specific loci, finding a stronger association with *ITGB3* in males and also finding male-specific association with *SLC6A4*, which encodes the serotonin transporter (SERT) (Weiss, et al., 2005).

Subsequent studies have considered how integrin $\beta 3$ may influence whole blood 5-HT levels and autism susceptibility. Two studies confirmed association between polymorphisms in *ITGB3* and whole blood 5-HT levels (Coutinho, et al., 2007; Weiss, Kosova, et al., 2006). Carneiro and colleagues in the Blakely lab demonstrated an interaction between integrin $\beta 3$ and SERT in the platelet. Their proteomic screen for proteins that interact with the C terminus of SERT identified a physical and functional interaction with integrin $\beta 3$ that may explain the earlier genetic findings (Carneiro, et al., 2008). We have now also identified a similar protein-protein interaction in the midbrain (JV, MC, RB, AC, paper in preparation). Two groups have reported nominally significant association at different *ITGB3* single nucleotide polymorphisms (SNPs) in ASD (Ma, et al.; Weiss, Kosova, et al., 2006). Reflecting the interaction between integrin $\beta 3$ and SERT, however, the most relevant analysis is likely to consider gene-gene interactions. Four independent populations have now reported interaction between *ITGB3* and *SLC6A4* in association with autism, albeit with different combinations of polymorphisms (Coutinho, et al., 2007; Ma, et al.; Mei, et al., 2007; Weiss, Ober, et al., 2006).

In the context of multiple lines of data pointing to *ITGB3* in association with an autism biomarker and perhaps with autism susceptibility itself, we endeavored to understand the

role of the corresponding protein in the mouse, focusing on the social and repetitive behaviors that may parallel autistic traits. A knockout mouse that completely lacks expression of a given gene is the classic tool for dissecting the biological role of the corresponding protein. This presents a complication for understanding the role of integrin $\beta 3$ in social and repetitive behavior, however, because the corresponding protein is a component of the fibrinogen receptor, integrin $\alpha \text{IIb}\beta 3$. Complete disruption of the integrin $\beta 3$ gene in the mouse causes impaired platelet aggregation, prolonged bleeding times, and hemorrhages in skin, gastrointestinal tract, and placenta (Hodivala-Dilke, et al., 1999). Hemorrhage leads to a significant loss of homozygous animals in the first 3 weeks of life and throughout the lifespan (Hodivala-Dilke, et al., 1999). In addition to $\alpha \text{IIb}\beta 3$, the integrin $\beta 3$ knockout mouse also cannot form the $\alpha \text{v}\beta 3$ receptor, important in placental implantation, bone resorption, and angiogenesis. Due to osteoclast dysfunction, homozygous knockout mice develop osteosclerosis (McHugh, et al., 2000). None of the reported phenotypes from the integrin $\beta 3$ null (-/-) mouse are reported in the hemizygous (+/-) mouse that maintains 50% expression.

A variety of behavioral tasks have been proposed as relevant to autism (Blundell, et al.; Etherton, et al., 2009; Lijam, et al., 1997; Moretti, et al., 2005; Moy, Nadler, Poe, et al., 2008; Moy, et al., 2007; Nakatani, et al., 2009; Roullet, et al.; Silverman, et al.; Young, et al.; Zhao, et al.). Given the bleeding phenotype in the *Itgb3* -/- animals, we avoided tests of direct social interaction that could include aggression. The 3-chamber sociability and preference for social novelty test (Moy, et al., 2004) allows the evaluation of a test mouse's preferences for proximity to a stimulus mouse while restraining the stimulus mouse within a wire cage. Activity level, anxiety-like behavior, and olfactory memory are potential confounds to this test, which we evaluated in the Open Field, Elevated Plus Maze, and Olfactory Habituation / Dishabituation test, respectively. Over the course of this behavioral testing, we observed that the *Itgb3* -/- animals appeared to exhibit increased grooming behavior, which was evaluated using both a standard 10-minute observation of grooming behavior and an extended automated monitoring of a subset of animals in their home cage environment. These initial behavioral analyses are intended as a preliminary assessment of ASD-relevant behavior in mice lacking integrin $\beta 3$ and motivate further study of this protein using conditional gene knockout strategies to remove the potential confounds of peripheral phenotypes in these mice.

Methods

Mice

All animal procedures were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. All procedures were approved by the Vanderbilt University Institutional Animal Care and Use Committee. All mice used in experiments were male progeny of pairings of *Itgb3* +/- mice on a C57BL/6J background (backcrossed at least 7 generations from 129S2/SvPas according to the Jackson Laboratory, Bar Harbor, ME) that were housed with 3-5 mixed genotype littermate animals per cage. Mice used within each experiment were greater than 8 weeks of age and were matched by birth date to test animals varying by no more than 4 weeks.

Order of Behavioral Experiments

The first cohort was composed of all littermates born sequentially to *Itgb3* +/- breeding pairs and underwent behavioral testing in the following sequence: elevated plus maze, open field testing, tail suspension test (TST, results to be reported elsewhere), 3-chamber sociability and preference for social novelty test. No *Itgb3* -/- mice were run on the TST due to concerns about possible bleeding. Mice with signs of active or recent bleeding were

excluded from experiments. Mice with open or infected wounds, hunched posture, or difficulty ambulating were euthanized, as well as any mice that would have been singly housed after loss of their cagemates. Of the 8 *Itgb3*^{-/-} mice that began in this cohort, 6 had died spontaneously, were euthanized, or were excluded due to signs of bleeding at the time of the 3-chamber sociability and preference for social novelty test. Due to this loss of sample size, 4 additional ^{-/-} animals that were the product of the same +/- breeding pairs were also evaluated on the 3-chamber sociability and preference for social novelty test. A second cohort of animals underwent olfactory habituation / dishabituation testing. When grooming was noted to be increased over the course of this testing, a subset of these animals underwent formal testing of grooming behavior along with additional matched littermate animals from the same +/- breeding pairs to increase the sample size.

Elevated Plus Maze (EPM)

The plus maze consists of four arms, 10 × 10 × 30 cm, connected in a plus configuration and elevated approximately 50 cm. Two of the arms have walls, 20 cm high, and two arms have no walls. This test was performed under full light conditions in the last 6 hours of the light period. Mice were placed in the center of the maze at the beginning of the 5-minute test session. The position of the mouse was tracked three times per second, and data were analyzed in real time on a Macintosh computer using Image EP (Miyakawa, et al., 2001) (O'Hara & Co., Tokyo, Japan), a modification of the public domain NIH ImageJ software (Abramoff, et al., 2004). The ratio of time spent in the open arms divided by total time spent in either open or closed arms was analyzed by one-way ANOVA as the primary measure of anxiety-like behavior. The number of entries was analyzed by one-way ANOVA as a secondary measure of activity.

Open Field Behavior

Exploratory locomotor activity was evaluated using activity monitors measuring 27.9 × 27.9 cm (MED Associates, Georgia, VT). Each apparatus contains 16 photocells in each horizontal direction, as well as 16 photocells elevated 4.0 cm to measure rearing. This test was performed under full light conditions in the last 6 hours of the light period. Mice were not previously habituated to the locomotor activity chamber. Mice were placed in the monitors for 15 minutes and allowed to explore freely. Horizontal beam breaks were automatically recorded and represented as distance travelled. Total distance travelled was analyzed by Kruskal-Wallis test as the primary measure of activity. Time spent ambulating, time spent at rest, and time spent vertical rearing were measured as secondary dependent variables and compared by one-way ANOVA.

Three-chamber sociability and preference for social novelty test (Moy, et al., 2004)

Social behavior was evaluated in a 3-chamber polycarbonate apparatus with 4" sliding gates separating the 7" × 9" chambers. The test mouse was initially allowed to explore all 3 chambers for 10 minutes to acclimate to the apparatus. The test mouse was then returned to the center chamber with the gates closed. For the sociability portion of the test, a stimulus mouse (novel mouse) was introduced inside a wire pencil cup (Spectrum Diversified Designs, Inc., Streetsboro, OH) in one side chamber with an empty pencil cup (novel object) introduced in the opposite side chamber. The gates were then opened, and the test mouse was allowed to explore all 3 chambers for 10 minutes. The test mouse was then returned to the center chamber with the gates closed. For the preference for social novelty portion of the test, the empty pencil cup was replaced by a second stimulus mouse (novel mouse), with the original stimulus mouse now becoming a familiar social stimulus (familiar mouse). The gates were again opened, and the test mouse was allowed to explore all 3 chambers for 10 minutes. Sessions were coded for the time that the test mouse spent within 1 cm of each

stimulus, which was analyzed by two-way repeated measures ANOVA with Bonferroni post-tests for each portion of the test.

Olfactory Habituation / Dishabituation Test (Yang & Crawley, 2009)

Water, almond, and banana extract were used as non-social odors, with each extract diluted 1:100 in water. Two dirty cages housing unfamiliar novel male mice of the same strain were used to generate two novel social odors. Each test mouse habituated to a new cage for 60 minutes in the testing area. After habituation, each non-social odor was introduced to the cage by dipping the end of a cotton swab in the odor, and then suspending the cotton swab about 4 – 5 cm from the bottom of the cage. Social odors were acquired by swiping the bottom of one of the two dirty cages identified earlier. Each odor was administered in three trials of two minutes per trial with one minute between trials. The odors were presented in the following order: water, almond, banana, social scent 1, and social scent 2. After completion, the mouse was returned to its home cage. An observer with a stopwatch recorded the seconds each mouse spent within 2 cm of each odor stimulus, which was analyzed by two-way repeated measures ANOVA with Bonferroni post-tests for each portion of the test.

Self-grooming in a novel environment (McFarlane, et al., 2008)

Self-grooming behavior was scored by placing each mouse in an empty cage, without bedding. Each mouse was allowed to habituate to this new environment for 10 minutes. An observer with a stopwatch recorded time spent grooming over the next 10 minutes. Total grooming time over 10 minutes was analyzed by Kruskal-Wallis test with Dunn's posttest.

Home Cage Grooming (Steele, et al., 2007)

Individual mice of each genotype were video recorded in their home cage for 24 hours while maintaining their 12:12 light-dark schedule. Automated video analysis was conducted using HomeCageScan (Clever Sys, Inc., Reston, VA) to measure grooming behavior. Time spent grooming in 24 hours was analyzed by one-way ANOVA.

Results

The *Itgb3* knockout animals showed no difference in distance traveled, the primary dependent variable, during the 15 minutes spent in the Open Field (OF) (Fig 1A, Kruskal-Wallis test statistic 1.96, $P = 0.38$; non-parametric analysis chosen due to trend for unequal variances on Bartlett's test, $P = 0.06$, $n = 13$ +/+, 23 +/-, 8 -/-). Similarly, no difference was seen on the secondary variables in the OF, including ambulatory time (Fig 1B, one-way ANOVA, $F = 0.91$, $P = 0.41$), resting time (Fig 1C, one-way ANOVA, $F = 2.10$, $P = 0.14$), and vertical time (Fig 1E, Kruskal-Wallis 3.47, $P = 0.18$); although significantly decreased variability was seen in *Itgb3* -/- animals on vertical time (Bartlett's test for unequal variances $P = 0.02$).

In the Elevated Plus Maze (EPM), the *Itgb3* knockout animals showed no difference on the primary dependent variable, the ratio of time spent in the open arms of in contrast with the total time spent in either the open or closed arms (Fig 2A, one-way ANOVA, $F = 0.51$, $P = 0.60$, $n = 13$ +/+, 23 +/-, 8 -/-). Likewise, there was no difference in the number of entries to the open arms (Fig 2B, one-way ANOVA, $F = 0.49$, $P = 0.61$). Consistent with the activity data from the OF, there was no difference in total entries on the EPM (Fig 2C, one-way ANOVA, $F = 0.84$, $P = 0.44$).

On the 3-chamber test of sociability, all three genotypes showed significant sociability, or preference for the novel mouse over the novel object, with no effect of genotype (Fig 3A,

two-way repeated measures ANOVA, genotype by stimulus interaction $F = 0.01$, $P = 0.99$; stimulus main effect $F = 42.64$, $P < 0.0001$; genotype main effect $F = 0.73$, $P = 0.49$; Bonferroni posttests of stimulus effect in all three genotypes, $P < 0.05$, $n = 10$ +/+, 20 +/-, 6 -/-). On the second portion of the test, however, there was a significant difference between the genotypes on preference for social novelty (Fig 3B, two-way repeated measures ANOVA, genotype by stimulus interaction $F = 4.56$, $P = 0.018$), with Bonferroni posttests showing a preference for social novelty in the wildtype (+/+) animals ($t = 4.13$, $P < 0.001$) and the heterozygous (+/-) animals ($t = 3.43$, $P < 0.01$) but no such preference in the null (-/-) animals ($t = 0.52$, $P > 0.05$).

Despite showing an absence of preference for social novelty on the 3-chamber test, the *Itgb3* -/- animals showed intact olfaction and recognition of novel non-social and social odors on the olfactory habituation / dishabituation test (Figure 3C, two-way repeated measures ANOVA, stimulus main effect $F = 7.60$, $P < 0.0001$; genotype main effect $F = 0.02$, $P = 0.89$; genotype by stimulus interaction $F = 0.77$, $P = 0.70$, $n = 11$ +/+, 8 -/-). Bonferroni posttests revealed no individual odor stimulus presentation that showed a significant difference between wildtype and *Itgb3* -/- animals.

Time spent grooming in a novel environment was significantly different across *Itgb3* genotypes (Figure 4A, Kruskal-Wallis test statistic 6.181 , $P = 0.046$; non-parametric analysis chosen due to trend for unequal variances on Bartlett's test, $P = 0.08$, $n = 6$ +/+, 6 +/-, 7 -/-). A significant difference in time spent grooming in the novel environment was observed between the wildtype and the *Itgb3* -/- animals (Dunn's posttest for multiple comparisons, difference in rank sum -7.38 , $P < 0.05$). In a subset of these animals, time spent grooming over 24 hours in the home cage was not different across *Itgb3* genotypes (Figure 4B, one-way ANOVA, $F = 0.38$, $P = 0.69$, $n = 6$ +/+, 3 +/-, 4 -/-).

Discussion

The pattern of diminished preference for social novelty and increased repetitive behavior in a novel environment in the *Itgb3* knockout mouse has face validity for some symptoms of autism – that is, it mirrors how we may imagine some human symptoms would be paralleled in a mouse. These preliminary findings raise a number of interesting issues, including the evaluation of a behavioral phenotype in the context of known peripheral phenotypes, the appropriate interpretation of these behavioral phenotypes, the use of a knockout mouse to evaluate the importance of this gene, and the possible biological mechanisms underlying the observed phenotypes.

The known peripheral phenotype in the *Itgb3* animals is of obvious concern in interpreting these data. The known anemia, bleeding time, or other peripheral phenotypes (Hodivala-Dilke, et al., 1999; McHugh, et al., 2000) in the *Itgb3* -/- mice complicated the behavioral testing, including requiring additional *Itgb3* -/- mice from the same breeding pairs to be included to achieve adequate sample size for the 3-chamber social test. The absence of littermate controls for these additional -/- animals is a potential confound given the possibility of parenting or litter effects on this social phenotype. On the other hand, the peripheral phenotypes are difficult to connect directly to the absence of preference for social novelty or the increase in grooming behavior in the novel environment. First, neither behavioral phenotype yields to a straightforward peripheral explanation. For example, if mice with a history of bleeding or other peripheral phenotype would hesitate to interact with a novel animal over a familiar animal due to past experience of aggression, we would also expect to see less sociability on the 3-chamber sociability test. Likewise, if mice with a history of bleeding or other peripheral abnormality were grooming more as a result of itching or wound-healing, we would expect them to show increased grooming at baseline,

not just in a novel environment. Second, at least in the case of grooming, the *Itgb3* +/- animals appeared to show an intermediate phenotype, supporting both a gene-dosage relationship and a lack of dependence upon bleeding or other peripheral phenotypes, which have not been reported in the hemizygous animals. Third, it is possible that a brain and behavior phenotype could be mediated directly by increased bleeding in the absence of the fibrinogen receptor, but we did not observe any evidence of previous stroke or brain hemorrhage in these mice in multiple fresh dissections of brain tissue or on Nissl stain (JV, MC, AC, paper in preparation). It remains possible that the peripheral phenotypes in the *Itgb3* null mouse do impact the observed behavioral changes, and further testing in a neuron-specific *Itgb3* null mouse would be necessary to fully rule-out this possibility. Alternatively, parallel testing could be conducted in mice with other causes of bleeding defects, anemia, or osteosclerosis could evaluate the contribution of these peripheral phenotypes to the observed behaviors.

Lack of preference for social novelty is a less straightforward social phenotype than lack of sociability, or the preference for a novel mouse over a novel object, which has been the focus of most behavioral studies of mouse models related to autism (Kwon, et al., 2006; Moy, Nadler, Young, et al., 2008; Silverman, et al., 2010; Tabuchi, et al., 2007). Indeed, absence of preference for people over objects is described in ASD dating back to Leo Kanner's original description of the disorder in 1943 (Kanner, 1943); although lack of sociability is not required for diagnosis of Autistic Disorder (American Psychiatric Association, 2000) and may pertain only to a portion of individuals with ASD (Waterhouse, et al., 1996). Instead, qualitative impairment in social interaction is described as the central deficit in ASD, which may be much more difficult to model in a mouse than absence of sociability itself (American Psychiatric Association, 2000). Lack of preference for exploring novel individuals over familiar individuals could fit as one possible component of this qualitative impairment. Indeed, Kanner included an absence of differentiation between individuals in his first description of autism: "The relation to the members of the household or to other children did not differ from that to the people at the office" (p. 247)(Kanner, 1943). Importantly, however, while this absence of differentiation between individuals may be true of some individuals with ASD, it is again not necessary for diagnosis and is not emphasized in the diagnostic assessment (Lord, et al., 1994).

A number of other mouse mutants have been described with an absence of preference for social novelty. Interestingly, the Dp(11)17/+ mouse model of Potocki-Lupski syndrome, which prominently includes symptoms of autism, lacks a preference for social novelty on the 3-chamber test (Molina, et al., 2008). Mice lacking the oxytocin receptor gene, which has been associated with autism in multiple studies (Gregory, et al., 2009; Jacob, et al., 2007; Lerer, et al., 2008; Liu, et al., 2010; Park, et al., 2010; Wermter, et al., 2010; Wu, et al., 2005), also show an absence of preference for social novelty in one of two studies (Crawley, et al., 2007; Takayanagi, et al., 2005). Using a paradigm of habituation to repeated exposure to a stimulus mouse, other disruptions of the oxytocin system, including knockout of the oxytocin gene itself (Ferguson, et al., 2000) and knockout of the oxytocin-regulating protein CD38 (Jin, et al., 2007), also result in apparent inability to differentiate a familiar from a novel animal. Finally, two mutant mouse lines with no clear genetic relationship to ASD show abnormalities on the preference for social novelty test portion of the 3-chamber test: mice lacking complexin 1 (*Cplx1*), which is important in synaptic vesicle docking and release (Drew, et al., 2007), and mice hemizygous for the schizophrenia-associated neuregulin-1 (*Nrg1*) (O'Tuathaigh, et al., 2007).

An increase in baseline grooming behavior has also been reported in a number of mutant mouse models (Chen, et al., 2010; Shmelkov, et al., 2010; Welch, et al., 2007), as well as in the BTBR inbred strain of mice, which show multiple behaviors with face validity for ASD

(McFarlane, et al., 2008). In each of these models, mice lose hair or develop skin wounds because of excessive grooming. In the *Itgb3* ^{-/-} mice, however, we observed increased grooming in novel environments but not in the home cage, at least in a subset of animals. Grooming in novel environments has been described as an indication of anxiety-like behavior in previous studies (Hart, et al., 2010; McNaughton, et al., 2008). The *Itgb3* ^{-/-} mice do not show any alteration in anxiety-like behavior in the Elevated Plus Maze, however, which would suggest that their increase in grooming behavior in novel environments could reflect an abnormal response to an anxiety-provoking environment, rather than an increase in anxiety-like behavior *per se*. Of note, increased repetitive behavior is seen in children with ASD in anxiety-provoking settings (Joosten, et al., 2009); although children with ASD might also be expected to have an increased baseline level of repetitive behavior (American Psychiatric Association, 2000).

The genetic link between the integrin $\beta 3$ gene, whole blood 5-HT levels, and autism susceptibility, led us to evaluate the behavior of the *Itgb3* knockout mouse. However, if the hypothesis is correct that increased integrin $\beta 3$ activity leads to increased SERT transport of 5-HT and therefore hyperserotonemia (Carneiro, et al., 2008), then mice with increased $\beta 3$ activity would be a better model of the alterations that we might expect to see in ASD. Although such a model of increased integrin $\beta 3$ function would be extremely interesting, the current data support a role for integrin $\beta 3$ in modulating social and repetitive behavior. Repeatedly, the ASD genetic literature has identified increases and decreases in expression or function of the same gene or chromosomal region as leading to autism susceptibility, including disruption or duplication of the Rett syndrome gene *MECP2* (Ramocki, et al., 2009), maternal duplication or deletion of chromosome 15q (Cook, et al., 1997), and duplication or deletion of chromosome 16p (Kumar, et al., 2008; Weiss, et al., 2008). Importantly, opposite alleles of the promoter polymorphism of SERT, which interacts with integrin $\beta 3$, are associated with different behavioral phenotypes within ASD (Brune, et al., 2006). As a whole, this literature suggests a narrow range within which activity of autism-associated genes is constrained, with risk alleles leading to expression that is either too high or too low.

The mechanisms underlying the behavioral phenotypes observed in the *Itgb3* ^{-/-} mice are not obvious. Unlike the neuroligin 3 knockout mouse, which shows absence of preference for social novelty in the context of an olfactory deficit (Radyushkin, et al., 2009), the *Itgb3* ^{-/-} mice have intact olfaction, as well as intact habituation to olfactory stimuli. Of note, the social scents used in the Olfactory Habituation / Dishabituation test were derived from cages holding multiple animals; therefore, the ability to distinguish the scents of individual animals cannot be directly inferred. The preference for spending time with a novel animal over a familiar animal over 10 minutes is also considerably more complex than the simple investigation of a single novel or familiar scent presented repeatedly. Further testing of social behavior could help clarify the cause of the absence of preference for social novelty, including a longer duration of co-habitation with the familiar stimulus mouse, multiple exposures to the familiar mouse, or tracking of interaction with each stimulus mouse over a longer duration of time. Follow-up testing could also include coding of grooming behavior during the three-chamber social task to evaluate the relationship between these two behavioral domains, which was not possible in this study. Additionally, further testing of cognition in these animals would clarify whether they have a more generalized deficit in preference for novelty, in social memory, in spatial memory, in reversal learning, or in object recognition that may underlie their absence of preference for social novelty.

Previous work on the role of integrin $\alpha v \beta 3$ as a cell adhesion molecule in the brain has been limited to neuronal culture and slice preparations of the hippocampus, where it is important in activity-dependent dendritic spine remodeling and scaling of excitatory synapses (Chavis

& Westbrook, 2001; Cingolani & Goda, 2008; Shi & Ethell, 2006). Neuronal activity-dependent genes have previously been implicated in ASD (Morrow, et al., 2008), as have synaptic cell adhesion molecules (Bourgeron, 2009; Buxbaum, 2009). It is tempting to interpret the lack of preference for social novelty as a hippocampal-associated memory deficit, but previous work has suggested that social memory may be more dependent upon the amygdala and the olfactory bulb (Adolphs, 2009; Ferguson, et al., 2001; Tobin, et al.). Increased grooming behavior is unlikely to implicate the hippocampus directly, as a corticostriatal circuit has been implicated in previous mouse mutants with increased grooming (Shmelkov, et al., 2010; Welch, et al., 2007).

In summary, these preliminary studies suggest social and repetitive behavior phenotypes in the *Itgb3* knockout mice that require further study. Targeted knockout of *Itgb3* in the brain is needed to demonstrate independence from the peripheral phenotypes previously described in these mice. Targeted knockout in individual brain regions may clarify the neuronal circuits underlying the particular behavioral abnormalities that we observed. Further behavioral characterization of these targeted knockout animals may be focused on potential memory deficits corresponding to hippocampal abnormalities.

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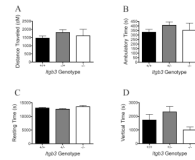


Figure 1. Open Field Activity

Mean and standard error of the mean are shown by genotype (13 +/+, 23 +/-, 8 -/-) for cumulative data over 15 minutes spent in the activity chamber for each genotype. A: Distance traveled in cm. B: Time spent in ambulatory movement. C: Time spent not moving. D: Time spent in the vertical position. No significant genotype effects were detected by one-way ANOVA on any of the measures of activity.

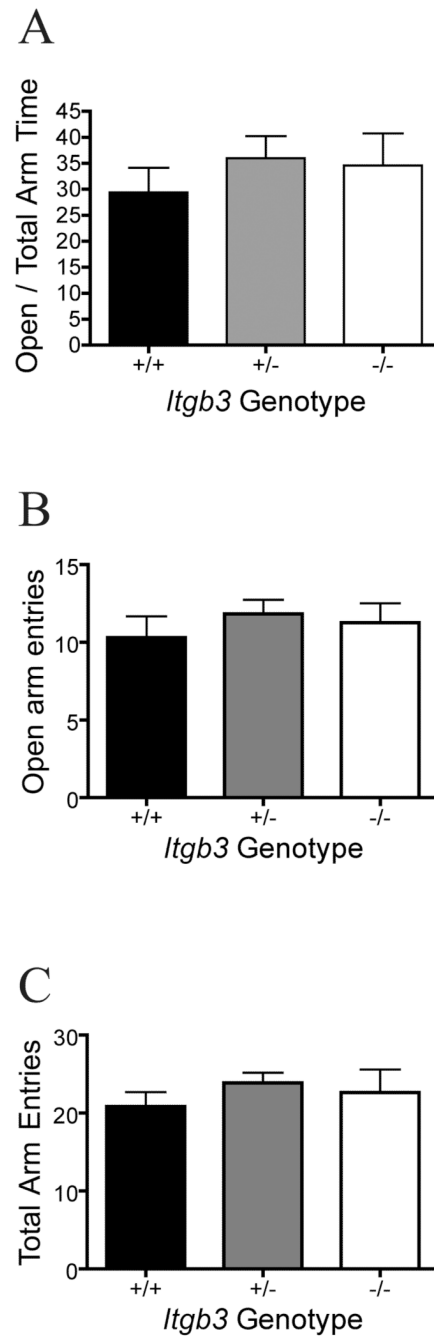


Figure 2. Elevated Plus Maze

Mean and standard error of the mean are shown by genotype (13 +/+, 23 +/-, 8 -/-) for cumulative data over 5 minutes spent in the elevated plus maze. A: Ratio of time spent in the open arms divided by the time spent in either the open or closed arms. B: Total entries into the open arms. C: Total entries into either open or closed arms. No significant genotype effects were detected by one-way ANOVA on any measure.

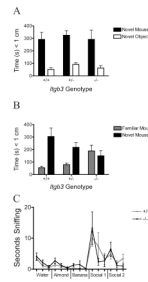


Figure 3. Social and Olfactory Behavior

A and B: Three-chamber social test. Mean and standard error of the mean are shown by genotype (10 +/+, 20 +/-, 6 -/-) for the cumulative data over each 10-minute test session. A: Time spent within 1 cm ('sniffing') of the wire cage containing the novel mouse or the novel object (empty wire cage). B: Time spent within 1 cm of the wire cage containing the familiar mouse or the novel mouse. C: Olfactory Habituation-Dishabituation. Mean and standard error of the mean are shown by genotype (11 +/+, 8 -/-) for the time spent within 2 cm of the cotton swab containing the odor stimulus over each 2-minute odor presentation.

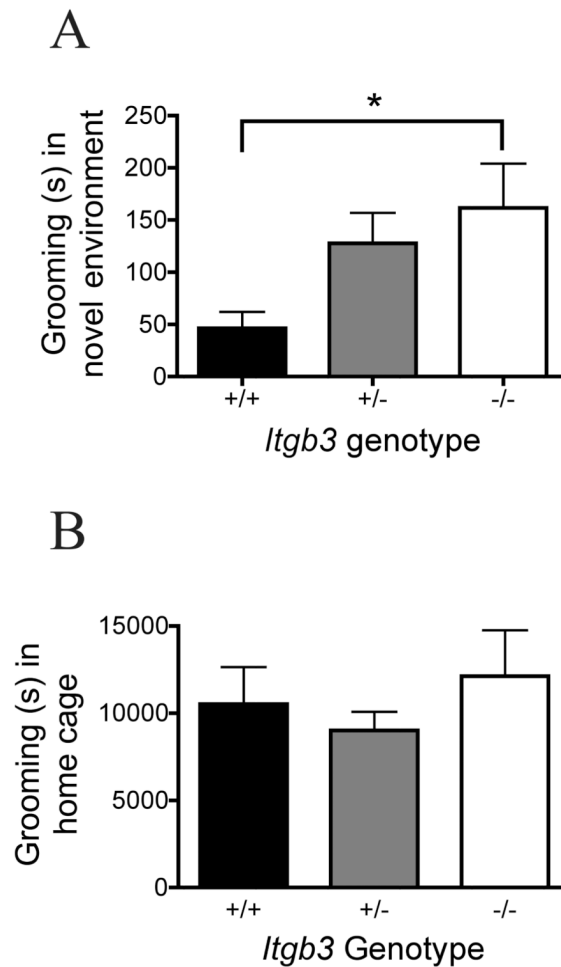


Figure 4. Grooming behavior

A: Grooming behavior in the novel environment. Mean and standard error of the mean are shown by genotype (6 +/+, 6 +/-, 7 -/-) for seconds spent grooming in a novel cage scored by stopwatch over a 10 minute observation period. B: Grooming behavior in the home cage. Mean and standard error of the mean are shown by genotype (6 +/+, 3 +/-, 4 -/-) for seconds spent grooming in the home cage as scored by the automated HomeCageScan software (Clever Sys, Inc., Reston, VA).