Gain-of-function glutamate receptor interacting protein 1 variants alter GluA2 recycling and surface distribution in patients with autism

Rebeca Mejias[a], Abby Adamczyk[a], Victor Anggono[b,c], Tejasvi Niranjan[a,d], Gareth M. Thomas[b,c], Kamal Sharma[b,c], Cindy Skinner[e], Charles E. Schwartz[e], Roger E. Stevenson[e], M. Daniele Fallin[f], Walter Kaufmann[g,h], Mikhail Pletnikov[b,h], David Valle[a], Richard L. Huganir[b,c,1], and Tao Wang[a,1]

[a]McKusick-Nathans Institute of Genetic Medicine and Department of Pediatrics, [b]Department of Neuroscience, [c]The Howard Hughes Medical Institute, [d]Predoctoral Training Program in Human Genetics, and [h]Department of Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, MD 21205; [e]Greenwood Genetic Center, Greenwood, SC 29646; [f]Department of Epidemiology, The Johns Hopkins University School of Public Health, Baltimore, MD 21205; and [g]The Kennedy Krieger Institute, Baltimore, MD 21205

Contributed by Richard L. Huganir, February 14, 2011 (sent for review December 13, 2010)

Glutamate receptor interacting protein 1 (GRIP1) is a neuronal scaffolding protein that interacts directly with the C termini of glutamate receptors 2/3 (GluA2/3) via its PDZ domains 4 to 6 (PDZ4–6). We found an association ($P < 0.05$) of a SNP within the PDZ4-6 genomic region with autism by genotyping autistic patients ($n = 480$) and matched controls ($n = 480$). Parallel sequencing identified five rare missense variants within or near PDZ4–6 only in the autism cohort, resulting in a higher cumulative mutation load ($P = 0.032$). Two variants correlated with a more severe deficit in reciprocal social interaction in affected sibling pairs from proband families. These variants were associated with altered interactions with GluA2/3 and faster recycling and increased surface distribution of GluA2 in neurons, suggesting gain-of-function because GRIP1/2 deficiency showed opposite phenotypes. *Grip1/2* knockout mice exhibited increased sociability and impaired prepulse inhibition. These results support a role for GRIP in social behavior and implicate *GRIP1* variants in modulating autistic phenotype.

Autism spectrum disorders are a group of highly heritable, heterogeneous disorders with 70% to 90% concordance in monozygotic twins (1, 2). Rare deleterious mutations of large effect are increasingly recognized as contributing to genetic predisposition in autism (3, 4). Point mutations, de novo deletions, and duplications associated with autism were found in genes controlling glutamate receptor clustering, synaptogenesis, axon guidance, and dendritic development (5–8). These results support the hypothesis that autism may be caused by an accumulation of individually rare mutations in multiple genes leading to disturbances in shared neurodevelopmental pathways and consequent behavioral phenotype. Identifying these genes and understanding the consequences of their mutations will shed light on the disease mechanism in autism (9).

Previous studies have implicated impaired glutamate signaling in autism. The levels of glutamate and *N*-acetyl-aspartate (10) and activities of glutamic acid decarboxylase—the rate-limiting enzyme that converts glutamate to GABA (11)—were lower in brains from individuals with autism. Abnormally increased transcripts of AMPA glutamate receptor 1, glial glutamate transporter 1, and glutamate receptor interacting protein 1 (GRIP1) were also found in the cerebellum of autistic patients (12). Multiple de novo deletions and duplications involving genes in the glutamate signaling pathway were identified in large autism cohorts (6, 13) These results highlight glutamate-signaling genes as promising candidates for genetic predisposition in autism.

GRIP1 was mapped to 12q14.3, a region that was implicated in autism in a study using Affymetrix GeneChip analysis (14). GRIP1 plays an important role in receptor trafficking, synaptic organization, and transmission in glutamatergic and GABAergic synapses (15–19). Interaction of PDZ domains 4 to 6 (PDZ4–6) with AMPA glutamate receptors, GluA2/3, is essential for receptor targeting and localization to the postsynaptic membrane and for activity-dependent synaptic reorganization of AMPA receptors (16, 20).

Results

We identified an association ($P = 0.048$) of a common SNP (rs7397862) in intron 15 of *GRIP1* within the region encoding PDZ4–6 (exons 12–16) in a case-control study of autism ($n = 480$) and ethnically matched normal controls ($n = 480$) (Tables S1 and S2). We built on this initial finding to perform parallel sequencing of all 25 exons and ~50-bp flanking introns of *GRIP1* in both cohorts. This analysis identified five missense variants involving highly conserved amino acid residues within or near PDZ4–6 only in autistic patients (Fig. 1*A*) (21). All five are transmitting variants and each was found in a single family (Table S3). The cumulative allele frequency of the five variants was higher ($P = 0.032$) in autism compared with ethnically matched controls (Table S2). Additionally, one missense variant, V54I in PDZ1, was found in two patients and two missense variants, Q821E and R869Q near PDZ7, were found in both autism and controls (Fig. 1*A*). Noncoding variants of interest within the immediate flanking intronic regions of exons 12 to 18 encoding PDZ4–6 include a single base-substitution (T > C) at position −3 of intron 13 in one patient, a three base (TTG) deletion at positions −10 to −12 of intron 11 in three patients and one control, and two common SNPs at positions +13 (rs7397862) and +34 (rs7397861) of intron 15 in both autism patients and controls (Fig. S1).

In particular, two variants, A625T and M794R, were found in families with two affected brothers who had different genotypes for respected *GRIP1* variants. This finding permitted a correlation study of the presence of these variants with the severity of autism phenotype, as defined primarily by Autism Diagnostic Interview-Revised (ADI-R) scores (Table 1). In the first family, the affected boy, who was heterozygous for A625T, had relatively (with respect to other components of these instruments) more impairment in ADI-R's Reciprocal Social Interaction, the Au-

Author contributions: R.M., D.V., R.L.H., and T.W. designed research; R.M., A.A., V.A., and T.N. performed research; V.A., G.M.T., K.S., C.S., C.E.S., R.E.S., and M.D.F. contributed new reagents/analytic tools; R.M., A.A., V.A., T.N., G.M.T., K.S., W.K., M.P., and T.W. analyzed data; and R.M., A.A., D.V., R.L.H., and T.W. wrote the paper.

Conflict of interest statement: Under a licensing agreement between Millipore Corporation and The Johns Hopkins University, R.L.H. is entitled to a share of royalties received by the University on sales of products described in this article. R.L.H. is a paid consultant to Millipore Corporation. The terms of this arrangement are being managed by The Johns Hopkins University in accordance with its conflict-of-interest policies.

[1]To whom correspondence may be addressed. E-mail: twang9@jhmi.edu or rhuganir@jhmi.edu.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1102233108/-/DCSupplemental.

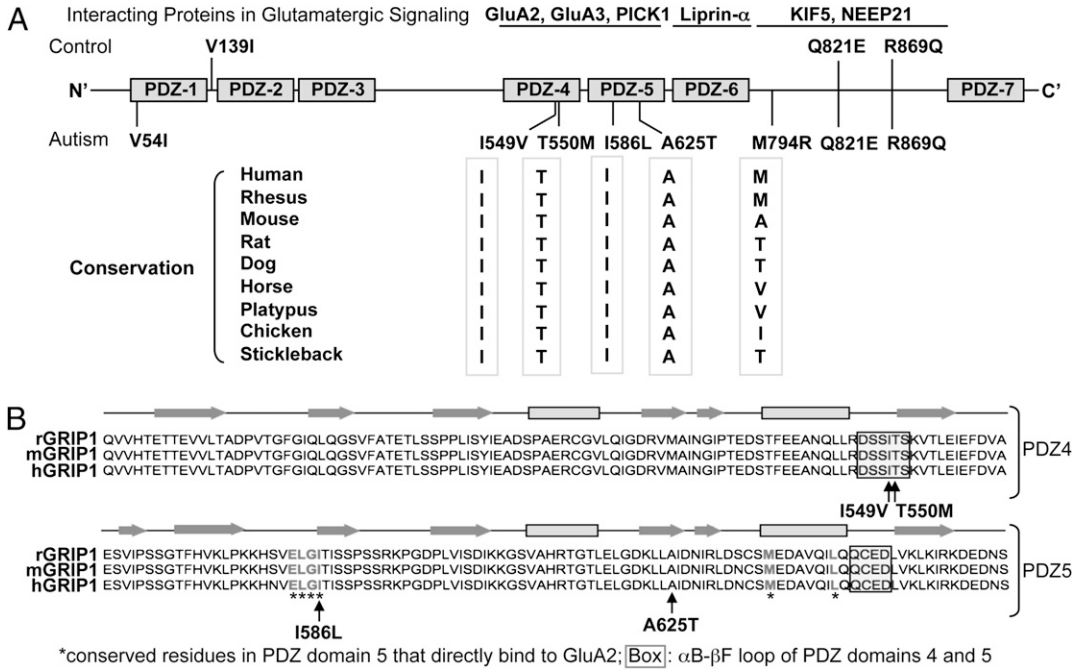

Fig. 1. Missense variants of GRIP1-PDZ4-6 in autism, evolutionary conservation, locations in an NMR structure of PDZ domains 4 and 5 (PDZ4–5), and known interacting proteins with PDZ4–6. (*A*) PDZ domain structure, locations of missense variants in both controls and autism, and alignment of nine species for the amino acid residues involved. PDZ4–6 domains interact with proteins involved in excitatory neurotransmission (GluA2 and GluA3, AMPA glutamate receptor 2 and 3 subunits; PICK1, protein interacting with protein kinase C, Liprin-α; KIF5, kinesin superfamily protein 5; NEEP21, neuron-enriched endosomal protein 21). (*B*) PDZ4-5 NMR structure overlapping with corresponding peptide sequence of rat, mouse, and human GRIP1 (modified from ref. 21). The nature and locations of the variants within PDZ4-5 domains are indicated by arrows.

tism Diagnostic Observation Schedule's Reciprocal Social Interaction domain, and Vineland Adaptive Behavior Scales' Socialization scale, compared with his affected brother, who did not carry this variant. In the second family, the boy who was homozygous for M794R also had relatively worse scores on ADI-R's Reciprocal Social Interaction domain, compared with his affected brother, who was heterozygous for this variant. Interestingly, their mother, who was also heterozygous for M794R but not diagnosed with autism, had a history of restricted interests, repetitive behavior, poor eye contact, and eccentricity.

Table 1. Neurological and behavioral phenotype of sibling pairs with different genotype in two families with *GRIP1* variants

Genotype and phenotype		GRIP-A625T		GRIP1-M794R	
		Wild-type	Heterozygous	Heterozygous	Homozygous
Subject	Affected with Autism	Yes	Yes	Yes	Yes
	Age (years at interview)	7	9	12	10
	Sex	Male	Male	Male	Male
	Ethnicity	Nonhispanic white	Nonhispanic white	Nonhispanic white	Nonhispanic white
	Relationship	Full sibling	Full sibling	Full sibling	Full sibling
Motor Milestones	Roll_month	3	3	7	4
	Sit_month	5	4	7.5	8
	Walk_month	15	10	14	15
PPVT	Standard scores	48	38	134	20
ADI-R	Reciprocal Social Interaction	20	24	22	26
	Verbal Communication	16	18	16	—
	Nonverbal Communication	—	—	(11)#	13
	Repetitive and Stereotypic Behaviors	4	6	10	8
Pedigree	Heterozygous for the variant */+				
	Homozygous for the variant **				
	Wild-type allele, no variant +/+				
	Open symbol, unaffected				
	Filled symbol, affected with autism				

Squares filled with black indicate individuals with a clinical diagnosis of autism and unfilled symbols (circles or squares) indicate individuals without a clinical diagnosis of autism; all individuals shown in the pedigrees were genotyped for respected *GRIP1* variants. Abbreviations: PPVT: Peabody Picture Vocabulary Test; ADI-R: Autism Diagnostic Interview-Revised. # , scores on ADI-R Nonverbal Communication component; */+, heterozygous for the indicated *GRIP1* allele; **, homozygous for the indicated *GRIP1* allele; +/+, WT allele.

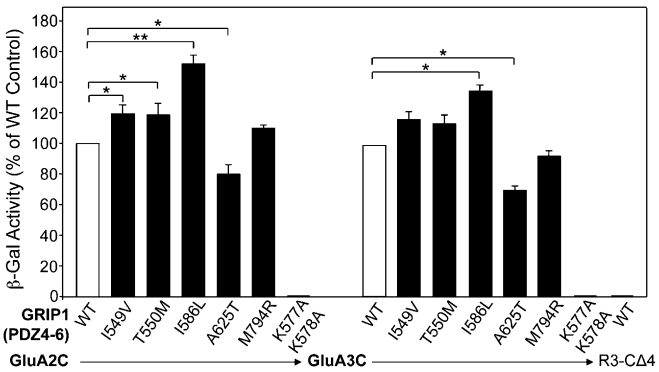

Fig. 2. Interaction of GRIP1 variants with the C terminus of GluA2/3 in a yeast two-hybrid assay. GluA2C or GluA3C (C-terminal 50 amino acids) and expression constructs for individual GRIP1 variants (containing PDZ4–6) were cotransformed into Y190. K577A-K578A: K577_K578 of GRIP1 were changed to A577_A578; R3-CΔ4: deletion of the last four amino acids at the C terminus of GluA3; both completely block the interaction between GRIP1 and GluA2/3. The Mean and SEM of β-galactosidase enzyme activities are shown. ANOVA followed by Student–Newman-Keuls method (n = 6 yeast clones). *$P < 0.05$, **$P < 0.01$.

The two aforementioned socially more-impaired subjects also had overall higher ADI-R scores and lower cognitive—but not motor—skills than their brothers.

The five GRIP1 variants (I549V, T550M, I586L, A625T, M794R), clustered around PDZ4–6 and found only in autism subjects, were further studied because these domains are known to directly interact with the C terminus of GluA2/3. The amino acid residues I549 and T550 within PDZ4, and I586 and A625 within PDZ5, are identical in a nine-species alignment, and the aliphatic/nonpolar nature of the amino acid at M794 is also conserved (Fig. 1*A*). Mapping the residues to a published NMR structure of PDZ4/5 (21) revealed that I549 and T550 are located within a six-amino acid motif (DSS**I**T**S**) of the αB-βF loop in PDZ4, which is essential for stabilizing the PDZ4/5 supermodule. Residue I586 is part of the four-amino acid motif (ELG**I**) that directly binds the C terminus of GluA2/3, and A625 is a highly conserved residue within a βB loop in PDZ5 (Fig. 1*B*). We hypothesized that these GRIP1 variants would display altered interactions with GluA2/3 and would affect GluA2/3 function and distribution in neurons.

To address the first possibility, we determined the in vitro interaction of the five variants with C-terminal domains of GluA2/3 (GluA2C and GluA3C) using a yeast two-hybrid assay. Deletion of the C-terminal four amino acids of GluA2/3 (R3-CΔ4) or artificial mutations, K577A_K578A, of GRIP1 are known to completely abolish GRIP1-GluA2/3 interaction. As shown in Fig. 2, we found a consistent increase in the interaction of three variants, I549V, I550M, and I586L with GluA2C, as reflected by β-galactosidase activity (WT, 100.0%; I549V, 120.5 ± 4.2%, $P = 0.023$; I550M, 119.3 ± 7.1, $P = 0.019$; I586L, 152.0 ± 5.3%, $P < 0.001$; A625T, 80.2 ± 5.9%, $P = 0.006$; M794R, 110.3 ± 2.0%; K577A_K578A, 0.0 ± 0.0%; $n = 6$ yeast clones) and GluA3C (WT 100.0%; I549V, 114.6 ± 5.7%; I550M, 114.0 ± 4.0; I586L, 133.7 ± 11.0% $P = 0{,}001$; A625T, 71.5 ± 2.6% $P = 0{,}01$; M794R, 91.7 ± 3.2%; K577A_K578A, 0.0 ± 0.0%; R3-CΔ4, 0.0 ± 0.0%; $n = 6$). The other two variants, A625T and M794R, showed moderately reduced interaction. Interestingly, I586L showed the most significant increase in the interaction, which is consistent with the NMR structure of PDZ4/5, in which I586 directly binds GluA2C and GluA3C (21).

GRIP1 was recently reported to modulate the activity-dependent trafficking of a pH-sensitive pHluorin-GluA2 fusion protein (pH-GluA2) (22). We therefore examined the effects of altered GRIP1-GluA2/3 interaction of three GRIP1 variants (T550M, I586L, A625T) on pH-GluA2 internalization and recycling in live, transfected hippocampal neurons (23). Expression of both WT and GRIP1 variants in neurons mimics the heterozygous state of these variants in individuals with autism. Compared with WT GRIP1, all three variants showed comparable amplitude of fluorescence changes, reflecting the rate of pHluorin-GluA2 internalization following NMDA stimulation (Fig. 3 *A*, *B*, and D) (amplitude, WT 0.70 ± 0.03; T550M 0.67 ± 0.03; I586L, 0.72 ± 0.02; A625T, 0.63 ± 0.03) but a faster recycling of pHluorin-GluA2 as reflected by shorter T1/2, the time for recovery of 50% of fluorescence. The differences in two variants, I586 and T550M, reached statistical significance (Fig. 3 *A–C*) [$t_{1/2}$(min), WT, 6.66 ± 0.64; T550M, 5.37 ± 0.64, $P = 0.174$; I586L, 3.97 ± 0.38, $P = 0.003$; A625T, 3.97 ± 0.83, $P = 0.026$; n = 6–13 neurons]. Consistent with the yeast two-hybrid data, I586L showed the most significant effect on GluA2 recycling. Because *Grip1/2*-deficient hippocampal neurons display slower

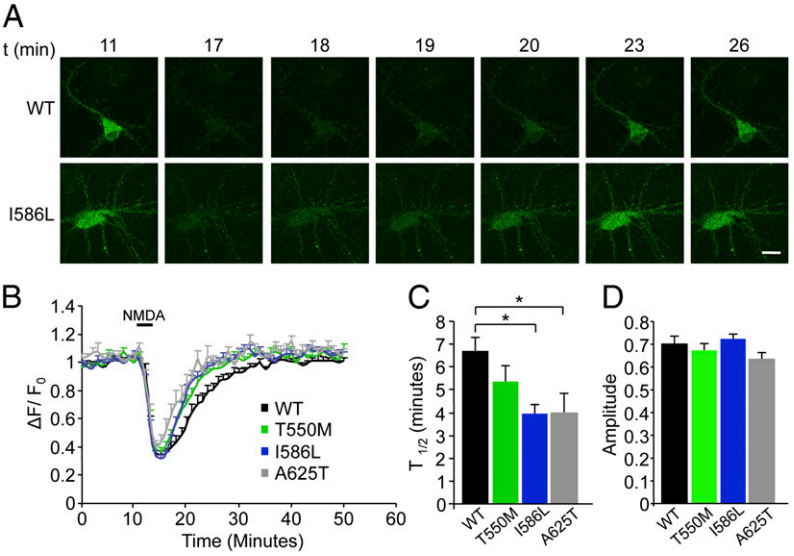

Fig. 3. GRIP1 variants alter GluA2 recycling process in hippocampal neurons. (*A*) Images from pHluorin assay of hippocampal neurons (d in vitro 16) transfected with wt GRIP1 and I586L GRIP1 variant during NMDA (3-min treatment: 11–14 min) perfusion/washout experiments. (Scale bar, 10 μm.) (*B*) Average pHluorin-GluA2 fluorescence time course from WT GRIP1 and T550M, I586L and A625T GRIP1 variants in response to 3-min NMDA treatment. (*C*), Histogram of $T_{1/2}$ from WT GRIP1 and T550M, I586L, and A625T GRIP1 variants after NMDA washout. (*D*) Histogram of pHluorin fluorescence change amplitude in response to NMDA treatment. Data presented as mean ± SEM (n = 6–13 neurons). Student's t test, *$P < 0.05$.

GluA2 recycling in the same assay (22), these results suggest a gain of GRIP1 function for these variants on GluA2 recycling.

Finally, we determined the relative abundance of surface versus total GluA2 in hippocampal neurons transfected with individual *GRIP1* variants. Among the five variants, three (I586L, A625T, M794R) increased the relative abundance of surface GluA2 (Fig. 4). The differences in two variants, I586L and A625T, reached statistical significance compared with WT GRIP1 (surface/total GluA2 (%), WT, 100.0; I549V, 100.6 ± 9.9; T550M, 106.0 ± 8.7; I586L, 163.9 ± 22.7, $P = 0.006$; A625T, 141.9 ± 10.8, $P < 0.001$; M794R, 123.3 ± 14.5; $n = 11$–22 neurons). These data are consistent with that from the pHluorin-GluA2 recycling studies (Fig. 3).

GRIP1 and GRIP2 are highly homologous proteins that are known to complement each other for certain neuronal functions (16). To understand the role of GRIP1/2 in modulating social behaviors, we studied the loss-of-function phenotype of *Grip1/2* double-knockout (DKO) mice. DKO mice were generated by crossing *Grip2* conventional KO mice with conditional *Grip1* (neuron-specific deletion via Nestin-Cre expression) KO mice on C57BL6/129 strain background (17) to circumvent the embryonic lethality of conventional *Grip1* KO mice (24), and *Grip1* deletion was then achieved by crossing with Nestin-Cre mice. Cre-dependent *Grip1* deletion was verified in brain lysates by Western blot analysis. Control mice (WT) were matched for age, sex, and strain background with DKO mice. A standard battery of behavioral tests, including an open-field, elevated plus maze, Y-maze, intruder-resident test, novel object recognition, sociability toward stranger mice, and prepulse inhibition (PPI) were used. Compared with WT, *Grip1/2* DKO mice showed a selective, significant increase in sociability toward stranger mice (empty cage in seconds: WT, 98 ± 9; DKO, 112 ± 11; with stranger mice in seconds: WT, 116 ± 10; DKO, 192 ± 23, $P = 0.012$; $n = 10$ mice), impaired PPI (percent of inhibition; WT, 54 ± 25 and DKO, 84 ± 18 at p74; WT, 27 ± 8 and DKO, 79 ± 18 at p78; $P = 0.012$; WT, 21 ± 5 and DKO, 59 ± 17 at p82; $P = 0.035$; WT, 16 ± 3 and DKO, 50 ± 15 at p86; $P = 0.034$; WT, 17 ± 4 and DKO, 41 ± 11 at p90, $P = 0.041$; $n = 10$ mice), and impairment in recognizing a novel object (time spent interacting with object in seconds: WT with familiar object, 7.1 ± 1.2; DKO with familiar object, 12.0 ± 1.9, $P = 0.043$; WT with novel object, 12.0 ± 2.4; DKO with novel object 12.8 ± 2.0) (Fig. 5). Lack of difference for other behavioral tests is depicted in Fig. S2. These results support a role for GRIP1/2 proteins in the modulation of social behaviors in mammals.

Discussion

We extended a previous linkage to chromosome 12q14 by identifying an association of a common SNP within the genomic region encoding PDZ4–6 of GRIP1 with autism. Sequencing of *GRIP1* in autism and matched control cohorts identified five rare missense variants located within or near PDZ4–6 only in the autism cohort. Importantly, these variants showed a higher cumulative mutation load in autism and were found to alter GRIP1 interaction with GluA2/3 in in vitro and neuron-based assays. A positive correlation of two variants with greater autistic behavioral severity, particularly of the core social-interaction domain, as measured by ADI-R, was found in families with affected sibling pairs. More severe genotypes in these families were also linked to greater cognitive impairment. Although three of the four parents in these two families are heterozygous for the respective variants, none of them was diagnosed with autism. Limited behavioral data were available only for the mother who was heterozygous for M794R and was noted having a history of

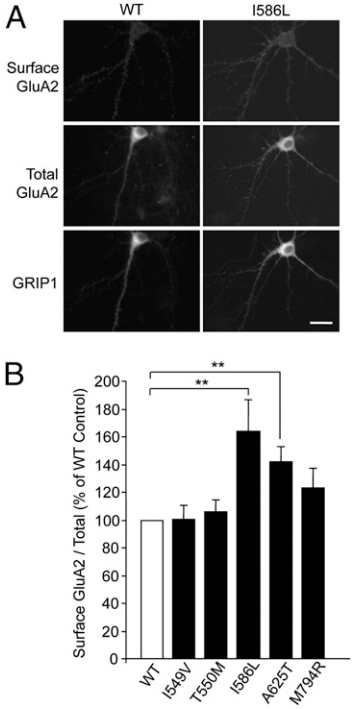

Fig. 4. GRIP1 variants increase GluA2 surface expression in transfected hippocampal neurons. (*A*) Representative microscope images of surface GluA2 (*Top*), total GluA2 (*Middle*), and GRIP1 (*Bottom*) immunofluorescence in hippocampal neurons (d in vitro 16) cotransfected with myc-WT GRIP1 or myc-I586L GRIP1 variant and GFP-GluA2 (for 48 h). (Scale bar, 10 μm.) (*B*) Average fluorescence (expressed in percentage of the WT control) coming from surface GluA2 immunostaining normalized by total amount of transfected GluA2 receptor in dendritic areas of neurons cotransfected with myc-WT GRIP1 or myc-GRIP1 variants (T550M, I586L, A625T, or M794R) and GFP-GluA2. Fluorescent intensities were quantified using ImageJ software (NIH) in 11 to 22 neurons (coming from two independent experiments) per condition using one to six dendritic areas per neuron analyzed. Data presented as mean ± SEM ($n = 11$–22 neurons). Student's *t* test, $^{**}P < 0.01$.

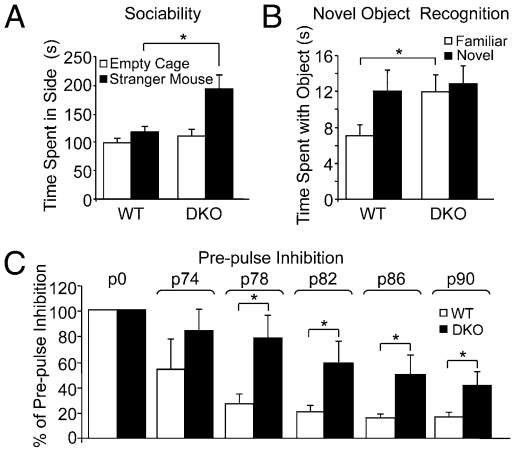

Fig. 5. Increased sociability and decreased prepulse inhibition of *Grip1/Grip2* DKO mice. *Grip1/Grip2* DKO were generated as described previously (17), and were subjected to a battery of behavioral tests following protocols from the Johns Hopkins Behavioral Core (www.brainscienceinstitute.org/index.php/cores/). (*A*) Mouse sociability testing for WT and *Grip1/Grip2* DKO mice with age-, and sex-matched WT controls ($n = 10$, 3–6 mo) revealed an increase in the time that DKO spent interacting with a reference mouse. (*B*), Time spent with a novel object compared with a familial object in the Object Recognition test. (*C*) Decrease in PPI of acoustic startle response in *Grip1/Grip2* DKO mice with regards to WT controls ($n = 10$, 3–6 mo). Data presented as mean ± SEM ($n = 10$ mice). Student's *t* test, $^{*}P < 0.05$.

GENETICS

restricted interests, repetitive behavior, poor eye contact, and eccentricity. Together, these results suggest that GRIP1 variants may modify the severity of the deficits in social interactions and cognitive function in autistic patients, but are not sufficient by themselves to cause autism.

Because most patients carrying *GRIP1* variants in this study are heterozygotes, we speculate that these variants affect neuronal and behavioral phenotype through a dominant mechanism. Consistent with this hypothesis, we found that three variants (I549V, T550M, and I586L) increased interaction with GluA2, two variants (I586L and A625T) are associated with a faster recycling of GluA2, and three variants (I586L, A625T, and M794R) increased surface GluA2 in hippocampal neurons. Because loss of GRIP1/2 function was associated with reduced interaction and slower GluA2 recycling (22), our data support a gain of GRIP1 function in these variants. Although disruption of GluA2-GRIP interactions in neurons also alters synaptic function and plasticity (19, 25), the effect of *GRIP1* variants on these readouts remains to be elucidated.

Among the five variants, I586L most strongly affected GRIP1 interaction with GluA2/3 in a yeast two-hybrid assay, GluA2 recycling, and surface distribution. This finding is consistent with the NMR structure of PDZ4/5, in which I586 is one of the four amino acid residues that bind directly to GluA2/3 (Fig. 1*B*). We recognize that not all variants exhibited the same profile of changes in different assay systems. This finding suggests that additional factors may be involved, which may not be satisfactorily explained based on a simple model of one-on-one interaction between GRIP1 and GluA2/3. These contributing factors may include the following: (*i*) PDZ4 and -5 form a protein supermodule that interacts with GluA2/3, but individual PDZ domains play different roles: I549 and T550 are located in PDZ4, which stabilizes the supermodule; I586L and A625T are located in PDZ5, which mediates the direct interaction of GRIP1 with GluA2/3; (*ii*) multimerizations of GRIP1 with GRIP1, and GRIP1 with GRIP2 likely affect GRIP function in neurons; (*iii*) PDZ4–6 are known to interact with several other proteins in the glutamatergic and GABAergic signaling pathways. It is possible that some effects of GRIP1 variants are exerted via interactions with one or more of these proteins. Studies of neurons from knock-in mice carrying specific Grip1 variants will be needed to further understand the underlying neuronal mechanisms.

The behavioral studies of *Grip1/2* DKO mice also suggest a role for GRIP proteins in the modulation of social behavior and possibly cognitive or memory function. *Grip1/2* DKO mice exhibited increased sociability, impaired PPI, and object recognition. Deficits in sociability are one of the core features of autism. Impaired PPI was observed in a small group of adult patients with autism (26) and in mice deficient in neurexin-1α, a gene associated with autism (27). Although we recognize that human autistic phenotypes consist of complex behaviors that may not be fully replicated or interpreted in mouse models, studies of knock-in mice carrying *GRIP1* variants may help to elucidate how GRIP1 regulates social behavior and cognitive and memory function.

Identification of rare functional variants is important in identifying molecular pathways that are disturbed in autism. GRIP1 binds key proteins that regulate glutamatergic signaling, including liprin-α (28), PICK1 (29), KIF5 (30), and NEEP21 (31), and also uses the same PDZ4–6 to bind the GABA receptor-associated protein, gephyrin (32) (Fig. 1*A*). We speculate that functional *GRIP1* variants in PDZ4–6 may alter not only glutamatergic but also GABAergic synaptic function, leading to an imbalance in selected neuronal circuits in autism (33). A role for synaptic imbalance in autism is supported by findings in mice carrying an autism-associated mutation (i.e., R451C) (5) in Neuroligin-3. These mice were found to have increased inhibitory synaptic markers, gephyrin, and vGABA transporter, and in the size of inhibitory synaptic responses and spontaneous inhibitory event frequency, suggesting an enhanced inhibitory synaptic transmission affecting a subset of inhibitory interneurons in forebrain through a gain of function mechanism (34).

Materials and Methods

Samples. DNA samples from patients with autism (n = 480) were obtained from the Autism Genetic Research Exchange (AGRE) and the South Carolina Autism Project (SCAP). Ethnically matched normal controls (n = 480), without the diagnoses of pervasive developmental disorder or autistic disorder, were obtained from Greenwood Genetic Center. An informed consent was obtained from each enrolled family at the respective institutions. All enrolled patients met *Diagnostic and Statistical Manual of Mental Disorders*, 4th edition diagnostic criteria for autistic disorder, which was supported by the ADI-R, and documented by clinical and behavioral phenotype. DNA samples from parents and other affected and unaffected relatives of the probands were available for the majority of the families from AGRE and SCAP. The study was approved by Institutional Review Board at the Johns Hopkins Medical Institutions.

Genotyping. SNP Genotyping was conducted by Sanger sequencing of individual samples from the autism and control cohorts. The SNP, rs7397862 located at +13 of intron 15 was selected for (*i*) its location near the center of the genomic region encoding PDZ4–6 (exons 12–18), (*ii*) on the same SNP linkage disequilibrium block encompassing exons 12 to 25 of *GRIP1*, and (*iii*) high minor-allele frequencies for all ethnic groups in the study cohorts with average heterozygosity of 0.458 ± 0.139 (mean ± STE) (dbSNP Build 132).

Sequencing. A Multiplexing Sample Preparation Oligonucleotide Kit (Illumina; PE-400-1001) was used to generate indexed libraries following the manufacturer's instructions. Sequencing was performed using a Genomic Analyzer II (Illumina) at the Johns Hopkins High-Throughput Sequencing Facility. Standard Sanger sequencing for variant detection and validation was conducted using a BigDye Terminator v3.1 Cycle Sequencing Kit and run using an ABI3100 automatic DNA analyzer (Applied Biosystems) following the manufacturer's instructions.

Yeast Two-Hybrid Assay. Yeast vectors containing PDZ4–6 domains from mutant *GRIP1* (I549V, T550M, I586L, A625T, M794R, or K577A-K578A PDZ4–6), in fusion with the GAL4 DNA binding domain, were generated by site-directed mutagenesis using pPC97-wt GRIP1-PDZ4-6 as a template. The C-terminal 50 amino acids from GluA2C or GluA3C were cloned into yeast vector pPC86 in fusion with the GAL4 activation domain (15). Next, pPC86-GluA2, pPC86-GluA3, or pPC86-GluA3-CΔ4 (with deletion of the last four amino acids at the C terminus of GluA3) were transformed into yeast strain Y190, using the lithium acetate method, together with either pPC97-GRIP1-WT or individual pPC97-GRIP1 variants. Positive yeast clones from cotransformation were selected on triple-deficient plates (Leu^-, Trp^-, His^-) containing 50 mM 3-aminotriazole and ONPG (*o*-nitrophenyl-β-D-galactoside). Yeast clones that grew on triple-selection plates and with a blue color, were selected to be cultured in triple-selection (Leu^-, Trp^-, His^-) liquid medium. β-Galactosidase activity was determined for six clones of each transformation following a standard protocol (Clontech).

Neuron Culture and Transfection. Low-density primary hippocampal cultures were prepared from embryonic day 18 (E18) rat brains, and maintained in serum-free medium, as described previously (22). Cells were fed twice per week and used for experiments after 14 to 18 d in vitro. Hippocampal neurons were transfected at 14 d in vitro using Lipofectamine 2000 (Invitrogen), following the manufacturer's instructions. Cells were used for experiments 48 h after transfection. Plasmids used for transfection were pH-GluA2 (23), pRK5-myc full length *GRIP1* WT, or pRK5-myc full-length *GRIP1* variants (generated by mutagenesis on pRK5-myc *GRIP1* WT and verified by sequencing), and pmCherry vector (Clontech).

Immunocytochemistry and Surface GluA2 Quantification. Cultured neurons were incubated with GFP antibody (JH4030, 1:2,000) in artificial cerebrospinal fluid buffer (containing 120 mM NaCl, 5 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 25 mM Hepes, 30 mM glucose, pH = 7.4) for 30 min. Neurons were fixed by Parafix (4% paraformaldehyde + 4% sucrose in PBS) for 15 min and permeabilized using 0.25% Triton in PBS for 10 min. Subsequently, neurons were incubated with myc primary antibody (9E10 clone, mouse monoclonal, 1:1,000), and fluorophore-conjugated secondary antibodies (Alexa Fluor goat anti-rabbit 555, and goat anti-mouse 647; Invitrogen). Coverslips were

mounted on slides using Fluoromount-G Media (SouthernBiotech). The cells were then observed using epifluorescence microscopy (Axiovert 200; Zeiss), and images were collected using a charge-coupled device camera (Hamamatsu Photonics) with axiovision (Zeiss) analysis software. Fluorescent intensities were quantified using ImageJ software (NIH, National Institutes of Health) in 11 to 22 neurons per condition using one to six dendritic areas per neuron analyzed.

pHluorin-GluA2 Recycling. Taking advantage of the pH difference between extracellular space (pH = 7.4) and intracellular vesicles (pH < 6.0), this assay use a pH-sensitive green fluorescent protein, pHluorin fused to the N terminus of GluA2 (pH-GluA2) as a reporter to visualize the dynamic process of GluA internalization and recycling (22, 23). Full details are in *SI Materials and Methods*.

Immunoblotting Analysis. Brain tissues were homogenized in lysis buffer (0.5% Triton, 5 mM EDTA in PBS) supplemented with protease inhibitor mixture tablets (Roche). Homogenates were incubated on ice for 30 to 45 min and centrifuged at 14,200 × *g* for 30 min at 4 °C. Supernatants were collected and 20 μg of total proteins were transferred to membranes and incubated with primary antibodies [GRIP1 mouse monoclonal antibody, 1:1,000 dilution (BD Transduction Laboratories) or GRIP2 rabbit polyclonal antibody, 1:1,000 dilution (16)], followed by horseradish peroxidase-conjugated secondary antibodies. The target proteins were visualized using Amersham ECL Western Blotting Detection Kit (GE Healthcare) and quantified using NIH ImageJ software.

Animal Breeding and Genotyping. All experimental procedures with mice and rats were approved by the Animal Care and Use Committee of the Johns Hopkins University School of Medicine, and were done in compliance with the relevant laws and institutional guidelines. The housing room was maintained at 23 °C on a 12-h light/dark cycle. Animals were provided with standard mouse chow and free access to water ad libitum. *Grip2* conventional knockout mice were bred to *Grip1* flox/flox mice (17). Mice were then crossed to Nestin-CRE transgenic mice (kindly provided by Paul Worley, Johns Hopkins University) for neuron-specific deletion of *Grip1*. *Grip1/2* DKO mice used for this study were *Grip1* flox/KO with *Grip2* KO/KO (conventional KO), and Nestin CRE-positive heterozygous. Control animals were wt for *Grip1* and *Grip2*, and Nestin CRE-positive heterozygous. *Grip1/2* DKO mice and WT controls were in mixed 129 × C57BL/6 background (17). Full details of genotyping are in *SI Materials and Methods*.

Mouse Behavioral Testing. Mice were subjected to a battery of behavioral tests following Behavioral Core User Manual of the Johns Hopkins Behavioral Core (www.brainscienceinstitute.org/index.php/cores/). Age- and sex-matched WT (n = 10) and *Grip1/2* DKO mice (n = 10) were generated as above, and tested between 3 and 6 mo of age. Full details are in *SI Materials and Methods*.

Statistical Analysis. Groups were first tested for normality and variance homogeneity. Student's t test was applied for comparison of two means and one-way ANOVA test for pair-wise multiple comparisons (Student-Newman-Keuls Method). SigmaStat software (SPSS) was used for data analyses. Data were presented as mean ± SEM and $P < 0.05$ was considered statistically significant.

ACKNOWLEDGMENTS. We thank Dr. Paul Worley for the Nestin-CRE transgenic mice, Jennifer Yocum for help with animal behavioral testing, Yilin Yu for help with animal genotyping, and Eva Andres-Mateos for help with the figures; and the resources provided by the South Carolina Autism Project and Autism Genetic Resource Exchange (AGRE) Consortium and the participating families. This study was supported in part by research Grant #2487 from the Autism Speaks Foundation and Grant R01HD052680 from the National Institute of Child Health and Human Development (to T.W.); a postdoctoral fellowship from the Ministry of Education and Science of Spain (to R.M.); and fellowships from the International Human Frontiers Science Program (LT00399/2008-L) and Australian National Health and Medical Research Council (ID477108) (to V.A.). R.L.H. is an investigator with the Howard Hughes Medical Institute. AGRE is a program of Autism Speaks and is supported, in part, by Grant 1U24MH081810 from the National Institute of Mental Health to Clara M. Lajonchere (PI).

1. Bailey A, et al. (1995) Autism as a strongly genetic disorder: Evidence from a British twin study. *Psychol Med* 25:63–77.
2. Rosenberg RE, et al. (2009) Characteristics and concordance of autism spectrum disorders among 277 twin pairs. *Arch Pediatr Adolesc Med* 163:907–914.
3. Abrahams BS, Geschwind DH (2008) Advances in autism genetics: On the threshold of a new neurobiology. *Nat Rev Genet* 9:341–355.
4. O'Roak BJ, State MW (2008) Autism genetics: Strategies, challenges, and opportunities. *Autism Res* 1:4–17.
5. Jamain S, et al.; Paris Autism Research International Sibpair Study (2003) Mutations of the X-linked genes encoding neuroligins NLGN3 and NLGN4 are associated with autism. *Nat Genet* 34:27–29.
6. Sebat J, et al. (2007) Strong association of de novo copy number mutations with autism. *Science* 316:445–449.
7. Durand CM, et al. (2007) Mutations in the gene encoding the synaptic scaffolding protein SHANK3 are associated with autism spectrum disorders. *Nat Genet* 39:25–27.
8. Bakkaloglu B, et al. (2008) Molecular cytogenetic analysis and resequencing of contactin associated protein-like 2 in autism spectrum disorders. *Am J Hum Genet* 82: 165–173.
9. Geschwind DH (2008) Autism: Many genes, common pathways? *Cell* 135:391–395.
10. DeVito TJ, et al. (2007) Evidence for cortical dysfunction in autism: a proton magnetic resonance spectroscopic imaging study. *Biol Psychiatry* 61:465–473.
11. Fatemi SH, et al. (2002) Glutamic acid decarboxylase 65 and 67 kDa proteins are reduced in autistic parietal and cerebellar cortices. *Biol Psychiatry* 52:805–810.
12. Purcell AE, Jeon OH, Zimmerman AW, Blue ME, Pevsner J (2001) Postmortem brain abnormalities of the glutamate neurotransmitter system in autism. *Neurology* 57: 1618–1628.
13. Pinto D, et al. (2010) Functional impact of global rare copy number variation in autism spectrum disorders. *Nature* 466:368–372.
14. Ma DQ, et al. (2007) Dissecting the locus heterogeneity of autism: Significant linkage to chromosome 12q14. *Mol Psychiatry* 12:376–384.
15. Dong H, et al. (1997) GRIP: A synaptic PDZ domain-containing protein that interacts with AMPA receptors. *Nature* 386:279–284.
16. Dong H, et al. (1999) Characterization of the glutamate receptor-interacting proteins GRIP1 and GRIP2. *J Neurosci* 19:6930–6941.
17. Takamiya K, Mao L, Huganir RL, Linden DJ (2008) The glutamate receptor-interacting protein family of GluR2-binding proteins is required for long-term synaptic depression expression in cerebellar Purkinje cells. *J Neurosci* 28:5752–5755.
18. Li RW, et al. (2005) GRIP1 in GABAergic synapses. *J Comp Neurol* 488:11–27.
19. Kim CH, Chung HJ, Lee HK, Huganir RL (2001) Interaction of the AMPA receptor subunit GluR2/3 with PDZ domains regulates hippocampal long-term depression. *Proc Natl Acad Sci USA* 98:11725–11730.
20. Li P, et al. (1999) AMPA receptor-PDZ interactions in facilitation of spinal sensory synapses. *Nat Neurosci* 2:972–977.
21. Feng W, Shi Y, Li M, Zhang M (2003) Tandem PDZ repeats in glutamate receptor-interacting proteins have a novel mode of PDZ domain-mediated target binding. *Nat Struct Biol* 10:972–978.
22. Mao L, Takamiya K, Thomas G, Lin DT, Huganir RL (2010) GRIP1 and 2 regulate activity-dependent AMPA receptor recycling via exocyst complex interactions. *Proc Natl Acad Sci USA* 107:19038–19043.
23. Lin DT, Huganir RL (2007) PICK1 and phosphorylation of the glutamate receptor 2 (GluR2) AMPA receptor subunit regulates GluR2 recycling after NMDA receptor-induced internalization. *J Neurosci* 27:13903–13908.
24. Takamiya K, et al. (2004) A direct functional link between the multi-PDZ domain protein GRIP1 and the Fraser syndrome protein Fras1. *Nat Genet* 36:172–177.
25. Daw MI, et al. (2000) PDZ proteins interacting with C-terminal GluR2/3 are involved in a PKC-dependent regulation of AMPA receptors at hippocampal synapses. *Neuron* 28: 873–886.
26. Perry W, Minassian A, Lopez B, Maron L, Lincoln A (2007) Sensorimotor gating deficits in adults with autism. *Biol Psychiatry* 61:482–486.
27. Etherton MR, Blaiss CA, Powell CM, Südhof TC (2009) Mouse neurexin-1alpha deletion causes correlated electrophysiological and behavioral changes consistent with cognitive impairments. *Proc Natl Acad Sci USA* 106:17998–18003.
28. Wyszynski M, et al. (2002) Interaction between GRIP and liprin-alpha/SYD2 is required for AMPA receptor targeting. *Neuron* 34:39–52.
29. Lu W, Ziff EB (2005) PICK1 interacts with ABP/GRIP to regulate AMPA receptor trafficking. *Neuron* 47:407–421.
30. Setou M, et al. (2002) Glutamate-receptor-interacting protein GRIP1 directly steers kinesin to dendrites. *Nature* 417:83–87.
31. Steiner P, et al. (2005) Interactions between NEEP21, GRIP1 and GluR2 regulate sorting and recycling of the glutamate receptor subunit GluR2. *EMBO J* 24:2873–2884.
32. Yu W, et al. (2008) Gephyrin interacts with the glutamate receptor interacting protein 1 isoforms at GABAergic synapses. *J Neurochem* 105:2300–2314.
33. Rubenstein JL, Merzenich MM (2003) Model of autism: Increased ratio of excitation/inhibition in key neural systems. *Genes Brain Behav* 2:255–267.
34. Tabuchi K, et al. (2007) A neuroligin-3 mutation implicated in autism increases inhibitory synaptic transmission in mice. *Science* 318:71–76.