**BRIEF COMMUNICATION**

**α5GABA_A receptor deficiency causes autism-like behaviors**

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**Abstract**

The prevalence of autism spectrum disorders (ASDs), which affect over 1% of the population, has increased twofold in recent years. Reduced expression of GABA_A receptors has been observed in postmortem brain tissue and neuroimaging of individuals with ASDs. We found that deletion of the gene for the α5 subunit of the GABA_A receptor caused robust autism-like behaviors in mice, including reduced social contacts and vocalizations. Screening of human exome sequencing data from 396 ASD subjects revealed potential missense mutations in GABRA5 and in RDX, the gene for the α5GABA_A receptor-anchoring protein radixin, further supporting an α5GABA_A receptor deficiency in ASDs.

**Introduction**

Autism spectrum disorders (ASDs) are complex neurodevelopmental conditions that are characterized by impaired social interactions, deficits in communication, repetitive behaviors, and reduced executive function.¹ ASDs occur in approximately 1 in every 68 children in the United States and 30% of cases are associated with genetic causes.²⁻⁴ Duplication of the q11.2–13 region on chromosome 15 is the most common duplication copy number variant associated with ASDs. Deletions of this region of the chromosome cause the neurodevelopmental disorders including Angelman syndrome and Prader–Willi syndrome.⁵⁻⁶ In humans, the q11.2–13 region of chromosome 15 contains genes that encode the α5, β3, and γ3 subunits of the γ-aminobutyric acid type A (GABA_A) receptor, as well as the ubiquitin protein ligase E3a.

Several lines of evidence have implicated α5 subunit-containing GABA_A receptors in ASDs. Postmortem analyses of brain tissue of individuals with ASDs have revealed reduced levels of both miRNA and protein for several GABA_A receptor subtypes including α5 and β3 subunits.⁷⁻⁹ Positron emission tomography studies have shown reduced binding of an α5GABA_A receptor-selective ligand in the amygdala and nucleus accumbens, brain regions that mediate social interaction and reward behaviors.¹⁰ Despite such compelling evidence, it remains uncertain whether reduced expression of α5GABA_A receptors contributes to the behavioral symptoms of ASDs.

The activity of GABA_A receptors is modified by proteins that regulate the trafficking and anchoring of GABA_A receptors to the plasma membrane. The anchoring of α5GABA_A receptors at extrasynaptic regions of neurons is regulated by the cytosolic protein radixin.¹¹
The role of radixin in ASDs has not been studied; however, exon deletions in the gene that encodes gephrin, another GABA<sub>A</sub> receptor-anchoring protein, have been linked to autism, schizophrenia, and seizures. Here, we studied whether deletion of the gene that encodes the α<sub>5</sub> subunit (Gabra<sub>5</sub><sup>−/−</sup>) in mice causes an autism-like behavioral phenotype. We also examined exome sequencing data from 396 human subjects to determine whether rare coding variants in the Gabra<sub>5</sub> gene or the radixin (RDX) gene were associated with autism.

**Materials and Methods**

**Experimental animals**

All experimental procedures were approved by the Animal Care Committee of the University of Toronto and were performed in accordance with guidelines of the Canadian Council on Animal Care. Gabri<sub>5</sub><sup>−/−</sup> mice were generated using a C57BL/6J and Sv129Ev background, as described previously. Male mice were used for all the behavioral assays except the measurements of ultrasonic vocalizations and pup retrieval. For these experiments, pups of both sexes were used and dams performed the pup retrieval. Age-matched 3- to 5-month-old mice were used to study social interaction, social preference, grooming, and executive function. Ultrasonic vocalization was measured on postnatal days 6–8. In the pup retrieval assay, the dams were greater than 3 months of age and studies were performed at postnatal days 6–8. Behavioral tests that have been previously used to study autism-like behaviors in mice were performed (Fig. 1A), as described in Data S1.

**Exome data from human probands**

The coding sequences of Gabra<sub>5</sub> (on human chromosome 15) and RDX (on chromosome 11) were examined for coding sequence variants. Next-generation exome sequencing data from 396 Canadian ASD probands was used to detect potential sequence variants, as previously described (see also Data S1). All subjects and/or parents consented to the study, which was approved by the Research Ethics Board of the Hospital for Sick Children. Following a general protocol that was similar to those used in previous studies, rare variants were defined as those with a frequency of less than 1% in population databases (The 1000 Genomes Project, NHLBI Exome Sequencing Project and the Exome Aggregation Consortium). All novel or rare nonsynonymous variants were validated using Sanger sequencing. Damaging missense single-nucleotide variants were defined as those predicted to be functionally damaging by SIFT and PolyPhen-2 prediction software.

**Results**

Reduced social contact is a common behavioral feature of ASDs. To study social contact, the social proximity assay was used to measure interactions between a test mouse and a conspecific. Gabra<sub>5</sub><sup>−/−</sup> mice exhibited significantly fewer social contacts than wild-type (WT) mice (<i>t</i>(24) = 2.28, <i>P</i> = 0.031 Fig. 1B). The numbers of nose-to-nose (<i>t</i>(24) = 4.68, <i>P</i> < 0.0001) and nose-to-head (<i>t</i>(24) = 4.14, <i>P</i> < 0.001) contacts were reduced in Gabra<sub>5</sub><sup>−/−</sup> mice. Other forms of social contact were similar between the genotypes (Fig. 1C).

Next, preference for social stimuli was assessed using the three-chamber social approach test. During the habituation phase of the study, WT and Gabra<sub>5</sub><sup>−/−</sup> mice spent equal time in the left and right chambers, indicating no inherent preference (genotype: <i>F</i><sub>1,72</sub> = 0.0, <i>P</i> = 1.0; interaction: <i>F</i><sub>2,72</sub> = 0.758, <i>P</i> = 0.472; center chamber: WT 148.0 ± 9.36 sec vs. Gabra<sub>5</sub><sup>−/−</sup> 133.58 ± 10.95 sec; left chamber: WT 364.29 ± 11.33 sec vs. Gabra<sub>5</sub><sup>−/−</sup> 372.75 ± 11.40 sec; right chamber: WT 387.71 ± 8.63 sec vs. Gabra<sub>5</sub><sup>−/−</sup> 393.67 ± 9.07 sec). During the testing phase, WT and Gabra<sub>5</sub><sup>−/−</sup> mice spent more time in the chamber that contained the conspecific. Thus, both genotypes exhibited a normal social preference in this test (chamber: <i>F</i><sub>1,72</sub> = 34.85, <i>P</i> < 0.0001; genotype: <i>F</i><sub>1,72</sub> = 0.02, <i>P</i> = 0.815; interaction: <i>F</i><sub>2,72</sub> = 0.33, <i>P</i> = 0.716; Fig. 1D).

To assess communication, we measured ultrasonic vocalizations (USVs) that were emitted by neonatal pups that had been separated from the dam. The latency to emit the first USV was increased in Gabra<sub>5</sub><sup>−/−</sup> dams relative to WTs (<i>t</i>(13) = 3.27, <i>P</i> = 0.006; Fig. 1E). The total number of calls was reduced in Gabra<sub>5</sub><sup>−/−</sup> mice (<i>t</i>(13) = 2.47, <i>P</i> = 0.029; Fig. 1E). In addition, the latency time recorded during the first minute of separation was reduced in Gabra<sub>5</sub><sup>−/−</sup> mice compared to WT mice, demonstrating a reduction in vocalization (Mann–Whitney <i>U</i> = 10.0, <i>P</i> = 0.04; Fig. 1E). The average length of individual USVs was no different between groups (<i>t</i>(13) = 1.14, <i>P</i> = 0.274; Fig. 1E).

To determine whether there were functional implications of the reduced USVs, the time required for the dams to retrieve five pups to the nest following the 3-min separation period was measured. The latency to retrieval was increased in Gabra<sub>5</sub><sup>−/−</sup> dams relative to WT dams (<i>t</i>(17) = 2.49, <i>P</i> = 0.024; Fig. 1F).

Next, repetitive behaviors, which are a common feature of ASDs, were studied. Such unusually long periods of self-grooming in mice are considered to be a spontaneous
form of motor stereotypy.\textsuperscript{20} \textit{Gabra5}\textsuperscript{-/-} mice spent more time self-grooming than WT mice during a 10-min observation period ($t_{(16)} = 3.25$, $P = 0.005$; Fig. 1G).

Executive function, which refers to problem solving and cognitive flexibility, is often impaired in ASDs.\textsuperscript{21} Executive function was assessed with the puzzle box. In this assay,
Figure 2. Executive function is impaired in Gabra5<sup>−/−</sup> mice. (A) Schematic of the puzzle box test. (B) Gabra5<sup>−/−</sup> mice and WT mice exhibited a similar latency at baseline, to enter the goal box through the open door and a similar latency on day 1, when they were required to use the underpass to enter the goal box. Gabra5<sup>−/−</sup> exhibited a longer latency than WT mice to burrow through bedding on day 2, or remove a cardboard plug on day 3 to gain access to the goal box. Two-way analysis of variance (ANOVA); n = 9–10; effect of genotype, P < 0.0001; effect of trial, P < 0.0001; effect of interaction, P < 0.001. Tukey’s HSD post hoc test; *P < 0.05, **P < 0.001. (C) Short-term memory (STM) on the puzzle box test, tested 2 min after first exposure to the task. Gabra5<sup>−/−</sup> mice exhibit impaired short-term memory and a longer latency to complete the short-term memory plug task. Two-way analysis of variance (ANOVA); n = 9–10; effect of genotype, P < 0.01; effect of trial, P < 0.0001; effect of interaction, P < 0.05; Tukey’s HSD post hoc test, **P < 0.001. (C) Long-term memory (LTM) on the puzzle box test, tested 24 h after first exposure to the task. Gabra5<sup>−/−</sup> and WT mice exhibit similar performance on the underpass and burrowing long-term memory tasks. Two-way analysis of variance (ANOVA); n = 9–10; effect of genotype, P = 0.238; effect of trial, P < 0.01; effect of interaction, P = 0.979. (E–G) Performance of WT and Gabra5<sup>−/−</sup> mice in the open-field test. (E) Gabra5<sup>−/−</sup> and WT mice exhibited a similar distance travelled in the open-field test over a 30-min test period. Gabra5<sup>−/−</sup> and WT mice spent a similar amount of time in the center (F) and perimeter (G) regions of the open field. Student’s t-test; n = 10. Data are presented as mean ± SEM.
mice were presented with progressively more difficult tasks to reach the goal (darkened) box (Fig. 2A). Relative to WT mice, Gabra5−/− mice required more time to reach the goal box and thus exhibited impaired performance on the first exposure to a new challenge that required burrowing (Tukey’s post hoc \( P < 0.05 \)) and for the removal of a plug that obstructed the underpass (post hoc test \( P < 0.001 \), genotype \( F_{1,68} = 20.31, P < 0.0001 \); interaction \( F_{3,68} = 6.56, P < 0.001 \); Fig. 2B). Short-term memory, an important element of executive function, was assessed by retesting the mice 2 min after the first exposure to the task. Latency for the plug task was longer in Gabra5−/− mice (genotype \( F_{1,51} = 9.91, P = 0.003 \); interaction \( F_{2,51} = 5.75, P = 0.006 \); post hoc test \( P < 0.001 \); Fig. 2C). Long-term memory tested 24 h after the first exposure to both the underpass and burrowing tasks was not impaired in Gabra5−/− mice (Fig. 2D).

In the open-field test, no differences were observed between WT and Gabra5−/− mice (Fig. 2E–G) suggesting normal locomotion and anxiety in Gabra5−/− mice.

Mutations in Gabra5 and Rdx in ASD probands

De novo and rare inherited sequence-level variants have been shown to contribute to ASD risk.\(^2\)\(^–\)\(^3\)\(^,\)\(^23\) Consequently, the coding sequences of Gabra5 and Rdx were screened for coding sequence variation using next-generation exome sequencing data from a cohort of 396 Canadian ASD probands. Two rare missense coding variants were identified in Gabra5, each in a single male ASD case. One of the variants was predicted to be functionally damaging as indicated by both PolyPhen-2 and SIFT prediction software (Table 1). Four missense coding variants were identified in Rdx. One of the variants (hg 19 chr11:110,104,062) was present in three male probands, whereas the remaining variants were present in single

ASD cases, two male and one female. Two of the variants in Rdx were predicted to be functionally damaging.

Discussion

Global deletion of the Gabra5 gene causes autism-like behaviors that are similar to those observed in other ASD mouse models, including the Tuberous sclerosis 1 mouse, the Shank1 null-mutant mouse, and inbred BTBR T+tf/J mice.\(^1\)\(^6\)\(^–\)\(^1\)\(^8\)\(^–\)\(^2\)\(^0\) Having identified a behavioral phenotype in Gabra5−/− mice, we sought to determine whether pathogenic variants in Gabra5 or Rdx might be found in human subjects with ASD. From a cohort of 396 cases analyzed by exome sequencing, we identified six rare missense variants (<1% frequency in population databases). Three of these missense mutations were predicted to damage protein function. Rdx encodes the anchoring protein radixin and damaging coding variants are predicted to decrease the number of zGABA\(_A\) receptors at extrasynaptic sites. Although rare missense coding variants of Rdx have not been previously reported in ASD cases, exonic deletions of the GABA\(_A\) receptor-anchoring protein gephyrin have been associated with psychiatric conditions, including autism.\(^1\)\(^2\) It remains to be determined whether the variants identified in this study contribute to ASD cases.

Individuals with autism frequently exhibit problems with learning and memory. Results from this study showed that Gabra5−/− mice exhibit deficits in short-term memory but only when the task became increasingly more difficult (i.e., the plug task). In contrast, no long-term memory deficits were observed in the Gabra5−/− mice. These experimental results are consistent with previous reports that show deficits depend on cognitive domain and demand of the task. For example, memory performance of Gabra5−/− mice is unimpaired for contextual fear memory, cued fear conditioning and novel

| Gene    | Position | Proband | Codon change | Substitution | Inheritance | PolyPhen-2 prediction | SIFT prediction |
|---------|----------|---------|--------------|--------------|-------------|----------------------|----------------|
| Gabra5  | chr15:27,182,361 | 1M | Gtc/Atc | V204I | Maternal | 0.005 benign | 0.41 tolerated |
| Gabra5  | chr15:27,128,545 | 1M | gGg/gGc | G113A | Maternal | 0.991 probably damaging | 0.04 damaging |
| Rdx     | chr11:110,104,002 | 1M | aCdaTtc | T516I | Paternal | 0.998 probably damaging | 0.02 damaging |
| Rdx     | chr11:110,104,138 | 1M | Cct/Act | P471T | Maternal | 0.585 possibly damaging | 0.51 tolerated |
| Rdx     | chr11:110,128,601 | 1F | Gct/Cat | D197H | Heterozygous in both | 0.999 probably damaging | 0.0 damaging |
| Rdx     | chr11:110,104,062 | 3M | gCt/gTt | A496V | 1 Maternal | 0.999 probably damaging | 0.52 tolerated |

The position of the mutation, the sex of the proband (M, male; F, female), the specific codon change, the resultant amino acid substitution, and the inheritance (maternal, paternal, or both) are listed. The prediction scores generated by PolyPhen-2 and SIFT software are listed for each mutation. A PolyPhen-2 score <0.5 denotes a mutation that is predicted to be benign, a score >0.5 denotes a mutation that is probably damaging, and a score = 1 denotes a mutation that is predicted to be damaging. A SIFT score <0.05 denotes a damaging mutation and a score >0.05 denotes a tolerated mutation.
object recognition. However, selective knockdown of Gabra5 in the dentate gyrus of the hippocampus caused impaired performance when mice were required to distinguish between an aversive context and a similar safe context. Reversal learning in the Morris Water Maze task was also impaired in these mice. Interestingly, Gabra5−/− mice show improved performance for trace fear conditioning and the Morris water maze compared with WT mice. Thus, only certain learning and memory tasks are vulnerable to reduced expression levels of z5GABA_A receptors.

The role of z5GABA_A receptors in memory formation is further supported by previous studies of long-term potentiation (LTP) of excitatory synaptic transmission in the hippocampus. LTP is widely considered to be a network substrate of memory and z5GABA_A receptors set the level of stimulation that is required to induce LTP in the CA1 subfield of the hippocampus. Specifically, stimulation of Schaffer collaterals at a low frequency (10 Hz) elicits long-term depression of excitatory transmission in slices from wild-type mice, whereas the same level of stimulation elicits LTP in Gabra5−/− slices. Thus, z5GABA_A receptors set the threshold for stimulating LTP and may therefore be involved in memory formation. Consistent with the above findings, a current theory suggests that autism-like behaviors result from an increase in the ratio of excitatory to inhibitory neurotransmission (E/I) in the brain. The autism-like behaviors observed in Gabra5−/− mice may result from an increased E/I ratio. Indeed, Gabra5−/− mice exhibit a reduced tonic inhibitory conductance and increased excitability of principal neurons in the hippocampus. In other brain regions, this increase in neuronal excitability may lead to autism-like behavioral deficits. Even transient depolarization of neurons using optogenetic techniques in the medial prefrontal cortex causes deficits in social behavior, and concomitant photostimulation of inhibitory, GABAergic neurons partially reverses these deficits. Similarly, treatment with a drug that increases GABAergic function reverses abnormal social behavior in the Scn1a+/− mouse model of autism.

The results from the current study suggest that drugs that act as positive allosteric modulators of z5GABA_A receptors may ameliorate autism-like behaviors. Certain positive allosteric modulators that reverse deficits in spatial memory in aged rats and locomotor hyperactivity in a mouse model of schizophrenia may reduce autism-like behavioral deficits.

Finally, reduced expression and function of GABRA5 and RDX may cause neurodevelopmental changes that contribute to ASD-like behavior. In future studies, it will be of interest to determine whether clinical disorders (e.g., seizures or cognitive defects) are observed in individuals with mutations of GABRA5 or RDX genes. Such an association would further strengthen the E/I hypothesis of autism. In summary, our results show that reduced expression of z5GABA_A receptors contributes to autism-like behaviors in mice and potentially damaging mutations of GABRA5 and RDX occur in ASD cases.

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Author Contributions

A. A. Z. conceived and designed the study, acquired and analyzed the data, and drafted the manuscript and figures. S. W. P. K. acquired and analyzed the data and contributed to the drafting of the manuscript. Z. A. acquired and analyzed the data. S. W. acquired and analyzed the data and contributed to the drafting of the manuscript. M. M., A. J. M., and E. S. contributed to the study design and drafting of the manuscript. B. A. O. contributed to the study design and drafting of the manuscript and figures.

Conflict of Interest

None declared.

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Supporting Information

Additional Supporting Information may be found online in the supporting information tab for this article:

Data S1. Detailed Methods