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Mice Lacking Full Length Adgrb1 (bai1) Exhibit Social Deficits, Increased Seizure Susceptibility, and Altered Brain Development

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Abbreviated title: *Bai1* influences behavior and seizure susceptibility

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Highlights

- BAI1/ADGRB1 is an adhesion GPCR that interacts with autism-relevant proteins.
- *Adgrb1*$^{-/-}$ mice show deficits in sociability and increased seizure susceptibility.
- *Adgrb1*$^{-/-}$ mice display reduced brain weight and neuron density.
- Loss of full length Bai1 is associated with a range of clinically relevant features.

Abstract

The adhesion G protein-coupled receptor BAI1/ADGRB1 plays an important role in suppressing angiogenesis, mediating phagocytosis, and acting as a brain tumor suppressor. BAI1 is also a critical regulator of dendritic spine and excitatory synapse development and interacts with several autism-relevant proteins. However, little is known about the relationship between altered BAI1 function and clinically relevant phenotypes. Therefore, we studied the effect of reduced expression of full length Bai1 on behavior, seizure susceptibility, and brain morphology in *Adgrb1* mutant mice. We compared homozygous (*Adgrb1*$^{-/-}$), heterozygous (*Adgrb1*$^{+/-}$), and wild-type (WT) littermates using a battery of tests to assess social behavior, anxiety, repetitive behavior, locomotor function, and seizure susceptibility. We found that *Adgrb1*$^{-/-}$ mice showed significant social behavior deficits and increased vulnerability to seizures. *Adgrb1*$^{-/-}$ mice also showed delayed growth and reduced brain weight. Furthermore, reduced neuron density and increased apoptosis during brain development were observed in the hippocampus of *Adgrb1*$^{-/-}$ mice, while levels of astrogliosis and microgliosis were comparable to WT littermates. These results show that reduced levels of full length Bai1 is associated with a broader range of clinically relevant phenotypes than previously reported.

Keywords

BAI1, seizures, epilepsy, autism, GPCR

Introduction

Brain-specific angiogenesis inhibitor (BAI1/ADGRB1) is a member of the adhesion G protein-coupled receptor (GPCR) family (Duman et al., 2016; Purcell and Hall, 2018). Besides sharing a well-conserved seven-transmembrane structure with other GPCRs, BAI1 also features a large N-terminal extracellular domain with five thrombospondin type 1 repeats (TSRs) and a GPCR-autoproteolysis-inducing (GAIN) domain (Cork and Van Meir, 2011; Stephenson et al., 2014). BAI1 suppresses angiogenesis (Cork et al., 2012; Kaur et al., 2009; Zhu et al., 2012), mediates engulfment of apoptotic cells and gram-negative bacteria (Das et al., 2011; Park et al., 2007; Sokolowski et al., 2011), promotes myogenesis (Hochreiter-Hufford et al., 2013), and serves as a brain tumor suppressor by stabilizing p53 (Zhu et al., 2018). Although BAI1 was first associated with non-neuronal functions, the receptor is most abundantly expressed in neurons and glia in the cortex, hippocampus, thalamus, amygdala, and striatum (Sokolowski et al., 2011; Zhang et al., 2014). *Adgrb1* mRNA expression peaks at postnatal day 10 (P10) in rodents and expression is maintained into adulthood (Kee et al., 2004).

Studies during the past decade indicate that *in vitro* and *in vivo* knockdown of Bai1 leads to the formation of more immature and unstable dendrites (Duman et al., 2019; Duman et al., 2013), while overexpression of Bai1 results in dendrite retraction (Duman et al., 2019). Mice lacking full-length Bai1 display reduced expression of post-synaptic density 95 (PSD-95) (Zhu et al., 2015), a protein that regulates synaptic stability and plasticity (Cheng et al., 2006). The continuous morphological modifications of dendrites and proper PSD-95 function are essential for learning and memory (Coley and Gao, 2018; Migaud et al., 1998) and are often altered in neurodevelopmental and neurological disorders (Lin and Koleske, 2010; Penzes et al., 2011; Tsai et al., 2012), primarily those characterized by impaired social interaction, communication deficits, and repetitive behaviors (D'Hooge and De Deyn, 2001). Consistent with this observation, *Adgrb1*$^{-/-}$ mice exhibit deficits in spatial memory and alterations in synaptic plasticity

that are reflected by enhanced long-term potential (LTP) and reduced long-term depression (LTD) (Zhu et al., 2015).

De novo rare variants in *ADGRB1* have been identified in patients with autism spectrum disorder (ASD) (Satterstrom et al., 2020). A substantial percentage of individuals with ASD (8-24%) have epilepsy and exhibit altered brain morphology and developmental delay (Amiet et al., 2008; Gabis et al., 2005; Ghacibeh and Fields, 2015). These behavioral phenotypes are also seen in animal models of ASD (Sierra-Arregui et al., 2020; Varghese et al., 2017). Furthermore, BAI1 interacts with autism-relevant proteins, including BAIAP2/IRSp53 and Neuroligin-1 (NLG1) (De Rubeis et al., 2014; Nakanishi et al., 2017; Oda et al., 1999), although little is known about the functional significance of these interactions. Therefore, in the current study, we investigated the *in vivo* physiological role of BAI1 by characterizing the behavioral and seizure phenotypes of homozygous mice lacking full-length Bai1 (*Adgrb1*$^{-/-}$) and heterozygous mice (*Adgrb1*$^{-/-}$) that express approximately 50% of wildtype levels. Our results indicate that *Adgrb1*$^{-/-}$ mice exhibit impairments in sociability, social discrimination, and increased seizure susceptibility. *Adgrb1*$^{-/-}$ mice also display increased apoptosis during brain development, reduced brain weight, and reduced hippocampal neuron density.

Material and methods

Animals

The generation and genotyping of mice lacking full-length Bai1 were previously described (Zhu et al., 2015). These mice were engineered with a deletion of exon 2 (where the ATG start codon is located) and fail to express full length Bai1. Heterozygous mutants (*Adgrb1*$^{+/-}$) on a C57BL/6J (000664, Jackson laboratory) background were bred to generate wildtype (WT), heterozygous (*Adgrb1*$^{+/-}$), and homozygous (*Adgrb1*$^{-/-}$) offspring. Mice were housed in groups of 3-5 on a 12-hour light/dark cycle with standard laboratory rodent chow (5001, Lab Diet) and water available

ad libitum. All experiments were performed in accordance with the Emory University Institutional Animal Care and Use Committee (IACUC) guidelines.

Survival and body growth curve analysis

Male and female *Adgrb1*$^{-/-}$, *Adgrb1*$^{+/-}$ and WT littermates were weighed once every two days from postnatal day 7 to 24 (P7 – P24) and then once a week until P65 (n=9-15/group).

Brain weight assessment

Male and female mice of all genotypes at three different ages (P1, 3 week, 2-3 month) were weighed and anesthetized with isoflurane. Brains were harvested and immediately weighed (n=8-18/group).

Immunofluorescence staining and imaging

Brains were postfixed in 4% PFA overnight at 4°C and then transferred to 30% sucrose solution in phosphate-buffered saline (PBS). Coronal sections (40 µm) were stained using with antibodies against GFAP (1:400, 13-0300, Thermo Fischer Scientific), IBA1 (1:400, Ab178846, Abcam), NeuN (1:1000, MAB377, Millipore), and cleaved-caspase-3 (CC3) (1:200, 9664, Cell signaling Technology). Goat anti-mouse IgG (1:500, ab150114, Abcam) and goat anti-Rat IgG (1:500, A-21434, Thermo Fischer) were used as the secondary antibodies for NeuN and GFAP, respectively. Goat anti-rabbit IgG (1:500, ab150077, Abcam) was used as the secondary antibody for IBA1 and CC3. Slides were washed multiple times with PBS and incubated with appropriate fluorescently labeled secondary antibodies for 1 h at 37°C. For NeuN, GFAP, and IBA1 staining, confocal images were acquired on an Olympus FV1000 inverted microscope using the Olympus Fluoview v4.2 software. For CC3 staining, an upright microscope (DM6000B Leica) was used. Three sections per mouse containing the hippocampus and primary somatosensory cortex (bregma –1.96 mm) were used for immunofluorescence staining. NeuN positive cells in the dentate gyrus (DG) and CA1 were counted per mm^2. CC3 positive (CC3+) cells were counted in the DG, CA1, and primary somatosensory cortex. GFAP and IBA1

immunoreactivity (IR) was calculated as the percentage area of the total region of interest (ROI) of the DG and CA1 using ImageJ. A threshold for IR was determined across all antibody images as previously described (Chalermpalanupap et al., 2018). The IR area within the ROI and the total area of the ROI were calculated using the "Measure" feature of ImageJ, and the percentage area of IR was determined (area of IR within ROI divided by the total area of ROI and multiplied by 100). Two sections per mouse containing the rostral hippocampus were used for P1 mice. Similarly, CC3+ cells were counted in the DG, CA1, and primary somatosensory cortex. The experimenter was blinded to genotype during quantification.

Western Blot

Whole brain was dissected from 5 month old WT, *Adgrb1*$^{+/-}$, and, Adgrb*1*$^{-/-}$ littermates, and the left hemisphere was used for western blot. Tissue extracts were prepared in RIPA buffer (89901, Thermo) containing protease and phosphatase inhibitor mix (1861280, Thermo), and total protein was quantified using the BCA protein assay. Laemmli sample buffer (1610747, BIORAD) was added after the BCA protein assay. Protein samples (75 µg) were loaded without boiling on a 10 % SDS-PAGE gel, resolved at 100 V/cm for 2.5 hours, and transferred to a PVDF membrane (1620177, BIORAD). Membranes were blocked with 5 % milk and incubated overnight at 4 ^{o}C with an anti C-terminal BAI1 antibody (1:1000, AP8170a, Abcepta; epitope: amino acids 1537-1567), followed by an anti-rabbit HRP-conjugated secondary antibody (1:5000; 31460, Thermo Fischer). The intensity of the Bai1 band was quantified using ImageJ (NIH) as previously described (Zhu et al., 2018) (n=3/genotype).

Behavioral analysis

Behavioral analysis was performed on 3-5 month old male *Adgrb1*$^{-/-}$, *Adgrb1*$^{+/-}$, and WT littermates. All behavioral assessments were videotaped and scored using the ANY-Maze Video Tracking System (Stoelting Co.) by an experimenter blinded to genotype. Behavioral analyses

were conducted one week apart in the following order: open field, novel object recognition, novel cage, and three-chamber social interaction.

Open field and novel object recognition

Novel object recognition was performed over 4 days as previously described (Dutton et al., 2017; Sawyer et al., 2016; Wong et al., 2021a; Wong et al., 2018). The apparatus consisted of an arena with opaque Plexiglas walls (60 cm x 60 cm x 60 cm). The center zone was a 30 cm x 30 cm area in the center of the chamber. On day 1, each mouse was placed in the empty arena and allowed to explore for 10 min. Locomotor activity, total distance traveled, average speed, and time spent in the center zone were scored. On days 2 and 3, two identical objects (cube or sphere) were placed in the center of the arena, and each mouse explored the arena for 10 min (n=13/genotype). On day 4, one of the objects was replaced with a novel object (cube was replaced with the sphere or vice versa). The objects and the location of the novel versus familiar object were counterbalanced. The time spent exploring each object was used to calculate a discrimination ratio (time exploring the novel object/ (time exploring the novel object + time exploring the familiar object) (n=7-8/genotype).

Three-chamber social interaction

Sociability and social discrimination were examined using the three-chamber social interaction paradigm (Dutton et al., 2017; Sawyer et al., 2016; Wong et al., 2021b). A partition separated each chamber (20 cm x 40 cm x 20 cm) with an opening to allow the mouse to move freely between them. The experiment consisted of three 10-minute sessions. The test mouse was first placed in the center chamber, with an empty cylindrical wire cage in the left and right chambers, and the mouse was allowed to freely explore for 10 minutes. In the second 10-minute session, an age- and sex-matched C57BL/6J mouse (stranger) was placed under one of the wire cages while the wire cage on the opposite side remained empty (object). The test mouse was again placed in the center chamber and allowed to explore freely. Time interacting with the ‘stranger’

mouse vs. the empty cage was calculated as a measure of 'sociability'. For the third 10-minute session, a second age- and sex-matched C57BL/6J mouse (novel mouse) was placed under the previously empty wire cage. The test mouse was again placed in the center chamber and allowed to explore freely. Time interacting with either the first (now 'familiar') mouse from the second session or the novel mouse introduced in the third session was calculated as a measure of 'social discrimination' (n=7-8/genotype).

Novel cage

Each mouse was placed into a novel standard mouse cage (33 cm × 18 cm× 15 cm) and observed for stereotyped behaviors for 10 minutes. The time spent grooming, digging, rearing, and circling was recorded (n=13/genotype).

Nestlet shredding

Nesting behavior was performed as previously described (Lustberg et al., 2020). Each mouse was placed into a novel standard mouse cage with a cotton nestlet square (5 cm × 5 cm, approximately 3 g). Nestlets were weighted before the experiment to calculate the percent shredded at the end of the task. Mice were left undisturbed between 1 pm and 3 pm, after which they were returned to their home cages. The weight of the remaining non-shredded nestlet material was recorded (n=13/genotype).

Buried food Test

Olfactory function was examined using the buried food test as previously described (Yang and Crawley, 2009). Two days prior to assessment of olfactory function, chocolate-flavored pellets (F05472-1, Bio-Serv) were introduced into the home cage to habituate animals to the novel but

highly palatable stimulus. Twenty-four hours prior to behavioral testing, all standard mouse chow and chocolate pellets were removed from the home cage. Mice were tested in the same room in which they were housed. On test day, chocolate pellets were placed in a randomly-selected corner of a clean mouse cage and buried under 3 cm of standard bedding. The latency for the mouse to find and eat (grasp and bite) the chocolate pellets was recorded. Mice that did not feed within 15 min were assigned a maximum feeding latency score of 900 s (n=13/genotype).

Seizure induction

Susceptibility to induced seizures was tested in 3-5 month old male and female *Adgrb1*$^{-/-}$, *Adgrb1*$^{+/-}$, and WT littermates.

6 Hz induced seizures

6 Hz psychomotor seizures were induced as previously described (Giddens et al., 2017; Shapiro et al., 2021; Wong et al., 2016; Wong et al., 2021b). Mice were given a topical analgesic to the cornea (0.5% tetracaine hydrochloride) before stimulation. Corneal electrical stimulation (6 Hz, 3 sec, 17 mA for male and 13 mA for female) was applied through a constant current device (ECT Unit 57800; Ugo Basile), and the mouse was moved immediately into a clean cage for behavioral seizure observation. Resulting seizures were scored on a modified Racine scale: RS0 = no abnormal behavior; RS1 = immobile ≥ 3 sec, RS2 = forelimb clonus, paw waving, RS3 = rearing and falling (n=9-11/group).

Flurothyl induced seizures

Seizure induction using flurothyl was performed as previously described (Martin et al., 2007; Shapiro et al., 2021; Shapiro et al., 2019; Wong et al., 2021a). Each mouse was placed in a plexiglass chamber (34 cm x 20 cm x 15 cm and exposed to flurothyl (bis[2,2,2-trifluoroethyl]

ether, 287571-5G, Sigma-Aldrich) at a rate of 20 µL/min. Latencies to the first myoclonic jerk (MJ) and generalized tonic-clonic seizure (GTCS) were recorded (n=8-11/group).

Statistical analysis

Prism v8.1.2 software (GraphPad) was used for statistical analyses. A 2-way ANOVA followed by Sidak's multiple comparisons test was used to compare the bodyweight of each genotype at the different time points of the growth curve, the time spent interacting with the stranger mouse and novel mouse, total entries into each side chamber during the three-chamber social interaction task, and brain/body weight measurements. For novel object recognition, a one-sample parametric t-test was used to compare the time spent with the novel object against 50% chance. A 1-way ANOVA followed by Tukey's multiple comparisons test was used to analyze the total distance traveled, speed, total time spent in the center of the open field, the latency to the MJ and GTCS during flurothyl seizure induction, the amount of nestlet shredded, the latency to grasp and bite the chocolate pellets in the buried food test, latencies to the first interactions in the three-chamber social interaction task, and immunofluorescence data. For the 6 Hz seizure induction paradigm, an unpaired nonparametric Mann-Whitney U-test was used to compare Racine scores between each genotype. Male and female mice were analyzed separately unless stated. All results are presented as mean ± SEM and a p-value <0.05 was considered significant.

Results

***Adgrb1*$^{-/-}$ mice show delayed growth and reduced brain weights**

Western blot analysis showed that *Adgrb1*$^{-/-}$ mice lack full length Bai1, and *Adgrb1*$^{+/-}$ mutants express approximately 50% of WT levels. (**Supplementary Fig. 1A-B)**. We next examined the developmental profile of *Adgrb1* mutants. We observed that *Adgrb1*$^{-/-}$ mice weighed significantly less than their WT littermates beginning at P15 for males (p=0.0097) (**Supplementary Fig. 2A**) and P9 for females (p=0.0112) (**Supplementary Fig. 2B**); however,

by P37, the body weights of *Adgrb1*$^{-/-}$ mice were comparable to same-sex WT littermates. In contrast, there were no significant differences in average body weights between *Adgrb1*$^{+/-}$ and WT littermates at any age.

As Bai1 is highly expressed in brain, we next focused on brain development. We compared brain weights of male and female mice with each genotype at three time points: P1, 3 weeks, and 2-3 months (**Fig. 1**). At P1, no significant differences were observed in either brain

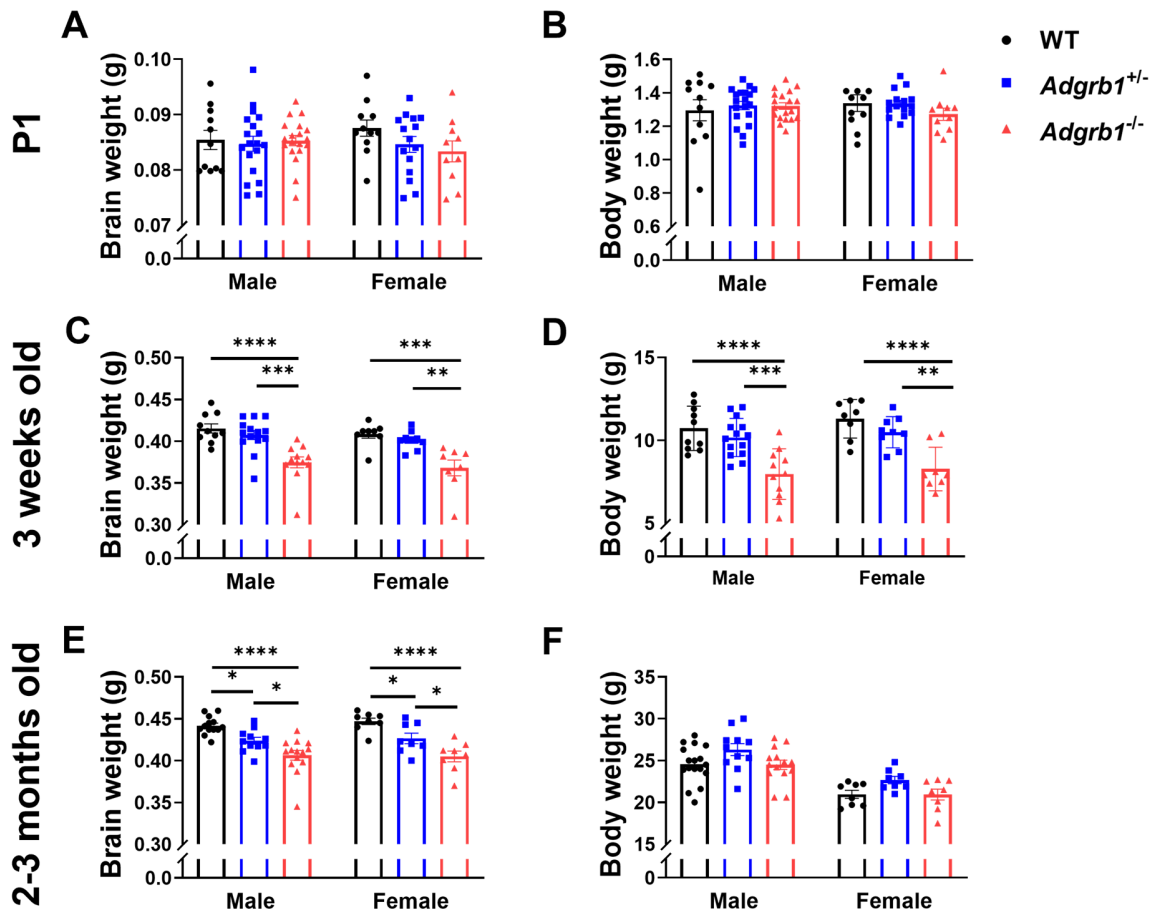

Figure 1. *Adgrb1*$^{-/-}$ mice exhibit reduced brain and body weight. (**A-B**). *Adgrb1*$^{-/-}$ male and female mice (P1) had comparable brain **(A)** and body weights **(B).** Male: WT, n = 11; *Adgrb1*$^{+/-}$, n = 19; *Adgrb1*$^{-/-}$, n = 19, Female: WT, n = 10; *Adgrb1*$^{+/-}$, n = 15; *Adgrb1*$^{-/-}$, n = 10. (**C-D**). *Adgrb1*$^{-/-}$ male and female mice (3 weeks old) had lower average brain **(C)** and body weights **(D)** compared to same-sex *Adgrb1*$^{+/-}$ mutants and WT littermates. Male: WT, n = 10; *Adgrb1*$^{+/-}$, n = 14; *Adgrb1*$^{-/-}$, n = 12, Female: WT, n = 8; *Adgrb1*$^{+/-}$, n = 9; *Adgrb1*$^{-/-}$, n = 8. **(E**) *Adgrb1*$^{-/-}$ and *Adgrb1*$^{+/-}$ male and female mice (2-3 months old) had lower average brain weights compared to WT littermates. **(F)** Body weights were comparable in 2–3 month old mice across all three genotypes**.** Male: WT, n = 14; *Adgrb1*$^{+/-}$, n = 11; *Adgrb1*$^{-/-}$, n = 14, Female: WT, n = 8; *Adgrb1*$^{+/-}$, n = 8; *Adgrb1*$^{-/-}$, n = 9. Mean ± SEM. **p*<0.05, ***p*<0.01, ****p*<0.001, *****p*<0.0001.

(p=0.3326) or body weight (p=0.6) (**Fig. 1A-B**). However, at 3 weeks of age, average brain weights of *Adgrb1*$^{-/-}$ mice of both sexes were significantly less than sex-matched *Adgrb1*$^{+/-}$ and WT littermates (p<0.001) (**Fig. 1C**). The average body weight of *Adgrb1*$^{-/-}$ mice was also significantly less than sex-matched *Adgrb1*$^{+/-}$ mice and WT littermates (p=0.0107) (**Fig. 1D**). At 2-3 months of age, no significant differences in average body weights between same-sex mice across all three genotypes were found (p=0.998) (**Fig. 1F**); however, average brain weights of *Adgrb1*$^{-/-}$ mice of both sexes were significantly less than same-sex *Adgrb1*$^{+/-}$ mice and WT littermates (p<0.0001) (**Fig. 1E**). In contrast to the other time points, at 2-3 months of age, the average brain weight of *Adgrb1*$^{+/-}$ mice was significantly lower than same-sex WT littermates (p<0.05) (**Fig. 1E**).

2-3 month old *Adgrb1*$^{-/-}$ mice exhibit reduced neuron density in the dentate gyrus and CA1

Since brain weight was lower in 2-3 month old *Adgrb1*$^{-/-}$ mice, we next examined whether brain morphology was altered. We focused on the hippocampus since *Adgrb1*$^{-/-}$ mice were previously shown to exhibit altered hippocampal LTP and LTD, and deficits in hippocampal dependent spatial memory (Zhu et al., 2015). We quantified neuron density in 2–3 month old male mice of each genotype in two hippocampal regions: the dentate gyrus (DG) and CA1. *Adgrb1*$^{-/-}$ male mice displayed significantly lower neuron density in both the DG (p=0.0402) (**Fig. 2A-D**) and

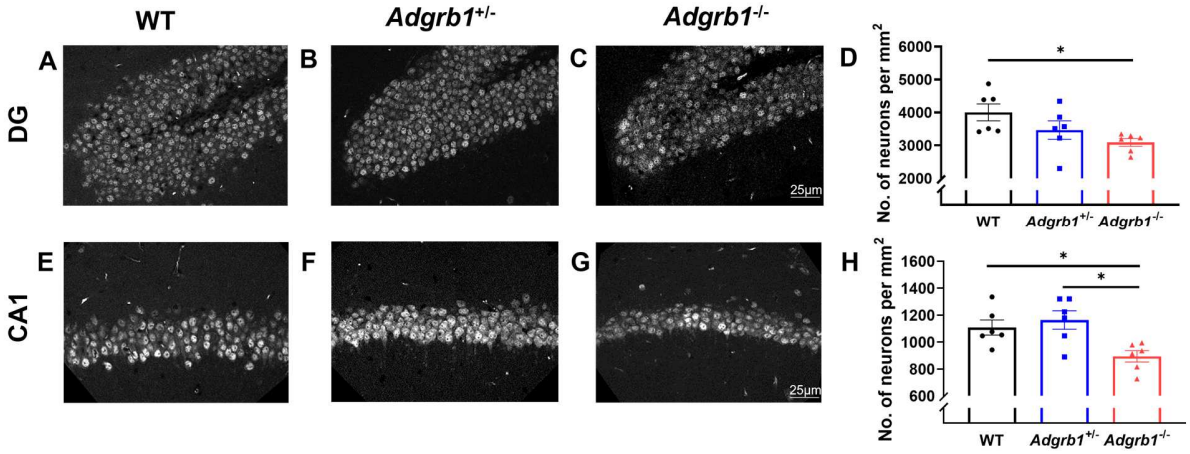

Figure 2. Reduced neuron density in 2-3 month old *Adgrb1*$^{-/-}$ mice. Representative images of NeuN positive cells in the DG **(A-C)** and CA1 region **(E-G).** *Adgrb1*$^{-/-}$ mice had significantly lower neuron density in the DG **(D)** and CA1 **(H)** than *Adgrb1*$^{+/-}$ and WT littermates. WT, n = 6; *Adgrb1*$^{+/-}$, n = 6; *Adgrb1*$^{-/-}$, n = 6. Mean ± SEM. *p<0.05.

CA1 (p=0.0103) (**Fig. 2E-H**) compared to WT littermates. We also compared the levels of GFAP (astrocyte marker) and IBA1 (microglia marker) expression in the DG and CA1 between male mice of each genotype (2-3 months old), and observed no significant differences (p>0.05) (**Supplementary Fig. 3**).

Adgrb1$^{-/-}$ mice exhibit higher levels of cleaved caspase-3-positive cells in the hippocampal CA1 and primary somatosensory cortex during early postnatal development

To determine whether the reduced neuron density observed in *Adgrb1*$^{-/-}$ mice was associated with a higher level of apoptosis, we used the cleaved casepase-3 antibody (Porter and Janicke, 1999) to compare apoptosis levels in all three genotypes at P1, 3 weeks, and 2-3 months of age. As expected, levels of cleaved caspase-3-positive (CC3+) cells were higher in P1 mice compared to 3 week old and 2-3 month old mice due to the greater level of programmed cell death (PCD) during early neurodevelopment (Yamaguchi and Miura, 2015). At P1, comparable levels CC3+ cells were observed in the DG of *Adgrb1*$^{-/-}$ mice and WT littermates (p=0.6801)

(**Fig. 3A-D**); however, we observed a significantly greater number of CC3+ cells in the CA1 region of *Adgrb1*$^{-/-}$ mice (p=0.0186) (**Fig. 3E-H**). No significant differences were detected between *Adgrb1*$^{+/-}$ mice and WT littermates. We also examined CC3+ cell levels in the primary somatosensory cortex, a region in which Bai1 is also highly expressed (Sokolowski et al., 2011). Similarly, we observed a greater number of CC3+ cells in the somatosensory cortex of P1

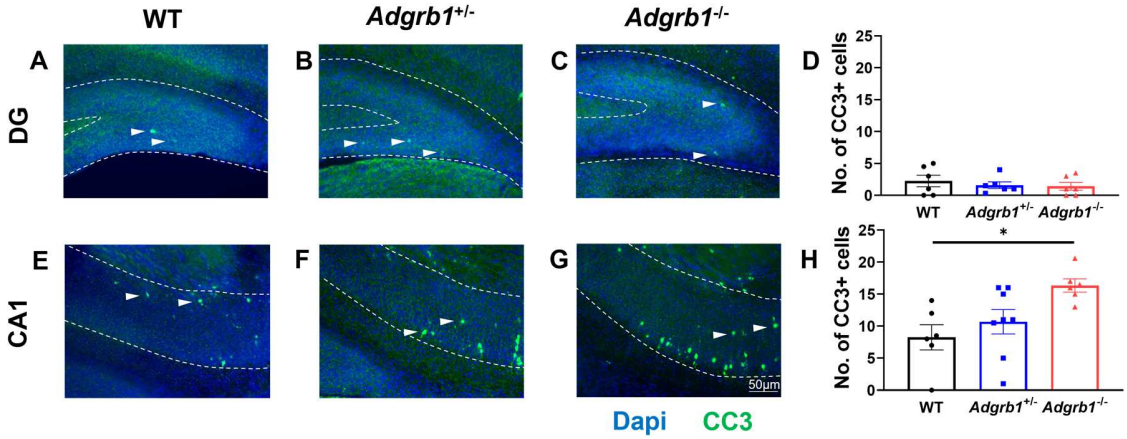

Figure 3. P1 *Adgrb1*$^{-/-}$ mice have more of CC3+ cells in the CA1. (A-C) Representative images of CC3+ cells in the DG. **(D)** Comparable numbers of CC3+ cells in the DG were observed across all three genotypes. **(E-G)** Representative images of CC3+ cells in the CA1. **(H)** *Adgrb1*$^{-/-}$ mice had more CC3+ cells in the CA1 than WT littermates. Dashed lines show the boundaries of the DG and CA1 regions. Examples of CC3+ cells are indicated with arrows. Mean ± SEM. WT, n = 6; *Adgrb1*$^{+/-}$, n = 6; *Adgrb1*$^{-/-}$, n = 6. *p<0.05.

Adgrb1$^{-/-}$ mice compared to WT littermates (p=0.023) (**Supplementary Fig. 4**). Comparable levels of CC3+ cells were observed in the CA1, DG, and primary somatosensory cortex of 3 week old and 2–3 month old mice of each genotype (**Supplementary Fig. 5**).

Adgrb1$^{-/-}$ mice exhibit deficits in social behavior and learning and memory

We used the three-chamber social interaction paradigm to examine sociability and social discrimination as BAI1 interacts with the autism relevant proteins, NLG1 and IRSp53, and mice lacking Nlg1 or Irsp53 show altered social behavior (Blundell et al., 2010; Chung et al., 2015).

WT littermates and *Adgrb1*$^{+/-}$ mice spent significantly more time exploring the stranger mouse than the empty cage (p=0.0008 for WT littermates, p=0.0099 for *Adgrb1*$^{+/-}$ mice). In contrast, *Adgrb1*$^{-/-}$ mice did not discriminate between the stranger mouse and empty cage (p=0.6627), suggesting a deficit in sociability (**Fig. 4A**). When presented with the choice between interacting with a novel or familiar mouse, WT littermates showed a significant preference for the novel mouse compared to the familiar mouse (p=0.0097). In contrast, *Adgrb1*$^{+/-}$ and *Adgrb1*$^{-/-}$ mice did

not show a statistically significant preference for the novel mouse (*Adgrb1*$^{+/-}$, p=0.0984; *Adgrb1*$^{-/-}$, p=0.7451), suggesting a deficit in social discrimination (**Fig. 4B**). There were no differences in total entries into each side chamber, or the latencies to the first interaction with the stranger mouse or the novel mouse in the 'sociability' and 'social discrimination' components of the task, respectively (**Supplementary Fig. 6)**. The similar performance of the mice of all three genotypes in the buried food test (**Fig. 4C**) demonstrates that the observed impairment in social

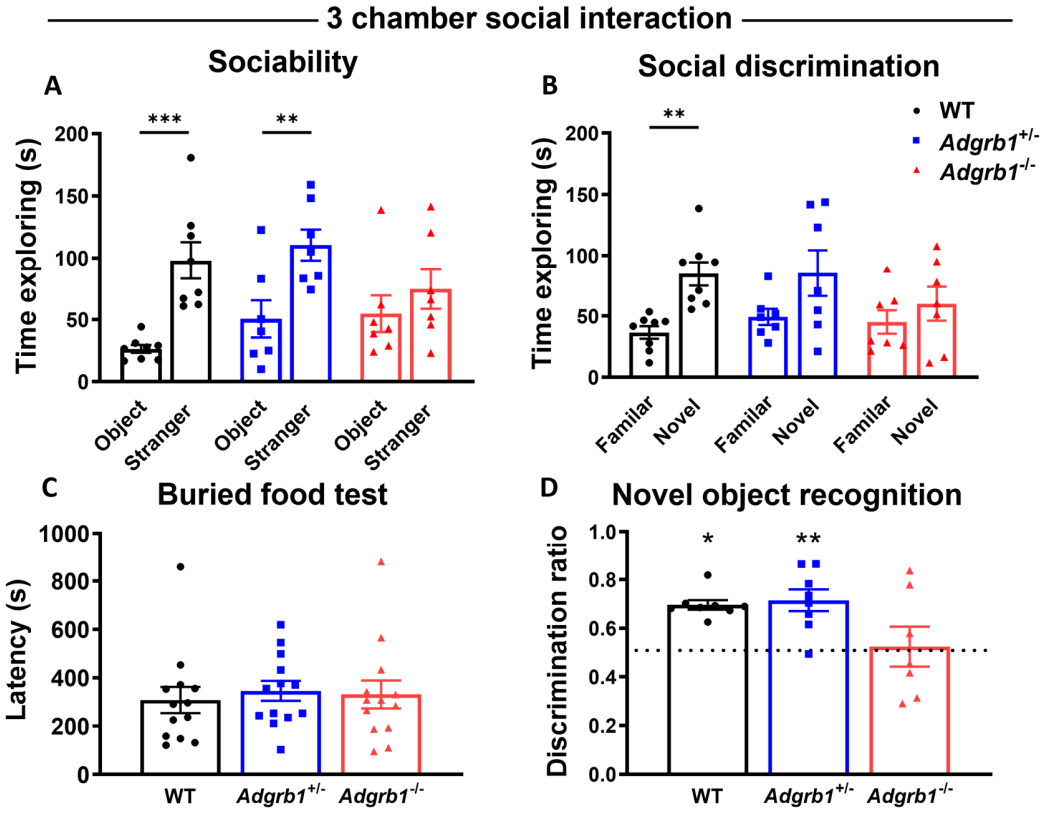

Figure 4. Male *Adgrb1*$^{-/-}$ mice exhibit deficits in sociability, social discrimination, and novel object recognition. (A) *Adgrb1*$^{-/-}$ mice did not significantly discriminate between a stranger mouse and an empty cage, demonstrating a sociability deficit. **(B)** *Adgrb1*$^{+/-}$ and *Adgrb1*$^{-/-}$ mutants did not exhibit a significant preference for a novel vs. a familiar mouse, indicating a deficit in social discrimination. WT, n = 8; *Adgrb1*$^{+/-}$, n = 8; *Adgrb1*$^{-/-}$, n = 7. **(C)** *Adgrb1*$^{-/-}$ mice spent similar amount of time to find buried food. WT, n = 13; *Adgrb1*$^{+/-}$, n = 13; *Adgrb1*$^{-/-}$, n = 13 **(D)** *Adgrb1*$^{-/-}$ mice did not discriminate between the novel and familiar object, suggesting a deficit in long-term recognition memory. WT, n = 8; *Adgrb1*$^{+/-}$, n = 8; *Adgrb1*$^{-/-}$, n = 7. Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

interaction is unlikely to be due to olfactory dysfunction.

In addition to abnormalities in social behavior, deficits in learning and memory have been described in patients and animal models of ASD (Pasciuto et al., 2015; Silverman et al., 2010). Therefore, we used the novel object recognition task to examine long-term recognition memory. WT littermates and *Adgrb1*$^{+/-}$ mice spent significantly more time exploring the novel object compared to 50% chance (WT, p<0001; *Adgrb1*$^{+/-}$, p=0.0019) (**Fig. 4D**). In contrast, *Adgrb1*$^{-/-}$ mice did not show a significant preference for the novel object (p=0.7765), suggesting a deficit in long-term recognition memory.

We also subjected the mice to the open field paradigm to examine locomotor activity and anxiety-like behaviors. Distance traveled (p=0.187) (**Supplementary Fig. 7A**) and average speed (p=0.1865) **(Supplementary Fig. 7B)** were found to be comparable between the three genotypes. *Adgrb1*$^{-/-}$ mice spent more time than WT littermates in the center of the open field (p=0.012), suggesting that loss of full length Bai1 is not associated with increased anxiety-like behavior (**Supplementary Fig. 7C**). Lastly, we examined nesting behavior (**Supplementary Fig. 7D)** and stereotypical behaviors (**Supplementary Fig. 7E)** and found that all three genotypes performed similarly (p= 0.294 and 0.328, respectively).

Adgrb1$^{-/-}$ mice are susceptible to induced seizures

Along with social deficits, many mouse models of ASD exhibit an increased vulnerability to seizures, an observation that is consistent with clinical observations that epilepsy is often comorbid with ASD (Ghacibeh and Fields, 2015; Hughes and Melyn, 2005). Therefore, we explored whether *Adgrb1* mutants might exhibit alterations in seizure susceptibility. In the 6 Hz paradigm, seizures were observed in all *Adgrb1*$^{-/-}$ mice (male: 3 RS1, 7 RS2; p=0.0185, female: 6 RS2, 2 RS3; p=0.0039) (**Fig. 5A and D**). In contrast, only 25% and 33% of male and female WT littermates seized, respectively (male: 6 RS0, 2 RS2; female: 6 RS0, 3 RS2). Additionally, when exposed to the proconvulsant flurothyl, *Adgrb1*$^{-/-}$ mutants displayed shorter average

latencies to the first myoclonic jerk (MJ) (male, p<0.0001; female, p=0.0002) (**Fig. 5B and E)** and generalized tonic-clonic seizure (GTCS) (male, p=0.0004; female, p=0.0014) when

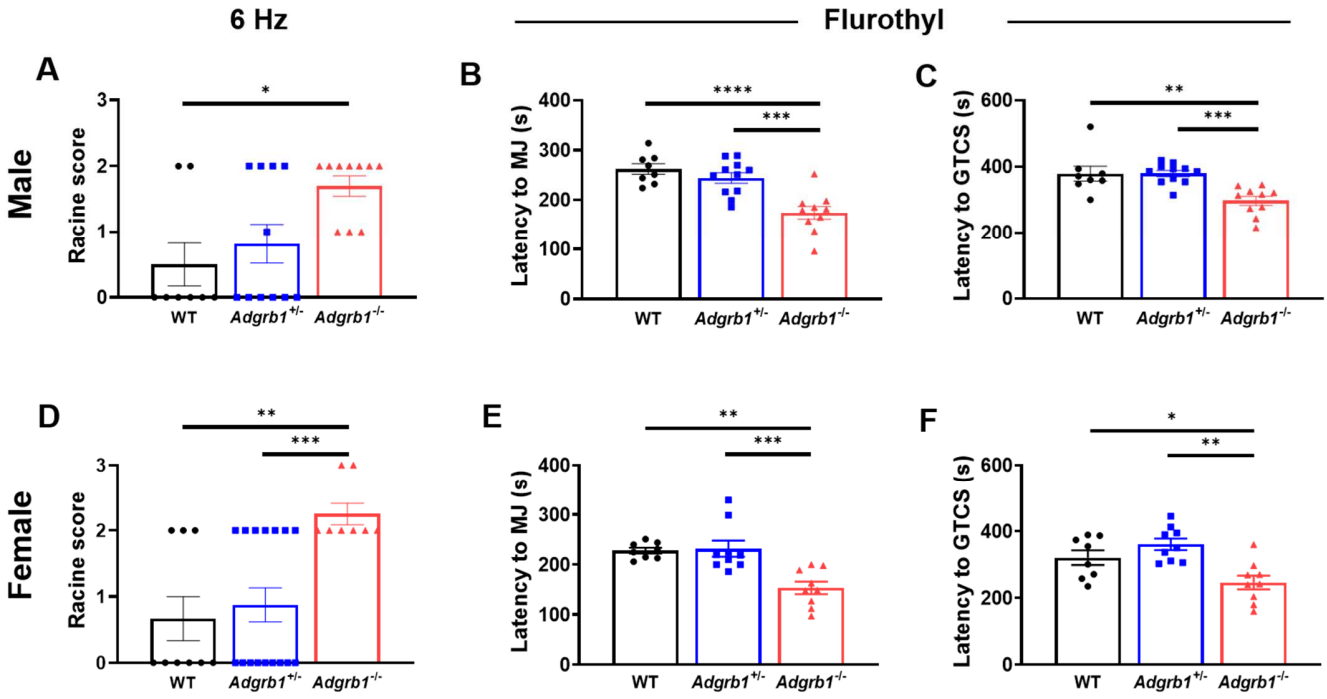

Figure 5. *Adgrb1*$^{-/-}$ mice are susceptible to induced seizures. (A and D) *Adgrb1*$^{-/-}$ male and female mice were more susceptible to 6 Hz seizures when compared to *Adgrb1*$^{+/-}$ and WT littermates. Male: WT, n = 8; *Adgrb1*$^{+/-}$, n = 11: *Adgrb1*$^{-/-}$; n = 10, Female: WT, n = 9; *Adgrb1*$^{+/-}$; n = 18, *Adgrb1*$^{-/-}$, n = 8. **(B and E)** *Adgrb1*$^{-/-}$ male and female mutants exhibited shorter latencies to the flurothyl-induced myoclonic jerk (MJ) and **(C and F)** the first generalized tonic-clonic seizure (GTCS) compared to *Adgrb1*$^{+/-}$ and WT littermates. Male: WT, n = 8; *Adgrb1*$^{+/-}$, n = 11; *Adgrb1*$^{-/-}$, n = 10, Female: WT, n = 8; *Adgrb1*$^{+/-}$, n = 9; *Adgrb1*$^{-/-}$, n = 9. Mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, ****p<0.0001.

compared to same-sex WT littermates **(Fig. 5C and F)**. In contrast, there were no differences in the average latency to the MJ or GTCS between same-sex heterozygous mutants and WT littermates**.**

Discussion

In the current study, we identified a wide range of phenotypes, including delayed growth, reduced brain weight, higher levels of apoptosis, deficits in social behavior, and increased

seizure susceptibility in mice lacking full length Bai1 expression. These observations expand the clinical features that could be potentially associated with BAI1 dysfunction.

We found that 3 week old *Adgrb1*$^{-/-}$ mice weighed significantly less than *Adgrb1*$^{+/-}$ and WT littermates; however, body weight was comparable between genotypes at 2-3 months of age. Lower body weight during early development has been previously reported for several mouse models of autism (Portmann et al., 2014; Yang et al., 2016). It is possible that competition with the WT littermates prior to weaning could have reduced milk intake in the mutants, thereby contributing to the slower initial weight gain. In contrast, at both the 3 week and 2–3 month time points, the average brain weight of *Adgrb1*$^{-/-}$ mutants was significantly less than WT littermates. Thus, the lower brain weight in *Adgrb1*$^{-/-}$ mutants was not simply due to the overall smaller size of the mutant mice. Consistent with the lower brain weights, we observed reduced neuron density in the DG and CA1 regions of 2-3 month old *Adgrb1*$^{-/-}$ mice. Since Bai1 is known to mediate the clearance of apoptotic cells (Mazaheri et al., 2014) (Sokolowski et al., 2011), we speculated that the absence of full-length Bai1 during early brain development may result in increased levels of uncleared apoptotic cells. In turn, this could contribute to secondary necrosis, neuron loss, and lower brain weight (Elliott and Ravichandran, 2010; Glass et al., 2010). In support of this prediction, we observed increased CC3+ cells in the CA1 region and somatosensory cortex of P1 *Adgrb1*$^{-/-}$ mice. Interestingly, the number of CC3+ cells in these regions were comparable between the three genotypes at the 3 week and 2-3 month time points, suggesting that Bai1 might play a greater role in the clearance of apoptotic cells early in brain development, a period that has the highest levels of apoptosis due to programmed cell death (Ahern et al., 2013; Yamaguchi and Miura, 2015).

While we observed reduced neuron density in both CA1 and DG of 2-3 month old *Adgrb1*$^{-/-}$ mice, similar levels of CC3+ cells were detected in the DG of P1 *Adgrb1*$^{-/-}$ mice and WT littermates. This suggests reduced clearance of apoptotic cells during early development is

unlikely to be solely responsible for the observation of reduced neuron density. Thus, additional studies will be required to fully resolve the underlying mechanisms.

Recent sequence analyses of ASD patients identified several *de novo* BAI1 variants (Satterstrom et al., 2020), suggesting that BAI1 might also play a role in ASD. BAI1 also interacts with autism-associated proteins, such as NLG1 and BAIAP2/IRSp53 (Nakanishi et al., 2017; Shiratsuchi et al., 1998; Toma et al., 2011; Tu et al., 2018). *Nlg1*$^{-/-}$ and *Irsp53*$^{-/-}$ mice also show abnormal social behavior, memory deficits, and altered synaptic plasticity (Blundell et al., 2010; Chung et al., 2015; Kim et al., 2009). However, while the phenotypes of *Adgrb1*$^{-/-}$ mutants overlap with *Nlg1*$^{-/-}$ and *Irsp53*$^{-/-}$ mice, the underlying mechanisms for the observed phenotypes might not be identical. Lack of full-length Bai1 leads to rapid degradation of Psd-95 due to the activation of the E3 ubiquitin ligase Mdm2 (Zhu et al., 2015), and mice lacking Psd-95 similarly demonstrate sociability and memory deficits (Coley and Gao, 2019; Migaud et al., 1998). However, no changes in Psd-95 protein levels were reported in *Irsp53*$^{-/-}$ and *Nlg1*$^{-/-}$ mice (Blundell et al., 2010; Kim et al., 2009), indicating other pathways might exist that cause the similar deficits observed in *Nlg1*$^{-/-}$ and *Irsp53*$^{-/-}$ mice.

In addition to the memory and social behavior deficits observed in the *Adgrb1* mutants, we also found that these mice are more seizure susceptible. While the mechanistic basis for this observation is currently unknown, it may be due, in part, to disrupted protein-protein interactions. For example, BAI1 interacts with BAI1 associated protein 3 (BAIAP3), which mediates endosome fusion within the trans-Golgi network (Zhang et al., 2017). BAIAP3 can modulate GABAergic neuronal firing (Wojcik et al., 2013), and *Baiap3*$^{-/-}$ mice also exhibit increased seizure susceptibility (Wojcik et al., 2013). Furthermore, *Adgrb1* mutants exhibit enhanced NMDA mediated long-term potential (LTP), which can also be an underlying cause of increased seizure susceptibility (Kapur, 2018).

While the alterations in social behavior, seizure susceptibility, and body weight were only observed in homozygous *Adgrb1*$^{-/-}$ mutants, brain weight was reduced in both the *Adgrb1*$^{+/-}$ and *Adgrb1*$^{-/-}$ mutants. These observations demonstrate that Bai1 haploinsufficiency can influence biological processes, and that some disease phenotypes associated with Bai1 dysfunction may be affected by gene dosage. The current study reveals previously undescribed roles for BAI1 in regulating social behavior, seizure vulnerability, and CNS development, thus implicating BAI1 in a range of clinically challenging neurological disorders, including ASD and epilepsy.

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Disclosure of conflict of interests

None of the other authors have any conflict of interest to disclose.

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