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Anxiety-like behaviors in mice lacking GIT2

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Abstract

G protein-coupled receptor kinase-interactor 2 (GIT2) is a signaling scaffold protein that also functions as GTPase-activating protein (GAPs) for ADP-ribosylation factor (Arf) small GTP-binding proteins. GIT2 has been implicated in the regulation of G protein-coupled receptor trafficking and cell adhesion and migration. To evaluate possible neurobehavioral functions of GIT2 *in vivo*, we evaluated GIT2-knockout (KO) mice for abnormalities in emotionality and mood. Male and female GIT2-KO mice presented with anxiety-like behaviors in the zero-maze and light-dark emergence tests. Immobility times in tail suspension were reduced in GIT2-KO males, but were normal in GIT2-KO females. Hence, GIT2-KO mice display anxiety-like behavior in an absence of depressive-like responses.

Keywords

GIT2; knockout mice; anxiety/mood regulation

GIT1 and GIT2 comprise one subfamily of the ADP-ribosylation factor (Arf) GTPase-activating proteins (GAPs), and serve as part of a signaling adaptor complex [5,20]. GIT proteins have been studied most extensively as regulators of G-protein coupled receptor (GPCR) internalization [13,14], focal adhesion dynamics and cell migration [8,28] as well as spine morphogenesis and synapse formation [21,26,27]. GIT1 and GIT2 proteins are structurally and functionally very similar, and homo- and hetero-dimerize when expressed within the same cell [6,11,15]. GIT proteins do not function alone, but form oligomeric complexes with the p21-activated protein kinase (PAK)-interacting exchange factor (PIX) proteins [15]. The two PIX proteins are guanine nucleotide exchange factors (GEFs) for members of the Rho family of GTP-binding proteins [9]. Thus GIT/PIX complexes function as a point of convergence for regulation of two small GTP-binding protein families (i.e., Arf

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and Rac1/Cdc42), and as recruitable multimeric scaffolds for a variety of signaling molecules, including protein kinases such as PAK [5].

Recently, a variety of proteins have been identified as GIT binding partners, including liprin-α, shank, piccolo, and huntingtin [5]. In hippocampal neurons, GIT1 localizes to both pre- and post-synaptic terminals [26] and its downregulation or mislocalization results in aberrant dendritic spine morphogenesis and synapse formation [26,27]. Furthermore, GIT1 facilitates AMPA receptor targeting in primary hippocampal neurons [7] and it mediates ephrinB signaling during spine formation [21]. In contrast, little is known about the neuronal functions of GIT2, despite widespread and predominantly overlapping expression of GIT2 and GIT1 throughout the brain [19]. To initiate the investigation into the *in vivo* neuronal functions of GIT2, we assessed the behavior of GIT2-KO mice.

GIT2-KO mice were obtained from intercrosses of heterozygous GIT2 genetrap mice [19]. Animals were group-housed in temperature- (22°C) and humidity- (45%) controlled rooms with a 12:12 light-dark cycle (lights on at 0700 h) with food and water available *ad libitum*. All experiments were conducted during the light phase of the light-dark cycle and with approved protocols from the Duke University Institutional Animal Care and Use Committee and in accordance with NIH guidelines.

For immunohistochemistry, mice were sacrificed and immediately perfused with 4% paraformaldehyde (PFA) in PBS. Isolated brains were post-fixed at 4ºC for 24 hours in 4% PFA/PBS followed by 48 hours in 4% PFA/20% sucrose in PBS. Brain were cut into 40μm sections using a sliding microtome. Floating sections were pretreated with 1% H_2O_2 in PBS at RT for 20 min, blocked with 3% goat serum in PBS-T for 1 hour and incubated with monoclonal anti-NeuN antibody (1:1000, Chemicon) in blocking solution overnight at 4ºC. Staining was visualized using the Vectastain universal ABC kit and ImmPACT DAB substrate (both from Vector Laboratories).

As an initial assessment, the mice were first submitted to a neurophysiological screen to evaluate sensory and motor performance as described [16,18]. Animals were next tested for exploratory behavior in the open field. Mice were acclimated to the room 2 hrs before testing, and spontaneous activity was monitored in 5 min segments over 30 min and analyzed using the Accuscan VersaMax system (Columbus, OH) [12,24].

Anxiety-like responses were assessed in naïve animals in the zero maze [12,22,24]. Mice were placed into one of two opposite quadrants enclosed by walls and videotaped while allowed to freely investigate the maze for 5 min. Videotape analysis was performed by trained observers blinded to genotype using Noldus Observer (Noldus Information Technologies, Leesburg, VA). Scored behaviors included percent of time in open areas, number of open area transitions, head-dips, stretch-attend postures, freezing, and latency to enter the open areas. In a second test, mice were subjected to the light-dark emergence test as previously described [24]. Testing was conducted in a two chambered apparatus (Med-Associates). The latency to emerge from the darkened into the lighted chamber and the percent time spent in the illuminated chamber were used as indices of anxiety-like behaviors.

For the assessment of depressive-like behavior, mice were subjected to the tail suspension test [4]. Mice were examined in a MedAssociates apparatus (St. Albans, VT) where body weight was used as a control for the magnitude of struggle activity. Behavior was scored as time spent in immobility over a 6 min period.

Statistical analyses were performed using SPSS-11 software (SPSS Inc., Chicago, IL) and results are presented as means and standard errors of the mean. For tests where behaviors were serially scored for the same animal, repeated measures ANOVA (RMANOVA) was used. Time

in the open field and time in immobility in tail suspension were used as the within subject effects. For all RMANOVA, genotype and sex were the between subjects effects. Comparisons between genotype and sex were assessed with ANOVA for cumulative open field activity, responses in the zero maze and tail suspension tests. T-tests were used to analyze response in the light-dark emergence test. When no sex effects were found, the data were collapsed into a single group. In all cases, $p < 0.05$ was considered significant.

Immunohistochemistry of coronal brain sections for the neuronal marker NeuN revealed normal overall brain morphology in homozygous GIT2 genetrap mice (Fig 1A). Western blot analysis revealed an almost complete loss of GIT2 immunoreactivity in cerebellum (Fig 1B) and other tissues (not shown) of homozygous GIT2 genetrap mice, indicating that the genetrap does interfere with normal GIT2 expression. We therefore refer to homozygous GIT2 genetrap mice as GIT2-KO mice. The levels of GIT1 protein were found to be unchanged in cerebellar lysates from GIT2-KO mice (Fig 1B), suggesting a lack of compensatory up-regulation of GIT1 expression after loss of GIT2. Pups from GIT2-Heterozygote breeding pairs were born in the expected Mendelian ratio (data not shown) and adult GIT2-KO mice showed normal overall appearance (Table I) and fertility (data not shown). In the neurophysiological screen, GIT2-KO animals displayed normal gross sensory and motor functions compared to WT littermates (Table I). GIT2-KO mice had mild “tremor” and reduced forepaw grip strength, but this did not appear to affect their responses on any behavioral tests. In the vertical pole test, the latency of GIT2-KO mice to climb up the pole was reduced relative to WT controls; all other spinocerebellar responses were undifferentiated by genotype.

In the open field, sex differences were observed between WT and GIT2-KO mice. During the first 5 min, locomotor activity was higher (Fig 2A) while rearing was lower in GIT2-KO males than in WT males (Fig 2C), and this appeared to be due to enhanced locomotion of GIT2-KO males in the center zone (Fig. 2E). When activities were collapsed over the 30 min test period, only rearing was significantly lower for the GIT2-KO males than their WT controls (Fig. 2C *inset*). With respect to females, locomotion was reduced over the first 20 min for GIT2-KO animals (Fig. 2B) and this appeared to be due to attenuated activities in both the central and peripheral zones (Fig. 2F,H). Rearing was decreased also in GIT2-KO females over the first 5 min in the open field (Fig 2D). When the data were collapsed over time, locomotion, rearing, and activities in the center and peripheral zones were decreased significantly in GIT2-KO females compared to WT females (Figs. 2B,D,F,H *insets*). Furthermore, GIT2-KO females also had reduced locomotion (Fig. 2B,*insert*), vertical activity (Fig. 2D,*insert*), and center activity (Fig. 2F,*insert*) compared to GIT2-KO males. Collectively, these data show that spontaneous exploratory activity is selectively influenced in GIT2-KO mice with females more affected than males.

When examined for anxiety-like behaviors, GIT2-KO mice responded with sex-specific differences in the zero maze. GIT2-KO males spent equal percent time in the open areas (Fig 3A), but they engaged in significantly more transitions (Fig 3C), spent less time in the open areas per visit (Fig. 3E), and displayed fewer head-dips than WT males (Fig 3G). In contrast, GIT2-KO females spent significantly less time in the open areas (Fig. 3B), had fewer transitions (Fig. 3D), spent less time in the open areas per entry (Fig. 2F), and engaged in fewer head-dips than WT females (Fig. 3H), and either WT or GIT2-KO males. No significant genotype or sex differences were observed for stretch-attend postures, freezing behavior, or latency to enter the open arms (data not shown). Anxiety-like behaviors may be differentially expressed in GIT2-KO males and females. In females it was evidenced by decreased time in the open areas, reduced transitions, open area visits, and head-dips, whereas for males is was represented as reduced open area visits and head-dips.

To further examine anxiety-like responses, animals were evaluated in the light-dark emergence test [3], which is related to behavioral indices of anxiety in the elevated plus [17] and zero [24] mazes. Since no sex differences were discerned in WT or GIT2-KO mice in this test, the data were collapsed across sex and analyzed as a function of genotype. The latency to enter the lighted chamber was significantly prolonged for GIT2-KO mice (Fig. 4A). Additionally, mutants spent less time in the lighted chamber (Fig. 4B), were less active in the lit chamber (Fig. 4C), and engaged in fewer crossings between lighted and darkened chambers than WT littermates (Fig. 4D). Activity in the darkened chamber was not different between GIT2-KO and WT mice (data not shown). The exaggerated preference for the darkened chamber over lighted chamber is consistent with an anxiety-like phenotype in these mice. Taken together, results from the zero maze and light-dark emergence tests indicate that the GIT2-KO mice display heightened anxiety-like behaviors.

Depressive-like behaviors were assessed in tail suspension [23]. In this test, increased immobility times are indicative of depressive-like responses, while decreased immobility may suggest resistance to this behavior [25]. GIT2-KO mice animals displayed a sex-specific phenotype. GIT2-KO males showed significantly decreased immobility times compared to WT males (Fig. 5A), whereas females did not differ from each other (Fig 5B). These findings suggest the GIT2-KO mice do not show depressive-like responses.

The results in this study provide the first evidence for a role of GIT2 in behavior, specifically in the regulation of anxiety-like behaviors in mice. The elevated zero maze and light-dark emergence tests impose a conflict for the animals between the propensity to explore a novel environment and the expression of defensive reactions due to the potential dangers inherent in unknown, novel contexts [2,10]. Deletion of the GIT2 gene caused behavioral abnormalities that may be expressed in a sex-specific manner in certain contexts. Although the reason for the sex-based differences in GIT2-KO mice in the zero maze is currently unclear, distinct behaviors of males and females in response to anxiety have been reported in animals [10] and humans [1]. A lack of a sex-based difference in the light-dark emergence test may reflect the limited behavioral choices presented in this test [2]. Consequently, the sex-based differences in anxiety responses for the GIT2-KO mice may reflect greater variability in behavioral responses between male and female animals.

In humans, depression and anxiety are often comorbid [29]. Immobility time is considered a good index of depressive-like behaviors in rodents [23]. However, because immobility times were not increased in GT2-KO animals, it appears that they may not possess a depressive-like phenotype. The increased struggle times in GT2-KO males suggests at least they may have some emotional dysfunction and they be more susceptible to stress than mutant females and WT controls. Collectively, the results show that GIT2 can exert effects on behavior and these appear to be manifested as alterations in anxiety-like responses.

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Figure 1.

Basic assessment of GIT2-KO mice. (A) Brain morphology appeared normal in GIT2-KO mice. Coronal brain sections (40 μm) were stained for neuronal marker NeuN. (B) Western blotting of cerebellar lysates from WT and GIT2-KO animals using the PKL (chicken GIT2) monoclonal antibody (BectonDickinson) detected GIT1 and GIT2 proteins. Actin was detected with a mouse monoclonal antibody (Santa Cruz Biotechnologies).

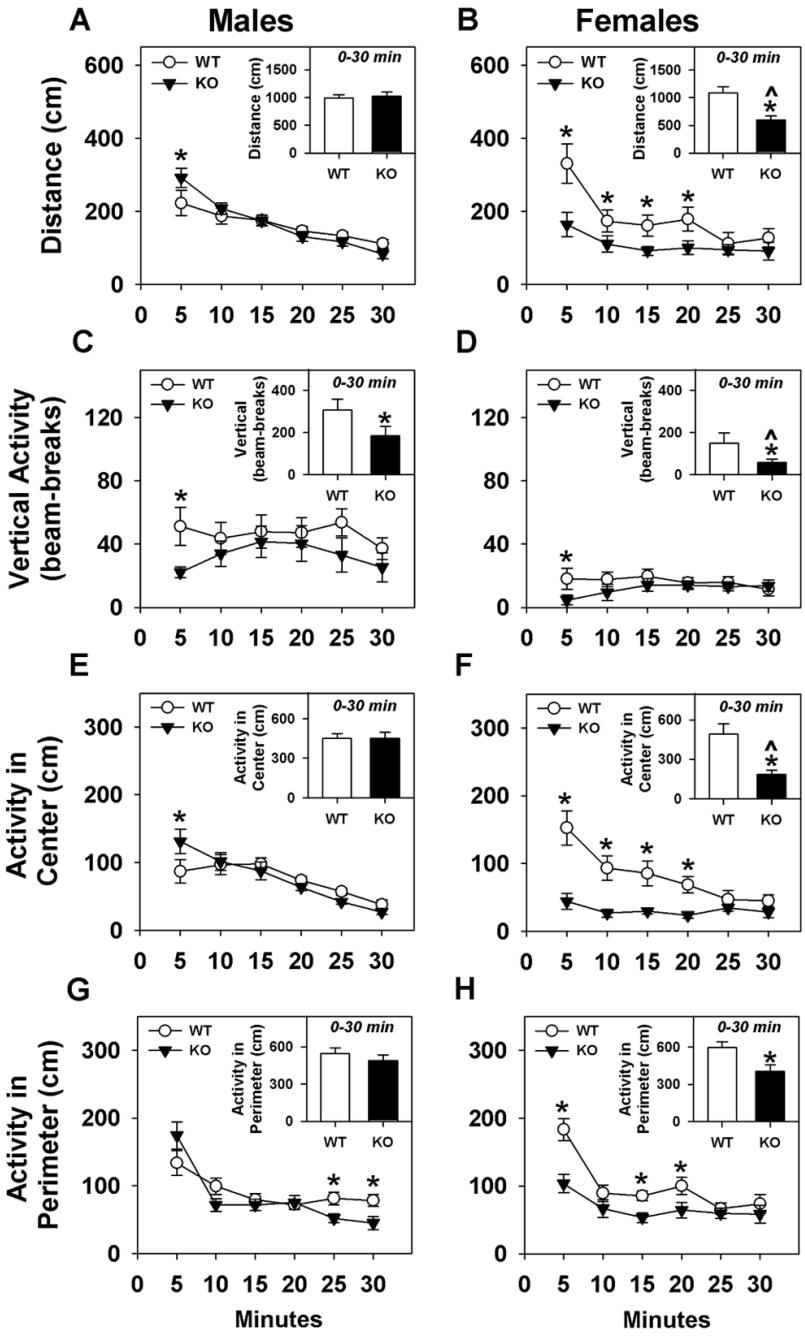

Figure 2.

Spontaneous activity in the open field. (A,B) Locomotor activities of male (A) and female (B) WT and GIT2-KO animals assessed in 5 min blocks over 30 min in the open field. (C,D) Rearing activities of male (C) and female (D) WT and GIT2-KO animals. (E,F) Locomotion in the center zone of male (E) and female (F) WT and GIT2-KO animals. (G,H) Locomotion in the peripheral zone for male (G) and female (H) WT and GIT2-KO animals. *Insets* show total activity over the 30 min test. n = 9–10 mice/genotype/sex; *p<0.05, WT versus KO animals; ^p<0.05, KO females versus WT and KO males.

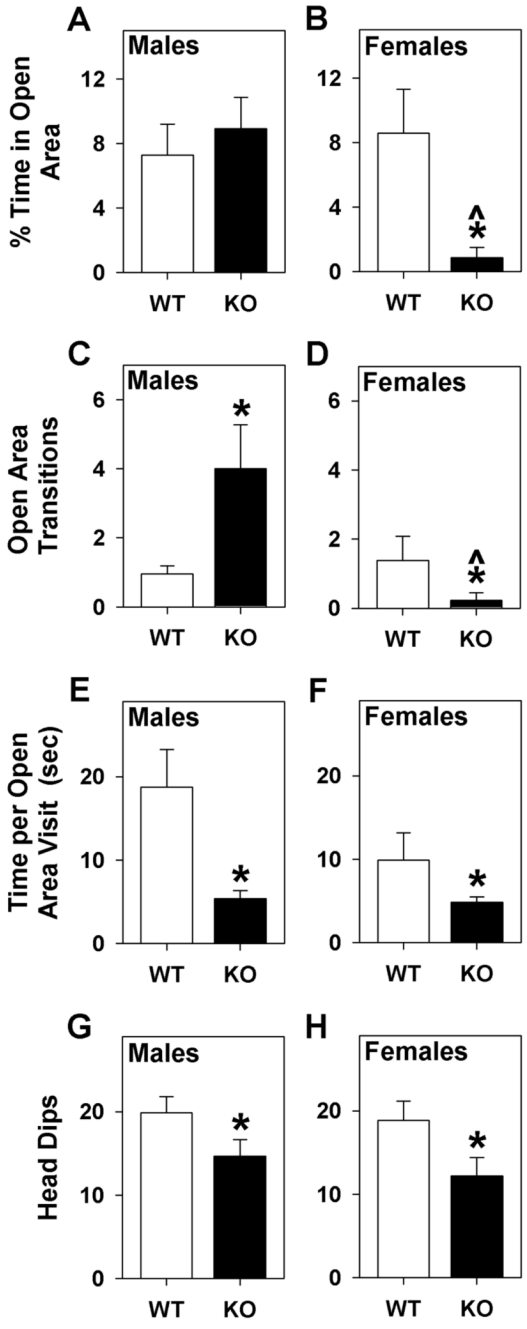

Figure 3.

Anxiety-like behaviors in the zero maze. (A,B) Percent time in the open areas for male (A) and female (B) WT and GIT2-KO animals. (C,D) Numbers of transitions from closed to open to closed areas for male (C) and female (D) WT and GIT2-KO animals. (E,F) Duration of open arm times per visit by male (E) and female (F) WT and GIT2-KO animals. (G,H) Number of head-dips for male (G) and female (H) WT and GIT2-KO animals. n=9–10 mice/genotype/sex; *p<0.05, WT compared to KO; ^p<0.05, KO male compared to KO female.

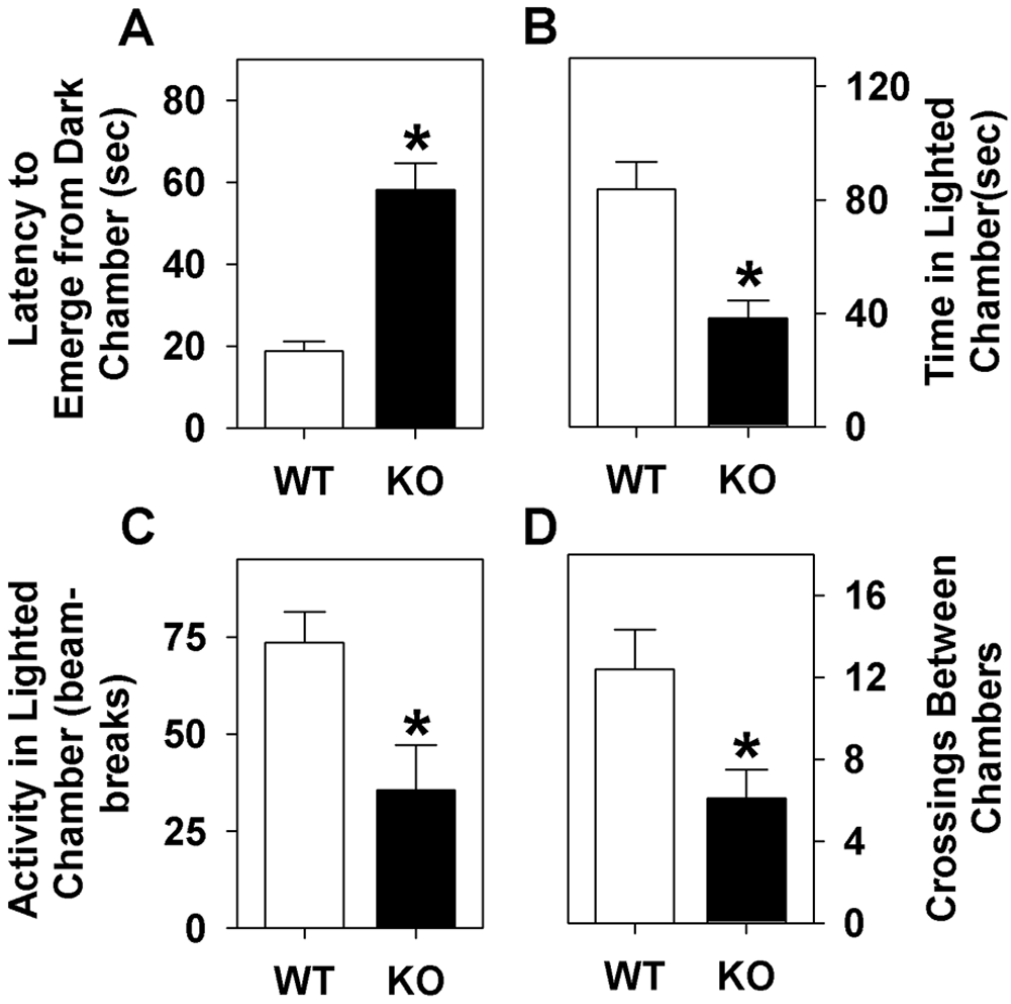

Figure 4.

Anxiety-like behaviors in the light-dark emergence test. (A) Latency to leave the darkened and enter the lighted chamber. (B) Time in the lighted chamber. (C) Activity in the lighted chamber. (D) Transitions between the darkened and lighted chambers. Since GIT2-KO males and females had similar responses, the data were collapsed across sex and analyzed for genotype differences by an independent measures t-test. n=9–10 mice/genotype/sex, *$p<0.05$, WT compared to KO.

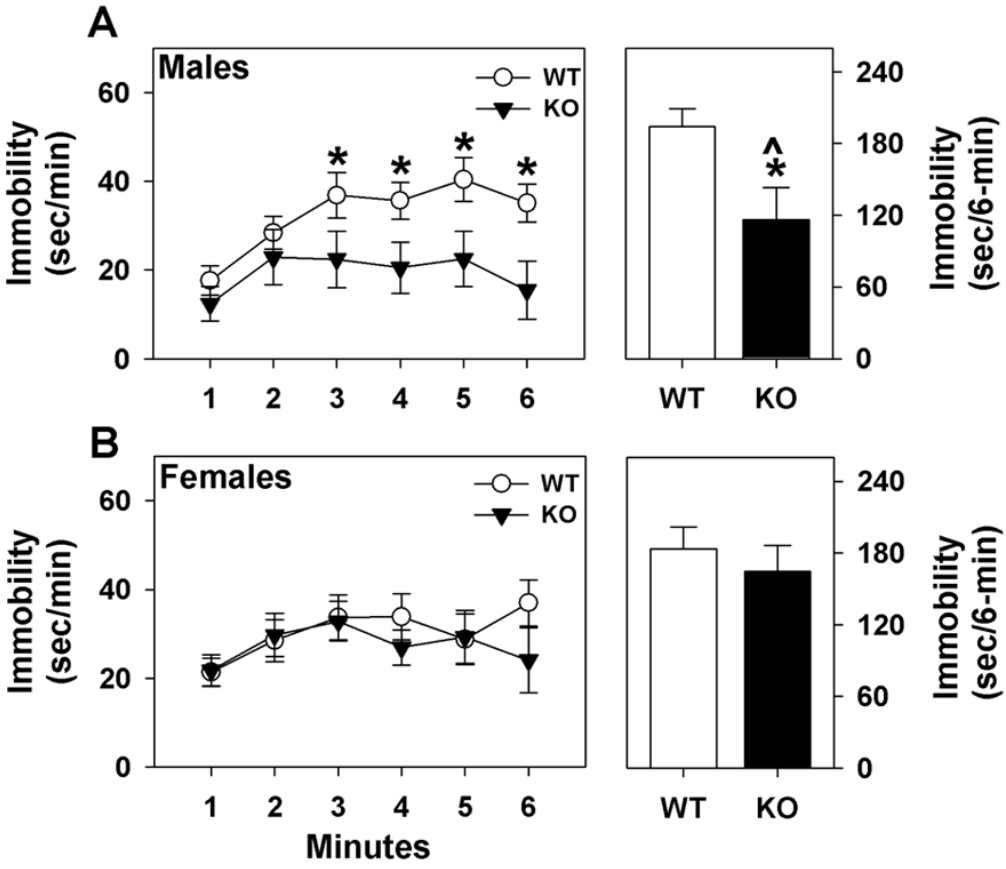

Figure 5.

Absence of depressive-like behaviors in GIT2-KO mice in tail suspension. Immobility times for (A) male and (B) female WT and GIT2-KO mice for each minute of the 6 min test (left), or aggregated over the entire period. n=9–10 mice/genotype/sex, *p<0.05, WT compared to KO; ^p<0.05, KO male compared to KO female.

Table I

Neurophysiological screen.

	WT	KO
Initial Evaluation and General Screen		
(a) Skin color	Normal	Normal
(b) Body tone	Normal	Normal
(c) Lacrimiation/Palperbral Closure	Normal	Normal
(d) Exopthalmus	Normal	Normal
(e) Convulsions/Tremor	Absent	Present - mild
(f) Heart Rate	Normal	Normal
(g) Respiration Rate	Normal	Normal
(h) Piloerection	Normal	Normal
(i) Barbering (% animals expressing)	Normal	Normal
Orientation and Reflexive Behavior		
(a) Visual orientation to object	Normal	Normal
(b) Visual Placement	Normal	Normal
(c) Whisker Stop	Normal	Normal
(d) Whisker Reflex	Normal	Normal
(e) Eye Reflex	Normal	Normal
(f) Pinna (ear) Reflex	Normal	Normal
Postural and Righting Reflexes		
(a) Postural – Vertical	Normal	Normal
(b) Postural – Horizontal	Normal	Normal
(c) Contact Righting	Normal	Slight delay[a]
Spinocerebellar Function (Front/Rear paw Strength, Coordination, and Balance)		
(a) Forepaw Grasp/Strength	48.1 ± 3.9	38.1± 3.2[b]
(b) Rearpaw Grasp/Strength	27.6 ± 1.9	26.2 ± 3.6
(c) Rearpaw Coordination	Normal	Normal
(d) Wirehang – Duration	40.26 ± 8.21	37.01 ± 8.43
(e) Pole Climbing Down		
Latency	2.01 ± 0.51	1.07 ± 0.71
Duration	17.73 ± 1.66	13.78 ± 1.18
(f) Pole Climbing Up		
Latency	5.86 ± 1.25	1.71 ± 0.35[c]
Duration	16.67 ± 1.23	15.69 ± 0.88
(g) Pole Walking		
Latency	1.73± 0.35	1.00 ± 0.01
Duration	6.38 ± 0.90	6.01 ± 0.78

[a] $x2$ analyses reveals no differences; mutants appear elevated but within normal limits

[b] $t\ (1,38) = 1.929,\ p < 0.054$

[c] $t\ (1,38) = 2.830,\ p < 0.009$