REPORT

A Defect in the Ionotropic Glutamate Receptor 6 Gene (*GRIK2*) Is Associated with Autosomal Recessive Mental Retardation

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Nonsyndromic mental retardation is one of the most important unresolved problems in genetic health care. Autosomal forms are far more common than X-linked forms, but, in contrast to the latter, they are still largely unexplored. Here, we report a complex mutation in the ionotropic glutamate receptor 6 gene (*GRIK2,* also called "*GLUR6*") that cosegregates with moderate-to-severe nonsyndromic autosomal recessive mental retardation in a large, consanguineous Iranian family. The predicted gene product lacks the first ligand-binding domain, the adjacent transmembrane domain, and the putative pore loop, suggesting a complete loss of function of the GLU_{K6} protein, which is supported by electrophysiological data. This finding provides the first proof that GLU_{K6} is indispensable for higher brain functions in humans, and future studies of this and other ionotropic kainate receptors will shed more light on the pathophysiology of mental retardation.

To date, only three genes have been found to be directly linked to nonsyndromic autosomal recessive mental retardation (NS-ARMR): *PRSS12* (also called "neurotrypsin" [MIM 606709]), which encodes a trypsin-like serine protease[1]; *CRBN* (also called "cereblon" [MIM 609262]),[2] which encodes an ATP-dependent Lon protease; and *CC2D1A* (MIM 610055), which encodes a putative signal transducer that participates in the positive regulation of the I-κB kinase/NF-κB cascade.[3] We recently reported eight genomic loci for NS-ARMR (*MRT4–MRT11*)[4] as the result of linkage studies of 78 consanguineous Iranian families. Here, we describe the first of the underlying gene defects, resolving the *MRT6* locus [MIM 611092], a 9.98-Mb interval on chromosome 6q16.1-q21 that we found in a family with moderate-to-severe NS-ARMR. The degree of mental retardation (MR) in the affected family members ranged from mild to severe (fig. 1). Patient V:1 had an intelligence quotient (IQ) of 30 at age 50 years. His youngest sister (V:5) had mild MR. Patients V:6 and V:12 also had mild MR, and patient V:11 had an IQ of 55 (moderate MR). The patients did not have neurological problems, congenital malformations, or facial dysmorphisms. Body height, weight, and head circumference were normal in all patients. For patient V:12, we performed a magnetic resonance imaging scan, which revealed no morphological abnormalities. Sample collection and clinical evaluation procedures were performed with the informed consent of the parents and have been described elsewhere.[4]

The *MRT6* locus contains 25 annotated genes; 8 of these (*GRIK2* [MIM 138244], *CCNC, COQ3, MCHR2, GPR63, KLHL32, POU3F2,* and *SIM1*) were considered to be plausible candidate genes for MR and were selected for mutation screening. We designed PCR primers for the amplification of exonic sequences and splice-site regions, using the Primer3 software.[5] PCR amplicons (primer sequences and reaction conditions are available on request) were analyzed by agarose-gel electrophoresis and direct sequencing. In DNA from patients, only a single nonpolymorphic sequence change was detected, apparently a deletion removing exons 7 and 8 of the *GRIK2* gene (see fig. 2*a* for physical coordinates and exon-intron structure), as suggested by our failure to amplify these exons by PCR. *GRIK2* encodes GLU_{K6}, a subunit of kainate receptors (KARs) that is highly expressed in the brain. Together with the intensively studied alpha-amino-3-hydroxy-5-methyl-4-isoxazole proprionic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors, KARs belong to the ionotropic receptors that are the targets of glutamate released in excitatory synapses.[6,7] Mutation or loss of NMDA and/or AMPA receptors is known to result in severe derangement of neuronal function. Still, extensive analyses of their structure, biophysical properties, and distribution[8–10] have so far failed to define a role for ionotropic KARs in higher brain functions. *GRIK2* has been associated with autism,[11] but this finding could not be substantiated by a recent genomewide linkage study,[12] and behavioral studies of *Grik2*-knockout mice did not yield conclusive results.[10] Thus, the involvement of this receptor in cognition has so far remained controversial.

Loss of exons 7 and 8 in *GRIK2,* as observed in our

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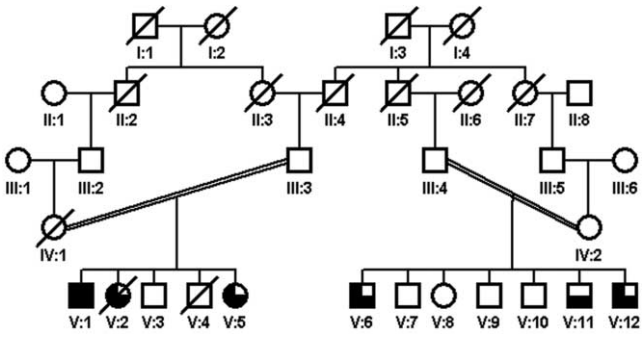

Figure 1. Family pedigree. Blackened symbols represent patients with severe MR, three-quarters-blackened symbols represent patients with moderate MR, and half-blackened symbols represent patients with mild MR.

patients with MR, results in an in-frame deletion of 84 aa between amino acids 317 and 402, close to the first ligand-binding domain (S1) in the extracellular N-terminal region of GLU_{K6}. To study whether this defect was sufficient to impair GLU_{K6} protein function, we cloned wild-type GLU_{K6} from human fetal brain RNA (BD Biosciences Clontech), using the pENTR Directional Topo Cloning kit (Invitrogen) in accordance with the manufacturer's instructions. We employed the resulting plasmid for subcloning *GRIK2* wild-type cDNA into modified (green fluorescent protein [GFP] replaced with c-Myc tag) pcDNA-DEST47 Gateway vectors (Invitrogen). We then engineered a $GLU_{K6}\Delta$ construct lacking exons 7 and 8 by site-directed mutagenesis with the QuikChange Site-Directed Mutagenesis Kit (Stratagene). For a cellular model system, we chose HEK293 cells, because they do not show endogenous *GRIK2* expression, and cotransfected them with either plasmid in combination with a green fluorescent transfection marker (1.8 μg cDNA encoding either wild-type *GRIK2* or *GRIK2*Δ plus 0.2 μg pEGFP-N1 vector [BD Biosciences Clontech]). The transfection reagent we used was DreamFect (OZ Biosciences) or Fugene reagent (Roche). For whole-cell patch-clamp recordings, cells were detached with 1 mM EDTA in cold PBS, 24 or 48 h after transfection, were transferred to poly-D-lysin–coated coverslips, and were allowed to settle for at least 4 h. We performed the recordings by using an intracellular solution containing 130 mM CsCl, 9.4 mM NaCl, 1 mM $MgCl_2$,

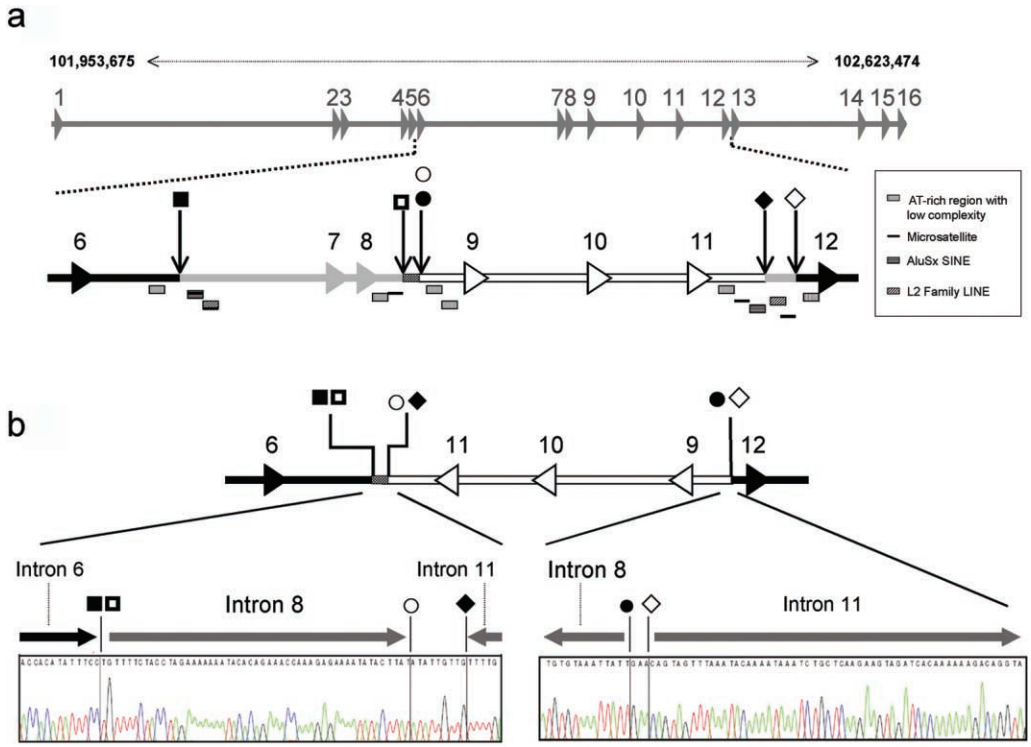

Figure 2. Genomic mutation in *GRIK2*. *a,* Schematic representation of the *GRIK2* wild-type allele. Physical coordinates (*top*) are according to NCBI build 36.1. Exons are depicted by numbered arrowheads. Black shading indicates unaffected sequence, gray shading indicates deleted regions, and the inversion is white outlined in black. A checkered pattern marks the uninverted sequence fragment 5′ of the deletion of exons 7 and 8. Squares, circles, and diamonds mark the positions of the deletion and inversion borders. Rectangular symbols below the schematic of *GRIK2* mark the locations of regions of low sequence complexity or repetitive elements. *b,* Schematic representation of the *GRIK2* mutant allele and the sequences of the 5′ and 3′ genomic junction fragments. LINE = long interspersed nuclear element; SINE = short interspersed nuclear element.

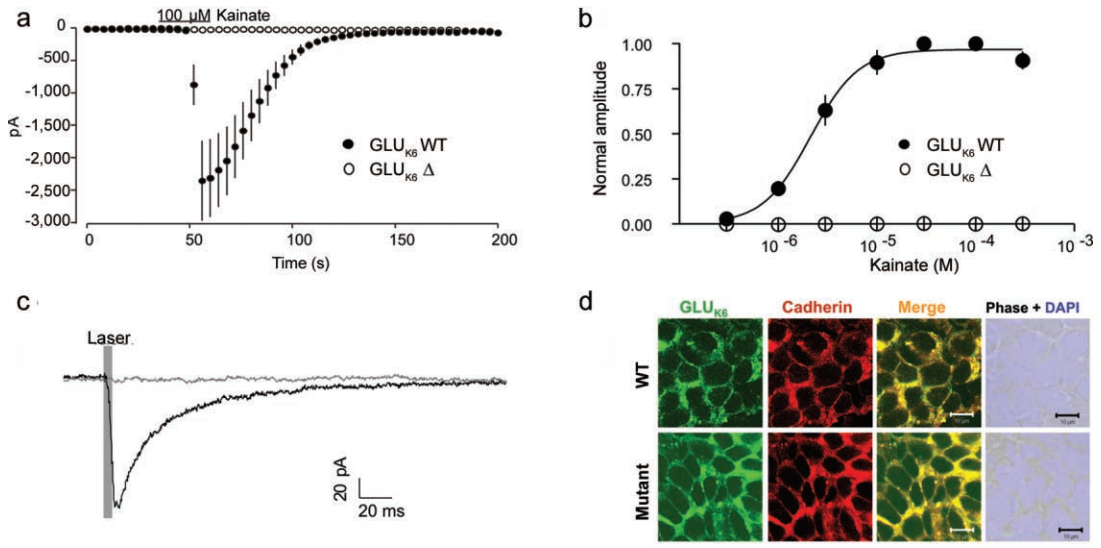

Figure 3. Loss of function of GLU$_{K6}$Δ. *a,* Mean currents evoked by 100 μM kainate (for GLU$_{K6}$ wild type [WT], $n = 8$; for GLU$_{K6}$Δ, $n = 9$). *b,* Dose-response curve for kainate, normalized to response in 100 μM kainate. Error bars represent SEM (for GLU$_{K6}$WT, $n =$ 6–8 per reading point; for GLU$_{K6}$Δ, $n = 3$–9 per reading point). *c,* Cells expressing GLU$_{K6}$WT show photolytically evoked glutamate responses without prior incubation in concanavalin A (*black line*), whereas GLU$_{K6}$Δ-expressing cells show no currents after fast glutamate application (*gray line*). Each trace represents the mean of five successive sweeps. *d,* Immunocytochemistry showing colocalization of both GLU$_{K6}$ WT and GLU$_{K6}$Δ with the endogenous plasma membrane protein cadherin. DAPI = 4′,6-diamidino-2-phenylindole.

0.2 mM guanosine triphosphate, 10 mM ethylene glycol tetraacetic acid, and 10 mM HEPES (pH adjusted to 7.3 with CsOH). The cells were superfused with HEPES-buffered solution (140 mM NaCl, 2.4 mM KCl, 10 mM glucose, 10 mM HEPES, 2.5 mM $CaCl_2$, and 1.3 mM $MgCl_2$ [pH adjusted to 7.3 with NaOH]). We treated the cells for 5 min with 200 μg/ml concanavalin A in glucose-free extracellular solution immediately before the recordings, to prevent lectin aggregation. Cells were voltage clamped at −60 mV, by use of an Axoclamp 700A. Recordings were digitized with 5 kHz and were analyzed online by use of IGOR Pro (Wavemetrics). All recordings were performed at room temperature. Our results show that GLU$_{K6}$Δ completely lost the ability to conduct ions when activated by kainate (fig. 3*a*) or glutamate (data not shown), independent of agonist dosage (fig. 3*b*). To exclude the possibility of altered receptor desensitization, we made use of a fast application system[13] for performing whole-cell patch-clamp experiments without concanavalin A. For this flash photolysis of glutamate, 40 μM 4-methoxy-7-nitroindolinyl–caged glutamate (Tocris) was perfused continuously on the cells in a volume of 10 ml. A 355-nm UV laser beam (DPSL-355-1W laser [Rapp Optics]) was coupled via a 200-μm quartz fiber optic into a spot illumination adaptor (Rapp Optics), mounted in the epifluorescence path of an Olympus BX51Wl microscope (equipped with a laser beam splitter and a 420-nm-long pass emitter [both from AHF Analysetechnik]) and was focused with a 60× water objective (numerical aperture 0.9 [Olympus]) onto a spot ~40 μm in diameter. The holding current was constant during washing of the caged glutamate. Again, we observed a complete loss of function of GLU$_{K6}$Δ compared with the wild-type GLU$_{K6}$ channels (fig. 3*c*). We then asked whether this might be caused by the absence of the receptor in the plasma membrane caused by a defect in receptor trafficking. Therefore, we performed GLU$_{K6}$ staining experiments in transfected HEK293 cells, fixed 48 h after transfection with paraformaldehyde (4%, for 10 min at room temperature) and permeabilized with Triton X-100 in PBS (0.1%, for 5 min at room temperature). The cells were subsequently blocked with normal goat serum (10%, for 1 h at room temperature). For detection of the membrane protein cadherin and GLU$_{K6}$, we used the antibodies panCadherin (ab6528 [Abcam]) (1:2,000, for 1 h at room temperature) and Anti-GLUR6/7 (05–921 [Upstate Biotechnology]) (1:2,000, for 1 h at room temperature), respectively. They were labeled by the secondary antibodies Alexa Fluor 488 chicken anti-mouse (Invitrogen) (1:1,000, for 1 h at room temperature) and Alexa Fluor 488 chicken anti-rabbit (Invitrogen) (1:1,000, for 1 h at room temperature). Confocal analysis of GLU$_{K6}$ stainings on a laser-scanning microscope (Zeiss Axiovert 100 M) with a 63× objective, however, clearly showed colocalization of transfected GLU$_{K6}$Δ with the endogenous membrane protein cadherin in HEK293 cells (fig. 3*d*). Biotinylation experiments confirmed that these cells had considerable amounts of GLU$_{K6}$ protein in the plasma membrane (fig. 4).

In parallel, we performed PCR experiments with various primer combinations to define the borders of the deletion

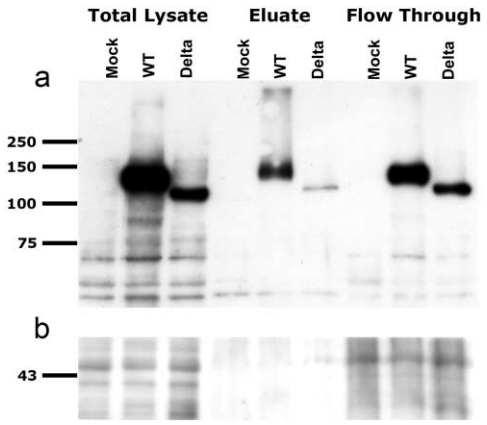

Figure 4. $GLU_{K6}\Delta$ present on the plasma membrane of transfected HEK293 cells. *a,* Membrane proteins biotinylated on HEK293 cells overexpressing GLU_{K6} wild type (WT) or $GLU_{K6}\Delta$ (Delta) at 48 h after transfection (Pinpoint [Pierce]). Nontransfected HEK293 cells served as a control (Mock). After cell lysis (Total Lysate samples), biotinylated membrane proteins were bound to a NeutrAvidin affinity column. Nonbiotinylated protein was separated, because it does not bind to the NeutrAvidin affinity column (Flow Through samples). Membrane proteins were eluted with sample buffer (Eluate samples). All fractions were separated on a 10% SDS gel, were blotted, and were probed for GLU_{K6} (with 05-921 [Upstate Biotechnology] at 1:800). GLU_{K6} WT and $GLU_{K6}\Delta$ are present in the membrane protein fraction (Eluate samples). *b,* Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) loading control (ab9482 [Abcam]).

but failed to amplify a junction fragment. We therefore investigated the 5′ region of the putative deletion in all available family members by Southern-blot analysis. Following standard procedures, we used 10 μg genomic DNA for restriction with *Bgl*II and a ^{32}P-labeled probe, directed against a 802-bp sequence upstream of the mutation region (forward primer: 5′-gccattgtgtaagatcatagtgctcagac-3′; reverse primer: 5′-gtatattagaaccactgggaagg-3′). The expected fragment size, corresponding to a *GRIK2* allele lacking exons 7 and 8, was 1.5 kb, yet we identified a 6.5-kb band in carriers of the mutant allele (fig. 5*a*), which pointed to the presence of a more complex genomic rearrangement. We then performed inverse PCR[14] on 1 μg of this fragment, isolated with the Qiaquick gel-extraction kit (Qiagen). The fragment was circularized by self-ligation in a 10-μl reaction volume by use of 1.5 U T4 DNA ligase and the reaction buffer provided by the manufacturer. After 16 h of incubation at 12°C, the enzyme was inactivated by increasing the reaction temperature to 65°C for 10 min.

For PCR amplification, we used reverse-oriented primers (forward primer: 5′-catgctcctagaggtgtgcaaatacc-3′; reverse primer: 5′-ctacaatggcaagcgaagtg-3′) located 5′ of the mutation in a region that could be amplified from patient DNA. Cloning and sequencing of the amplicon confirmed the deletion of exons 7 and 8 by uncovering a sequence junction between the remnants of introns 6 and 8. This junction fragment was joined to a sequence fragment belonging to intron 11 in reverse orientation. Through further PCR and sequencing analyses and Southern-blot experiments (fig. 5*b*), with use of *Eco*RV for DNA digestion and a 930-bp upstream probe (forward primer: 5′-tggaaattcatagtaaaggtatgtgg-3′; reverse primer: 5′-ctgagtagaaagagagagaaaacaatg-3′), we were able to elucidate the full extent of the observed mutation. This is illustrated in figure 2, which shows that, in addition to the ~120-kb deletion (spanning exons 7 and 8), the mutation comprises an inversion of ~80 kb, including exons 9, 10, and 11 in combination with a deletion of ~20 kb of intron 11. At the protein level, this rearrangement can be expected to result in the loss not only of the first ligand-binding domain but also of the adjacent transmembrane domain and the putative pore loop of GLU_{K6} (fig. 6).

Cosegregation of the mutation with MR in the family was shown by array-based comparative genomic hybridization (array CGH) and Southern-blot experiments (figs. 5 and 7). We also performed Southern blots on a panel of 172 individuals with university education. Neither these nor any of 390 individuals from a random population sample (healthy blood donors) screened by PCR carried the mutation.

To check for *GRIK2* expression, we extracted total RNA from Epstein-Barr virus–transformed lymphoblastoid cell lines of all available patients and healthy siblings by use of the TRIzol reagent (Invitrogen). We isolated mRNA (Dynabeads Oligo (dT)25 [Dynal Biotech]) and generated cDNA by using the SuperScript III Reverse Transcriptase (Invitrogen) together with random hexamers. This cDNA was used to perform PCRs, by use of different combina-

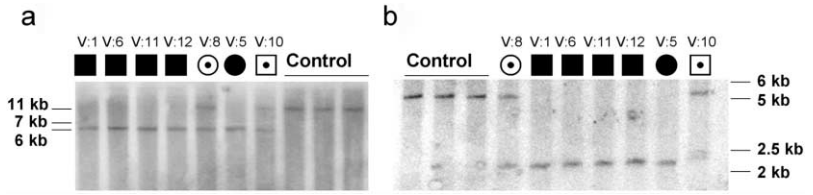

Figure 5. Southern blots for patients and heterozygous carriers of the mutation, characterizing the 5′ border (*a*) (by use of *Bgl*II) and the 3′ border (*b*) (by use of *Eco*RV) of the mutation.

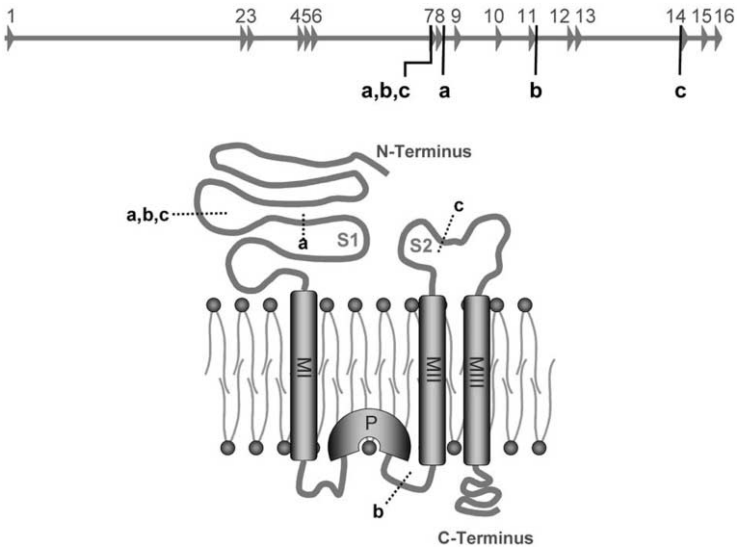

Figure 6. Impact of *GRIK2* mutation on GLU_{K6} protein structure. Shown is a schematic representation of the *GRIK2* wild-type allele and a cartoon of a GLU_{K6} monomer. MI–MIII = transmembrane domains; P = pore loop. Letters indicate the start and end of different alterations in the genomic sequence and their corresponding impact on the resulting protein. a = deletion of exons 7 and 8; b = complete genomic rearrangement, as observed in patients with MR; c = region lacking in the putative transcript with a junction between exons 6 and 14.

tions of primers from exon 6 with primers from exons 12, 13, and 14. With a combination of primers from exon 6 (5′-gaaaagtggtcgatggaacg-3′) and exon 14 (5′-ggcccacattttg-tcatacg-3′), we obtained an aberrant fragment in the cDNA from homozygous and heterozygous carriers of the mutation (data not shown). Compared with the transcript expected from the deletion-inversion, this corresponds to a transcript that translates into a GLU_{K6} protein, which lacks an additional transmembrane domain (fig. 6). In view of our finding that the deletion of exons 7 and 8 is already sufficient to cause a complete loss of GLU_{K6} function, we conclude that the patients do not express any *GRIK2* gene product with the capacity to form functional ion channels.

GLU_{K6}-containing receptors are present at the presynaptic level,[18,19] as well as at the postsynaptic level,[20] and the primary involvement of GLU_{K6} in excitatory synaptic activity suggests that defective GLU_{K6} might cause cognitive impairment through alterations in local brain circuitry. An attractive hypothesis, in this respect, is that KARs might be involved in the maturation of microcircuits and network formation in brain areas that are important for higher brain functions, such as the hippocampus. In fact, it has been shown that, in mice, the expression level of KAR-subunit mRNAs in the hippocampus is very high during early stages of development, whereas adult animals show lower levels.[21,22] Furthermore, it has been found that, in the rodent neonatal hippocampus, KARs are activated by ambient glutamate and thereby regulate spontaneous network activity and functional maturation of hippocampal synapses.[23,24]

At the cellular level, it is conceivable that defective GLU_{K6} could also have structural effects. Indeed, GLU_{K6} has been found to be involved in profilin II–mediated interactions with actin,[25,26] through which it might contribute to the stabilization of dendritic spine morphology[27] and normal neuritogenesis.[28] Further studies of this aspect of GLU_{K6} function in model organisms will be particularly interesting, because abnormal morphogenesis of dendritic spines is observed in a variety of MR disorders, such as fragile X syndrome (MIM 300624) (for review, see, e.g., the work of Grossmann et al.[29]). A third avenue to be explored might be the investigation of a putative impact of GLU_{K6} deficiency on synaptic plasticity. In this context, it is noteworthy that GLU_{K6} also seems to interact with synapse-associated protein 102,[30] for which mutations in the coding gene (*DLG3*) were found in families with severe X-linked MR.[31]

By identifying *GRIK2* as a likely NS-ARMR gene, we provide strong evidence that functional integrity of this ionotropic glutamate receptor might be a sine qua non for normal human brain function. Given the unclear phenotype of *Grik2*-deficient mice, this study also indicates that genotype-phenotype studies in man are indispensable for identifying the genes that play a role in complex behavioral traits. Finally, we expect that our results will stimulate research into the role of other KARs in cognition and behavior. *GRIK2* is the first gene for NS-ARMR with a known function in the CNS. Its identification is part of an ongoing systematic effort to unravel the molecular causes of recessive MR, which will shed more light on the function of the human brain in health and disease.

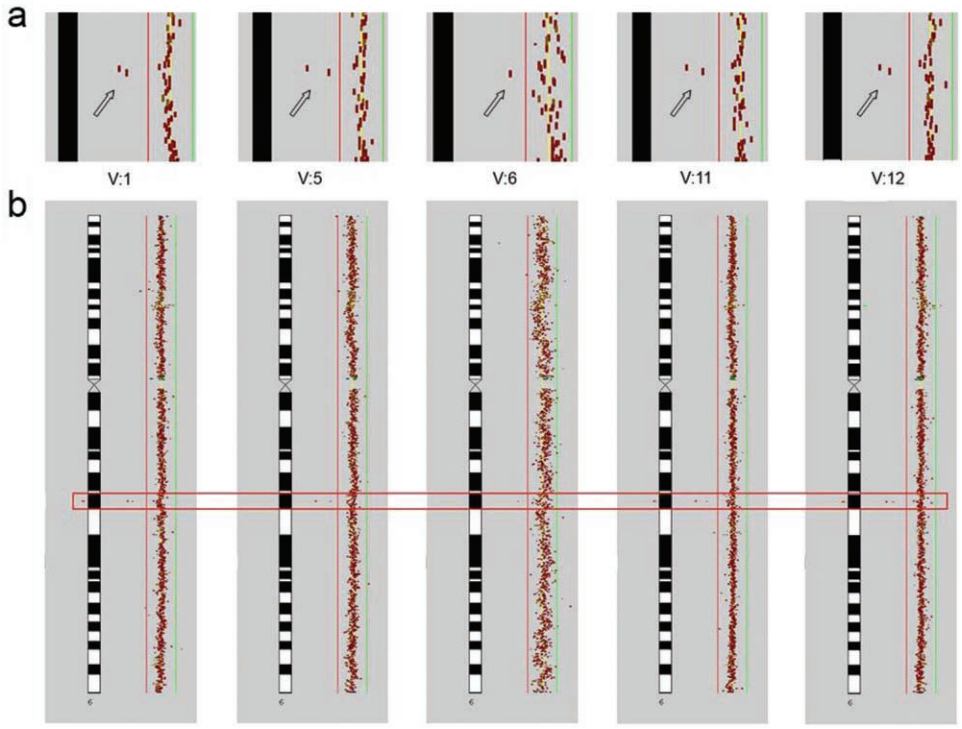

Figure 7. Array CGH results for patients with MR. Submegabase-resolution array CGH was performed as described elsewhere.[15] After normalization by subgrid LOWESS, Cy3:Cy5 intensity ratios of each clone were plotted in a size-dependent manner along the chromosome ideograms, by use of CGHPRO.[16] Red and green bars indicate the $\log_2$ ratio thresholds of −0.3 (loss) and 0.3 (gain), respectively. A homozygous deletion of 6q16.3 was the only aberration that cosegregated with the disease; it was present in all affected probands and was absent in all unaffected probands tested. Array CGH data shown here have been deposited in NCBI Gene Expression Omnibus (GEO)[17] and are accessible through GEO series accession number GSE 7886. *a,* Close-up of findings for patient 6q16.3, demonstrating a deletion of two BAC clones (RP11-226C08 and RP11-543M18 [*arrows*]) encompassing *GRIK2*. (Because of lower hybridization quality for V:6, only one BAC represents the DNA copy-number loss.) *b,* Overall view of chromosome 6 in the corresponding patients. A red rectangle highlights the region depicted in panel a.

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Web Resource

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for neurotrypsin, cereblon, *CC2D1A, MRT6, GRIK2,* and fragile X syndrome)

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