The function of glutamatergic synapses is not perturbed by severe knockdown of 4.1N and 4.1G expression

Christian Wozny1,*,‡, Jörg Breustedt1,*, Friederike Wolk2,*, Frédérique Varoqueaux2, Susann Boretius3, Aleksandar R. Zivkovic1, Antje Neeb4, Jens Frahm3, Dietmar Schmitz1, Nils Brose2 and Aleksandra Ivanovic2,‡

1Neuroscience Research Center, Charité—Universitätsmedizin Berlin, Charitéplatz 1, D-10117 Berlin, Germany
2Max-Planck-Institut für Experimentelle Medizin, Abteilung Molekulare Neurobiologie, DFG Center for Molecular Physiology of the Brain, Hermann-Rein-Str. 3, D-37075 Göttingen, Germany
3Biomedizinische NMR Forschungs GmbH, Max-Planck-Institut für Biophysikalische Chemie, Am Fassberg 11, D-37077 Göttingen, Germany
4Institut für Toxikologie und Genetik, Forschungszentrum Karlsruhe, Hermann-von-Helmholtz-Platz 1, D-76344 Eggenstein-Leopoldshafen, Germany

*These authors contributed equally to this work
‡Authors for correspondence (e-mails: ivanovic@em.mpg.de; cwozny@mrc-lmb.cam.ac.uk)
§Present address: MRC Laboratory of Molecular Biology, Hills Road, Cambridge CB2 0QH, UK

Accepted 6 November 2008
Journal of Cell Science 122, 735-744 Published by The Company of Biologists 2009
doi:10.1242/jcs.037382

Summary
AMPA-type glutamate receptors mediate fast excitatory synaptic transmission in the vertebrate brain. Their surface expression at synapses between neurons is regulated in an activity-dependent and activity-independent manner. The protein machinery that regulates synaptic targeting, anchoring and turnover of AMPA receptors consists of several types of specialized scaffolding proteins. The FERM domain scaffolding proteins 4.1G and 4.1N were previously suggested to act jointly in binding and regulating synaptic trafficking of the AMPA receptor subunits GluR1 and GluR4. To determine the functions of 4.1G and 4.1N in vivo, we generated a mutant mouse line that lacks 4.1G entirely and expresses 4.1N at 22% of wild-type levels. These mice had combined 4.1G and 4.1N protein expression in the hippocampus at 12% of wild-type levels (equivalent to 8-10% of combined GluR1 and GluR4 expression levels). They show a moderate reduction in synaptosomal expression levels of the AMPA receptor subunit GluR1 at 3 weeks of age, but no change in basic glutamatergic synaptic transmission and long-term potentiation in the hippocampus. Our study indicates that 4.1G and 4.1N do not have a crucial role in glutamatergic synaptic transmission and the induction and maintenance of long-term plastic changes in synaptic efficacy.

Supplementary material available online at http://jcs.biologists.org/cgi/content/full/122/5/735/DC1

Key words: Knockout, Mouse, Hippocampus, GluR1, GluR4, Synaptic plasticity

Introduction
The excitatory neurotransmitter glutamate activates α-amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) receptors, N-methyl-D-aspartate (NMDA) receptors and kainate receptors. Fast excitatory transmission in the brain is mainly mediated by AMPA receptors (AMPARs), and its dynamic alteration is thought to be involved in several cognitive processes, including learning and memory.

One of the most extensively studied models of activity-dependent plastic changes in synaptic strength is long-term potentiation (LTP), which is thought to be crucial for learning and memory. NMDA-type receptors (NMDARs) are important for the induction of LTP, which involves long-lasting changes in synaptic strength caused by activity-dependent modulation of AMPAR-mediated transmission (Malenka and Bear, 2004). In the course of LTP generation, high-frequency stimuli activate AMPARs and NMDARs, leading to subsequent changes in trafficking of AMPARs to the synapse (Barry and Ziff, 2002; Bredt and Nicoll, 2003; Collingridge et al., 2004).

Several scaffolding proteins are known to regulate AMPAR trafficking, including the transmembrane AMPAR-regulatory proteins (TARPs), the ATPase N-ethylmaleimide-sensitive factor (NSF), members of the membrane-associated guanylate kinase protein-family (MAGUKs) and other PDZ-domain-containing proteins, such as the glutamate receptor-interacting protein (GRIP) or the protein kinase C α (PKCα)-binding protein PICK1. Stargazin (CCG2) and γ-8 (CCG8) are members of the TARP family and were shown to be essential for the surface expression and clustering of AMPARs at synapses (Nicoll et al., 2006). Stargazin-deficient mice lack synaptic AMPARs in cerebellar granule cells, but show normal synaptic transmission in the hippocampus (Chen et al., 2000). In γ-8-knockout mice, hippocampal synaptic transmission and plasticity are reduced (Rouach et al., 2005). Overexpression of the MAGUK PSD95 increases AMPAR-mediated transmission (Beique and Andrade, 2003; Ehrlich and Malinow, 2004; Nakagawa et al., 2004; Schnell et al., 2002), and PSD93 PSD95 double-knockout mice show a severe impairment in fast excitatory transmission (Elias et al., 2006). GRIP, NSF and PICK1 interact directly with the cytoplasmic tail of the GluR2 subunit of AMPARs (Kim et al., 2001; Luthi et al., 1999; Setou et al., 2002).

The founding member of the protein 4.1 family, 4.1R (official mouse protein symbol, Epb4.1), was originally identified as a stabilizer of erythrocyte shape, connecting the transmembrane protein glycoporphin C (GLPC) and the MAGUK p55 with the spectrin cytoskeleton (Marfatia et al., 1995). In addition to 4.1R,
the 4.1 family includes the members 4.1B (Epb41L3), 4.1G (Epb41L2) and 4.1N (Epb41L1). All four 4.1 proteins share a highly conserved domain structure characterized by the presence of an N-terminal FERM (four point one, ezrin, radixin, moesin) domain, a spectrin-actin-binding domain and a C-terminal domain, all of which interact with multiple transmembrane proteins. The ability of 4.1 proteins to bind very structurally diverse interaction partners via their different protein domains enables them to participate in many different physiological processes in a variety of cell types and tissues.

Proteins 4.1G and 4.1N have been shown to be expressed in neuronal and non-neuronal cells in the brain (Lu et al., 2004; Ohara et al., 2000; Ohno et al., 2005). Protein interactions with the GluR1 and GluR4 subunits of AMPARs were described for the MAGUK SAP97 and the 4.1 proteins 4.1N and 4.1G (Cai et al., 2002; Coleman et al., 2003; Rumbaugh et al., 2003; Shen et al., 2000). The Drosophila 4.1 protein homologue coracle interacts with GluRIIA (Chen et al., 2005) and binding of 4.1N and 4.1G is suggested to regulate surface expression of GluR1 and GluR4 (Coleman et al., 2003; Shen et al., 2000).

In the present study, we examined the role of the 4.1 paralogues 4.1G and 4.1N in hippocampal neurotransmission and synaptic plasticity using mutant mice in which combined 4.1N and 4.1G protein expression in the hippocampus is reduced to 12% of wild-type levels, which is equivalent to 8-10% of combined GluR1 and GluR4 expression levels.

**Results**

**Targeting of the mouse 4.1G and 4.1N genes**

The 4.1G and 4.1N double-mutant (4.1G/N) mice (abbreviated as DKO in figures) were generated by a conditional mutagenesis strategy. In both cases, two LoxP sites flanking the first coding exon and a neomycin-resistance gene flanked by flt sites were introduced by homologous recombination in embryonic stem cells (129/ola) (Fig. 1A,D). A fragment representing bp 25,131,023-25,131,494 on band A3 of Chromosome 10 and bp 1-471 in the 4.1G mRNA (AJ542537) was floxed in the mouse 4.1G gene Epb41L2 (GeneID 13822). The 4.1N gene Epb41L1 (GeneID 13821) is located on band H2 of Chromosome 2. The region representing bp 156,185,360-156,185,551 in the 4.1N gene and bp 206-396 in 4.1N mRNA (AF061283) was floxed. Of 192 screened embryonic stem (ES) cell clones in both cases, nine clones with the desired 4.1G recombinant and 15 clones with the desired 4.1N recombinant were identified by Southern blot analysis with a 5′/H11032 external probe (probe 1 for 4.1G, Fig. 1A; probe 2 for 4.1N, Fig. 1D). Two ES cell clones of each gene were injected into C57BL/6 blastocysts. The offspring of chimeric males and subsequent generations were genotyped by PCR (Fig. 1C,F,G). The resulting recombinant single 4.1G/– and 4.1N/– mice were bred with EIIaCre deleter mice (Lakso et al., 1996), which resulted in the loss of the first coding exon in both cases (Fig. 1G). The 4.1G-knockout mice were then crossed with 4.1N mutant mice to breed 4.1G/N double-mutant animals. The offspring were bred to homozygosity and selected for the absence of the Cre transgene.

**Fig. 1.** Targeting of genes encoding 4.1G and 4.1N. (A,D) Schematic representations of the mouse 4.1G and 4.1N genomic loci, targeting vectors, targeted alleles, and excision of the first coding exon by Cre recombinase. LoxP sites are indicated by grey triangles, the neomycin selection cassette is flanked by flt sites (black arrowheads) for possible excision by flip recombinase. Ex, exon. (A) Targeting strategy for gene encoding 4.1G. The first coding exon has been excised. The positions of AccI restriction sites and the 5′/H11032 Southern probe (Probe 1) are shown. (B) Southern blot analysis of 4.1G. Probe P1 was used for hybridisation. The signal for the 4.1G WT allele resulted in a 6598 bp band and the recombinant allele resulted in a 4569 bp band. (C) Genotyping of 4.1G mutants. Genotyping of mouse tail DNA with primers p1 and p2 resulted in a 243 bp WT band and no band was synthesized after knockout of 4.1N (F). In genomic PCRs with primers p7 and p8, a 1239 bp band for the recombinant allele (flox/flox–) and a 708 bp band after Cre recombination (Cre+−) were detected (G).
Role of 4.1G and 4.1N in synaptic transmission

Anatomy and cage behaviour of 4.1G/N double mutants
Neither single mutants nor homozygous 4.1G/N double mutants showed obvious phenotypic alterations. The 4.1G/N double-mutant mice were born at the expected mendelian frequency, and no gross behavioural abnormalities were apparent. The life spans were similar to those of control animals (data not shown).

Examination of the double-mutant animals by MRI using T1- and T2-weighted images showed only slight changes in the gross anatomy of the brain in 3-week-old animals (Fig. 2A). Quantitative volumetric analyses revealed mild, although significant, reductions in whole brain volume and cerebellar volume in young 4.1G/N double-mutant mice (Fig. 2C,D). These changes seem to be compensated during development, as they were absent in 7-month-old double-mutant mice (Fig. 2B-E).

Immunohistochemical staining was performed to determine the number of matched and mismatched pre- and postsynaptic components of glutamatergic synapses in the stratum radiatum of hippocampal CA1 area of 4.1G/N double mutants and wild-type (WT) controls at the age of 3 weeks. Neither the spatial distribution nor the densities of puncta positive for VGLUT1/VGLUT2, ProSAP1 or both were altered significantly in 4.1G/N double-mutant hippocampus (Fig. 3A,B). Moreover, the ultrastructure of synapses formed in double-mutant animals was indistinguishable from that of WT controls (Fig. 3C). Mature synaptic specializations were observed in the CA1 region of 3-week-old mice in both the mutant and WT animals. Synaptic parameters, such as the number of synapses per 100 μm², the length of the postsynaptic density and the width of the postsynaptic density, were similar in both groups (Fig. 3D) (WT, n=3; DKO, n=3). Taken together, these data indicate that the expression of proteins 4.1G and 4.1N is not critical for proper formation of glutamatergic synapses.

Expression of 4.1 proteins in 4.1G/N double-mutant mice
Western blot analyses confirmed that 4.1G was completely absent in 4.1G/N double-mutant mice (Fig. 4A), whereas the short variant of two 4.1N isoforms was still present (Fig. 4B; Fig. 5B,C), albeit reduced to a level of 30% of that in control preparations (Fig. 5D). The level of 4.1B protein was unchanged in the 4.1G/N double-mutant animals (supplementary material Fig. S1). The ratio between the short and the long variant of 4.1N in the hippocampus is 2.6±0.7 (n=6, calculated based on a densitometric analysis of the data shown in Fig. 5B,C). Thus, a 70% reduction of the short form of 4.1N and a complete elimination of the long 4.1N variant in our mutants is equivalent to a 78% reduction of total 4.1N expression in the hippocampus of 4.1N/G double-mutant mice. The ratio between 4.1N and 4.1G protein expression is 1:0.78 in rat total hippocampus (Yamakawa and Ohara, 2000) and 1:0.94 in mouse hippocampus postsynaptic density preparations (Trinidad et al., 2008). Thus, the reduction of combined 4.1G/N protein expression in our homozygous double-mutant hippocampus is estimated to be 88-89% compared with WT controls.

To deduce the primary structure of the residually expressed 4.1N isoform, we performed western blot analyses with three domain-specific antibodies: anti-4.1N exon 2 directed against the first 15 amino acids encoded by exon 2, anti-4.1N monoclonal antibody (BD Biosciences), which is specific for a region of the spectrin-actin-binding domain, and an anti-CTD antibody against the C-terminal domain of 4.1N (Fig. 5E). Fig. 5A shows that the anti-
4.1N exon 2 antibody detected only the upper band in the WT, whereas in the 4.1G/N double-mutant mice, no signal was recognized at all (n=3). By contrast, the anti-4.1N monoclonal antibody and the anti-4.1N CTD antibodies detected two bands in the WT, whereas only the lower band was identified in the 4.1G/N double mutants (n=3) (Fig. 5B,C).

To characterize the residual 4.1N splicing variant in the 4.1G/N double mutants, we combined RACE (rapid amplification of cDNA ends) experiments and database analyses. The disappearance of the lower WT 4.1N band in western blots stained with the anti-4.1N exon 2 antibody (Fig. 5A, left panel) indicated an alternative use of the first coding exon, as previously reported for 4.1R and 4.1B (Conboy, 1991; Gascard, 2004). There are indications of alternative start codon use in human (NM_177966) and equine (XM_001501911) 4.1N mRNA, but no corresponding 4.1N mRNA or ESTs could be identified for the mouse.

To define the full brain-specific use of 4.1N exons in WT and 4.1G/N double mutants, we generated 5′/H11032 RACE and 3′/H11032 RACE transcript sequences (Fig. 5G). All transcripts cloned experimentally by 5′RACE from WT (12 clones) included exon 2. Exon 3 never contributed to the 5′RACE sequences in the WT.

The 3′ RACE-PCRs generated two different populations of transcripts. Four of nine clones encoded the high molecular weight class of 4.1N including exon 17B. The other five clones corresponded to the lower 4.1N form. To our surprise, in the 4.1G/N double mutants all 5′RACE-PCR transcripts (nine clones) started with exon 3, which is usually removed from mature mRNA, and the first initiation codon was localized in exon 4. All 3′RACE PCR clones from the 4.1G/N double mutants lacked exon 17A and exon 17B. As the anti-4.1N exon 2 peptide antibody is directed against the first 15 amino acids of the N-terminus, it is possible that this epitope is not accessible for the antibody in the WT 4.1N short variant. The absence of a band in the 4.1G/N double mutants can be explained by the genetic knockout of exon 2, which includes the epitope of the anti-4.1N exon 2 antibody. We conclude from the results obtained by western blots and RACE experiments that the residually expressed protein in 4.1G/N double mutants is similar to the short form expressed in the WT, but differs in its N-terminal sequence.

Next, we determined expression levels of GluR1, GluR2/3, GluR4 and NR1 in young adult 4.1G/N double-mutant mice by quantitative western blot analyses of synaptosome preparations from

**Fig. 3.** Synaptic morphology in the hippocampus of 4.1G/N double-mutant mice. (A) Properly aligned pre- and postsynaptic specializations in stratum radiatum of hippocampal area CA1. The left panel shows representative micrographs of the CA1 area of control (WT, n=3) and 4.1G/N double-mutant (DKO, n=3) sections after double labeling for glutamatergic excitatory postsynapses (stained for ProSAP1, red) and presynapses (stained for VGLUT1/2, green). Scale bars: 8 μm. (B) Quantification of isolated and colocalized ProSAP1 and VGLUT1/2 puncta in the CA1 region of control (WT, white, n=3) and 4.1G/N double-mutant mice (DKO, grey, n=3). The total numbers of synapses were not significantly different in the two experimental groups. Error bars indicate s.d. (C,D) Ultrastructural analysis of CA1 neurons in 4.1G/N double-mutant mice and WT controls at the age of 3 weeks. Mature synaptic specializations were observed in both groups; quantification was performed in the stratum radiatum. The number of synapses per 100 μm² and the length and the width of PSDs were similar in both experimental groups. Error bars indicate s.d. The numbers within the bars in D indicate the number of synapses. Scale bar: 500 nm.
Role of 4.1G and 4.1N in synaptic transmission

whole-brain homogenates (Fig. 6A). Here, we found significantly reduced synaptic GluR1 protein levels in synaptosomes from 4.1G/N double-mutant mice (WT, \( n = 6 \); DKO, \( n = 6 \); **\( P < 0.01 \)). Furthermore, we measured GluR1, GluR2/3 and GluR4 concentrations in Triton-X-100-insoluble postsynaptic density (PSD) preparations of hippocampi obtained from young adult 4.1G/N double-mutant mice and WT controls. Supporting our findings in synaptosomes, we found significant changes in the levels of the GluR1 subunit (Fig. 6B) (WT, \( n = 7 \); DKO, \( n = 7 \); *\( P < 0.05 \)). GluR2/3 levels were also significantly decreased in PSD preparations, although a direct interaction between 4.1G or 4.1N and GluR2/3 has not been described. In vivo, AMPARs usually form heteromers composed of different subunits. A reduction in the GluR1 subunit might therefore be accompanied by a reduction of other GluR subunits such as GluR2/3.

Synaptic and extrasynaptic AMPAR responses are normal in young 4.1G/N double-mutant mice

The Schaffer collateral synapses in the hippocampus are very well characterized with respect to their short- and long-term synaptic plasticity. It is generally accepted that the induction of long-term potentiation (LTP) in area CA1 is NMDAR dependent (Malenka and Bear, 2004). A leading hypothesis states that LTP in area CA1 is activity dependent and relies on insertion of AMPARs as GluR1/GluR2-containing receptors are driven into the synapses after the induction of LTP (Hayashi et al., 2000).

Fig. 4. Expression pattern of 4.1G and 4.1N in WT and 4.1G/N double-mutant brains. (A) Western blot analysis using a 4.1G-specific antibody detecting an epitope located within the first coding exon. 4.1G is expressed in all examined regions of the central nervous system (upper panel). OB, olfactory bulb; CX, cortex; CPu, striatum; Hi, hippocampus; Thal, thalamus; Hthal, hypothalamus; Co, colliculus; Ch, cerebellum; BS, brain stem; SpC, spinal cord. No signal was detected in 4.1G/N double-mutant mice (lower panel). (B) Western blot analysis using the 4.1N monoclonal antibody. 4.1N is expressed in all examined brain regions with minor expression in BS and SpC. The short variant of the two 4.1N isoforms was still present at low levels in the 4.1G/N double-mutant animals.

Fig. 5. Residual expression of a shorter 4.1N form in the 4.1G/N double-mutant hippocampus. (A-C) Hippocampal preparations from 3-week-old WT (left panel) or 3-week-old 4.1G/N double mutant (right panel) were loaded and stained on western blots with the following antibodies. (A) Anti-4.1N exon 2, which is directed against the first 15 amino acids of the N-terminus; (B) Anti-4.1N monoclonal antibody, which recognizes a C-terminal region of the spectrin-actin-binding domain; (C) Anti-4.1N CTD, which is detecting the very C-terminus of 4.1N. (D) Densitometric quantification of data pooled from B and C. The error bars indicate s.e.m. **\( P < 0.01 \); ***\( P < 0.001 \); Student’s t-test. (E) Schematic representation of the murine full-length 4.1N protein. The positions of the epitopes of the antibodies used in A-C are indicated. U1, unique region 1; FERM, Four-point-one, ezrin, radixin, moesin homology domain; FA, FERM adjacent region; U2, unique region 2; SABD, spectrin-actin-binding domain; U3, unique region 3; CTD, C-terminal domain. Unique regions are shown in yellow, conserved areas are in blue. (F) Exon usage map of the mouse 4.1N gene in brain. Numbering is derived from mouse Epb41l1 as described (Ramez et al., 2003). The colour code is modelled on the domain structure shown in E. Dotted boxes denote nontranscribed exons. The positions of the specific 5’ RACE primers and the specific 3’ RACE primers are indicated by arrows. (G) Schematic representation of the obtained RACE transcripts from WT and 4.1G/N double mutants.
As 4.1 proteins were shown to interact with the GluR1 subunit and because we found a reduction of GluR1 expression in synaptosomes of 4.1G/N double-mutant mice (Fig. 6A), we tested whether the loss of protein 4.1G together with the strong reduction of protein 4.1N leads to alterations in synaptic transmission and changes in LTP in young adult 4.1G/N double mutants. To measure synaptic responses in hippocampal brain slices of 4.1G/N double-mutant mice, we stimulated the Schaffer collaterals in area CA1 and recorded postsynaptic field potentials (fEPSPs) in the stratum radiatum of area CA1. Comparing the size of the presynaptic afferent volley (representative of presynaptic excitability), with the slope of the fEPSP (representing postsynaptic responsiveness), we found no significant differences in the fEPSP slopes at various afferent volley amplitudes (WT, \( n = 10 \); DKO, \( n = 13 \)) (Fig. 7A). Using whole-cell patch-clamp recordings, we found that the ratio of AMPAR- to NMDAR-mediated excitatory postsynaptic current (EPSC) is not altered in 4.1G/N double mutants (AMPA/NMDA ratio: WT, 4.8±0.9, \( n = 13 \); DKO, 6.1±1.1, \( n = 11 \); \( P = 0.39 \); AMPAR-mediated EPSC: WT, 241±20 pA; DKO, 54±9 pA, \( P = 0.35 \)) (Fig. 7B).

Paired-pulse facilitation (PPF) is a form of short-term plasticity and generally assumed to be of presynaptic origin. Using a paired-pulse stimulation protocol, the second response is facilitated compared with the first response within a short time-window. Using different stimulation intervals varying from 50 mseconds to 500 mseconds, we found that PPF in field recordings is not significantly changed in 4.1G/N double mutants (WT, 1.47±0.07, \( n = 15 \); DKO PPF, 1.47±0.04, \( n = 15 \); \( P = 0.97 \)) (Fig. 7C,D). We also recorded miniature EPSCs (mEPSCs) in the presence of the sodium channel blocker tetrodotoxin (TTX, 1 \( \mu \)M) and the AMPAR desensitization inhibitor cyclothiazide (CTZ, 100 \( \mu \)M). AMPAR-mediated mEPSCs showed neither a difference in frequency nor a difference in amplitude recorded at –60 mV between WT and 4.1G/N double-mutant animals. The mean mEPSC frequency was 0.9±0.1 Hz in WT mice and 1.2±0.1 Hz in 4.1G/N double-mutant mice (WT, \( n = 5 \); DKO, \( n = 9 \); \( P = 0.11 \)) and the mean amplitude of the AMPAR-mediated mEPSCs was 18±2 pA in WT animals and 16±1 pA in 4.1G/N double-mutant mice (Fig. 8) (\( P = 0.31 \)). In summary, basal synaptic properties of

Fig. 6. GluR levels are altered in 4.1G/N double-mutant mice. (A) Reduced GluR1 levels in whole brain synaptosomal preparations. Protein levels in synaptosomal fractions of 3-week-old mice expressed as percentage of WT levels. Error bars indicate s.d. (WT, \( n = 6 \); DKO, \( n = 6 \); ** \( P < 0.01 \)). (B) Reduced GluR1 and GluR2/3 levels in PSD preparations from young adult hippocampi. Protein levels are expressed as percentage (± s.d.) of WT levels (WT, \( n = 7 \); DKO, \( n = 7 \); * \( P < 0.05 \)).
AMPARs are not significantly changed in 4.1G/N double-mutant mice.

Next, we tested for alterations in the biophysical properties of AMPAR-mediated currents by measuring the rectification index, which was not significantly different between WT and 4.1G/N double-mutant neurons (2.8±0.4, n=6 and 2.7±0.4, n=6, respectively, P=0.8) (see supplementary material Fig. S2). Also, pharmacological challenging of AMPARs with the relatively selective GluR2 antagonist Naphthyl-acetyl-spermine (100 μM) revealed no differences in the slope of fEPSP recordings between the two genotypes (WT slope, 97.7±0.5% of control; DKO slope, 99.0±3% of control, n=4). The effectivity of the drug was confirmed by EPSC recordings from interneurons, where a substantial amplitude reduction was observed (see supplementary material Fig. S3). We thus did not find any indication for a change in AMPAR subunit composition in 4.1G/N double-mutant neurons.

As it is known that 4.1 proteins interact with AMPARs, we tested whether extrasynaptically located AMPAR are altered in 4.1G/N double-mutant mice. In the presence of TTX and CTZ, we bath-applied S-AMPA (100 nM) for 5 minutes and recorded the whole-cell current. By this procedure AMPARs are activated in the synaptic cleft, on the dendrites and on the somata. Application of S-AMPA led to a robust inward current of approximately 400-500 pA in WT and 4.1G/N double-mutant cells, with no significant differences between the tested genotypes (WT, 510±47 pA, n=4; DKO, 462±51 pA, n=5; P=0.52) (Fig. 9). Taken together, these results show that neither synaptic nor extrasynaptic AMPAR function is affected in 4.1G/N double mutants.

Long-term potentiation is normal in young 4.1G/N double-mutant mice

We studied long-term synaptic plasticity in WT and 4.1G/N double-mutant mice using field potential recordings. After recording stable fEPSPs in area CA1 for 10-20 minutes, the Schaffer collaterals were stimulated tetanically (four 1-second trains of 100 Hz, separated by 20 seconds). LTP was stable for 30-40 minutes after the tetanic stimulation in WT and 4.1G/N double mutants. No significant differences between WT and 4.1G/N double-mutant mice were found in the fEPSP slope 30-40 minutes after induction of LTP (Fig. 10) (WT, 137±6%, n=10; DKO, 135±7%, n=11; P=0.8). These data show that neither basal synaptic transmission nor short-term or long-term plasticity are altered in 4.1G/N double-mutant mice.

Discussion

In the present study, we used 4.1G/N double-mutant mice to assess the role of proteins 4.1G and 4.1N in glutamate receptor trafficking and synaptic anchoring, in hippocampal synaptic transmission and in synaptic plasticity. We found that (1) in young adult mice the GluR1 expression levels in 4.1G/N double mutants are reduced in synaptosomal and PSD fractions (Fig. 6); (2) GluR2/3 expression levels in 4.1G/N double mutants are reduced in PSD fractions (Fig. 6); (3) AMPAR-mediated glutamatergic synaptic transmission in...
4.1G/N double mutants are not changed (Figs 7-9), which is not consistent with the previously published notion that proteins 4.1G and 4.1N bind GluR1 and tether it to the postsynaptic cytoskeleton (Shen et al., 2000); and (4) LTP at CA3-CA1 Schaffer collaterals in 4.1G/N double mutants is not altered (Fig. 10), which argues against a crucial role of proteins 4.1G and 4.1N in the activity-dependent insertion of GluR1-containing AMPARs into potentiated synapses (Hayashi et al., 2000).

The two AMPA receptor subunits that were shown to bind 4.1 proteins, GluR1 and GluR4, bind to both 4.1N and 4.1G (Shen et al., 2000; Coleman et al., 2003). Thus, 4.1N and 4.1G can be viewed to have a joint role in GluR1 and GluR4 trafficking. Our knockdown strategy eliminated 4.1G expression entirely and 4.1N expression very strongly. The combined 4.1N/G protein expression in double-mutant hippocampus was reduced to 11-12% of WT levels. If 4.1G and 4.1N acted jointly as crucial stoichiometric interaction partners and scaffold proteins of GluR1 and GluR4 under normal circumstances, the residual expression of 4.1N in the 4.1G/N double-mutant mice is unlikely to be sufficient for the maintenance of normal function, because the relative expression levels of 4.1G, 4.1N, GluR1 and GluR4 in mouse hippocampus are 0.89-0.99, 0.95-1.05, 1.48-1.61, and 0.87-0.95, respectively (Trinidad et al., 2008). Thus, total deletion of 4.1G and a reduction of 4.1N expression by 78% would lead to a massively sub-stoichiometric ratio between combined 4.1G and 4.1N, and combined GluR1 and GluR4 expression in mouse hippocampus of at least 0.08:1, and 0.1:1, at most. Such a massive deficiency of 4.1G and 4.1N expression compared with GluR1 and GluR4 expression in the double-mutant hippocampus would be expected to have severe functional consequences on glutamatergic transmission if 4.1G and 4.1N had a crucial role in GluR1 and GluR4 trafficking and anchoring, but our data show that this is not the case.

The fact that GluR1 levels are reduced in 4.1G/N double mutants supports the notion that proteins 4.1G and 4.1N bind GluR1 and stabilize it (Shen et al., 2000). However, the finding that neither basal AMPAR-mediated synaptic transmission nor synaptic plasticity in the hippocampus are altered in 4.1G/N double mutants makes an essential role of proteins 4.1N and 4.1G in the trafficking, function, and dynamics of AMPARs in vivo rather unlikely. This lack of phenotypic changes in 4.1G/N double mutants is either due to the fact that previously published in vitro experiments on the role of 4.1G and 4.1N in GluR1 trafficking and anchoring detected cell-culture specific phenotypes that are not relevant in vivo, or is caused by the fact that multiple AMPAR scaffold proteins operate in parallel at synapses and can substitute for the loss of the two scaffold proteins studied here. We favour the latter notion, as outlined below.

Striking functional redundancy has been reported for other postsynaptic AMPAR scaffold protein families that have been studied in detail. Targeted truncation of PSD95 in mutant mice, for example, does not affect AMPAR-mediated synaptic transmission in the hippocampus (Migaud et al., 1998). Similarly, single deletions of PSD95 or PSD93 do not cause functional changes in basal synaptic transmission (Elias et al., 2006). Only the simultaneous deletion of both PSD93 and PSD95 leads to marked reductions in glutamatergic synaptic transmission (Elias et al., 2006). By contrast, individual knockdown of either PSD93 or PSD95 using an shRNA strategy causes impaired glutamatergic transmission in hippocampal slice cultures (Elias et al., 2006). These findings indicate that PSD93 and PSD95 are functionally redundant and can compensate for the loss of the other MAGUK if sufficient time is available for these compensatory mechanisms to take place, as is the case in the deletion mutant mice, but apparently not when the proteins are knocked down by shRNAs.

Two recent studies found the interaction of stargazin and other TARPs with AMPARs to be the most robust and least transient compared with other putative interactions, including those with 4.1 proteins (Fukata et al., 2005; Vandenberghhe et al., 2005). Indeed, TARPs function as auxiliary subunits of AMPARs and are important determinants of AMPAR trafficking and synaptic anchoring. An 80-90% reduction in hippocampal AMPAR protein levels and a 30-40% reduction in synaptic transmission are observed in γ8-knockout mice, and extrasynaptic AMPARs were also reduced by 80-90% (Rouach et al., 2005). Similarly, cerebellar granule cells from mice lacking Stargazin lack synaptic and extrasynaptic AMPARs (Chen et al., 2000). These data show that γ8- and Stargazin determine AMPAR stability and synaptic recruitment. Furthermore, the data obtained from γ8-deficient mice indicate that even strong reductions in overall AMPAR levels do not cause similarly severe reductions in synaptic AMPAR function. This apparent lack of a linear correlation between overall AMPAR levels and AMPAR function at synapses might explain why, in the case of the 4.1G/N double-mutant mice described here, a 20-30% reduction of GluR1 levels at postsynapses does not cause a concomitant reduction of synaptic transmission in the hippocampus.

In summary, the present study indicates that proteins 4.1G and 4.1N are generally dispensable for proper function of glutamatergic...
supernatant fraction was collected, and the membranes were washed with ice-cold 
PBS and solubilized in detergent lysis buffer (PBS containing 1% Triton X-100). 
Protein concentration was determined by the Lowry method. Western blotting was 
performed as described above. The sequences of antibodies used were as follows: 
NMDA receptor subunits (BD Biosciences), synaptotagmin (Chemicon), PSD-95, 
PSD-93 and PSD-99 (Chemicon). The intensity of protein bands was determined by 
chemiluminescence (ECL Plus; Amersham). Quantitative densitometric analysis was 
performed using ImageJ software. The intensities of the signals were normalized to 
actin. Statistical analyses were performed using Student’s t-test.

**Electrophysiology**

WT and 4.1G/N double-mutant mice (3- to 5-weeks old) of both genders were 
decapitated under anesthesia and the brains were quickly removed. Horizontal 
hippocampal mouse brain slices (300 μm thick) were prepared in a vibratome 
under ice-cold conditions. Only coronal sections (3 to 6) were used. The slices were 
sliced in oxygenated ACSF solution (125 mM NaCl, 2.5 mM KCl, 25 mM NaHCO3, 
5 mM glucose, saturated with 95% O2 and 5% CO2 at pH 7.4) and kept at room 
temperature. Field potential recordings were performed with low-resistance 
micro-electrodes (filled with 2 M NaCl and 1 M NaF, 0.5 M NaCl, 25 mM sucrose, and 25 mM glucose. After perfusion with ACSF, the slices were incubated with 100 μM tetrodotoxin (TTX) at 37°C for 30 minutes. The recordings were performed using a CED 1401plus system (Cambridge Electronic Design) and analyzed with Clampfit software (Molecular Devices). The input resistance was monitored throughout the experiment.
patch-clamp electrodes filled with ACSF. Field EPSPs were recorded in stratum radiatum of CA1 from freely moving rats. The field EPSPs were stimulated with a frequency of 0.05 Hz. Single cell recordings were performed in whole cell patch-clamp mode. Patch-clamp electrodes (electrode resistance 2-5 MΩ) were filled with 117.5 mM Cs-glucuronate, 2.5 mM CsCl, 8 mM NaCl, 10 mM HEPES, 10 mM TEA, 0.2 mM EGTA, 4 mM Mg-ATP, 0.3 mM Na-GTP and QX-314 2; pH was adjusted to 7.2 with CsOH. Access resistance ranged from 6 to 20 MΩ and was continuously monitored throughout the experiment. Recordings were discarded when the series resistance changed more than 20%. All patch-clamp experiments were done in the presence of the GABA<sub>A</sub> receptor-antagonist gabazine (95 5351, 1 μM; Sigma) and 4 mM MgSO<sub>4</sub> and CaCl<sub>2</sub>. AMPA/NMDA ratios were obtained by evoking single-component EPSCs at ~60 mV and a dual-component EPSCs at ~40 mV. The NMDA-mediated portion of the dual-component current at +40 mV was measured 100 msec after the stimulus artefact. AMPAR-rectification index was determined (in the presence of 40 μM APV) as current amplitude at a holding potential of ~60 mV divided by the amplitude at ~40 mV. Miniature EPSCs were recorded in the presence of tetrodotoxin (TTX; 1 μM) and cyclothiazide (100 μM). AMPA-mediated whole-cell currents were obtained by bath application of 100 nM S-AMPA for five minutes in the presence of TTX and cyclothiazide. In field recordings, LTP was induced by four tetani of high-frequency stimulation at 100 Hz for 1 second with 20 second intertrain intervals. The magnitude of LTP was determined by averaging the responses collected during the last 5 minutes of each experiment. Paired-pulse facilitation (P2/P1) in the field and whole-cell recordings was investigated by analysing the ratio of the second to the first synaptic response. Data are expressed as mean ± s.e.m., and statistical comparison was done by applying Student’s t-test (Excel, Microsoft). The significance level was set to P<0.05.

We thank I. Thanhäuser, D. Schwerdtfeger, and F. Benseler (Göttingen, Germany) for oligonucleotide synthesis and DNA sequencing, and the staff of the Transgenic Animal Facility at the Max-Planck-Institut für Experimentelle Medizin (Göttingen, Germany) for excellent technical support. We are also grateful to S. Walden and A. Schönherr for excellent technical assistance (Berlin, Germany). This work was funded by the Max Planck Society (Munich, Germany) and by the Deutsche Forschungsgemeinschaft (Exc 257, GRK 1125 and SFB 665).

References

Barry, M. F. and Ziff, E. B. (2002). Receptor trafficking and the plasticity of excitatory synapses. Curr. Opin. Neurobiol. 12, 279-286.

Beique, J. C. and Andrade, R. (2003). PSD-95 regulates synaptic transmission and plasticity in rat cerebral cortex. J. Physiol. 546, 859-867.

Boeckers, T. M., Kreutz, M. R., Winter, C., Zuschatter, W., Smalla, K. H., Sammartini-Vila, L., Wes, H., Languinea, K., Boekmann, J., Garner, C. E. et al. (1999). Protein-synch-associate protein-1: a putative binding protein (ProSAP/ContBP1) in a PDZ domain protein highly enriched in the postsynaptic density. J. Neurosci. 19, 6506-6518.

Bredt, D. S. and Nicoll, R. A. (2003). AMPA receptor trafficking at excitatory synapses. Nature 40, 361-364.

Cai, C., Conklin, S. K., Niemi, K. and Keinanen, K. (2002). Selective binding of synaptic-associated protein 97 to GluA-alpha-aminoo-5-hydroxy-3-methyl-4-isoxazole propionate receptor subunit is determined by a novel sequence motif. J. Biol. Chem. 277, 31484-31490.

Chen, K., Merino, C., Sigrist, S. J. and Featherstone, D. E. (2004). The 4.1 protein coracle mediates subunit-selective anchoring of droshplia glutamate receptors to the postsynaptic actin cytoskeleton. J. Neurosci. 25, 6667-6675.

Chen, L., Chetkovich, D. M., Petralia, R. S., Sweeney, N. T., Kawasaki, Y., Wastholt, R. J., Bredt, D. S. and Nicoll, R. A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408, 936-943.

Coleman, S. K., Cai, C., Mottershead, D. G., Haapalahti, J. P. and Keinanen, K. (2003). Surface expression of GluD-AMPAR receptor is dependent on an interaction between its C-terminal domain and a 4.1 protein. J. Neurosci. 23, 798-806.

Conboy, J. G., Chan, J. Y., Chasis, J. A., Kan, Y. W. and Mohandas, N. (1991). Glutamate receptor GRP1 directly steers kinesin to dendrites. Nature 347, 83-87.

Conboy, J. G., Shan, Y., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2002). Glutamate receptor GRP1 directly steers kinesin to dendrites. Nature 417, 321-327.

Rouach, N., Byrd, K., Petralia, R. S., Elias, G. M., Adesnik, H., Tomita, S., Kimmeth, S., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2003). Protein 4.1 G localizes in rodent microglia. J. Cell Sci. 116, 371-379.

Nicholls, D. G. (1978). Calcium transport and porton electrochemical potential gradient in mitochondria from guinea-pig cerebral cortex and rat heart. Biochem. J. 170, 511-519.

Sulzer, D. and Nicoll, R. A. (2006). Auxiliary subunits assist AMPA-type glutamate receptors. Science 311, 1253-1256.

Ohara, R., Yamakawa, H., Nakayama, M. and Ohara, O. (2000). Type II brain 4.1 (4.1BKA9A987), a member of the protein 4.1 family, is localized to neuronal paraneuronal react. Brain Res. Mol. Brain Res. 85, 41-52.

Ohno, N., Terada, N., Tanaka, J., Yokoyama, A., Yamakawa, H., Fujii, Y., Baba, T., Ohara, O. and Ohno, S. (2005). Protein 4.1G localizes in rodent microglia. J. Neurosci. 25, 8370-8376.

Sulzer, D. and Nicoll, R. A. (2000). Stargazin regulates synaptic targeting of AMPA receptors by two distinct mechanisms. Nature 408, 936-943.

Rouach, N., Byrd, K., Petralia, R. S., Elias, G. M., Adesnik, H., Tomita, S., Kimmeth, S., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2003). Protein 4.1 G localizes in rodent microglia. J. Cell Sci. 116, 371-379.

Rouach, N., Byrd, K., Petralia, R. S., Elias, G. M., Adesnik, H., Tomita, S., Kimmeth, S., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2003). Protein 4.1 G localizes in rodent microglia. J. Cell Sci. 116, 371-379.

Rouach, N., Byrd, K., Petralia, R. S., Elias, G. M., Adesnik, H., Tomita, S., Kimmeth, S., Seog, D. H., Tanaka, Y., Kanai, Y., Takei, Y., Kawagishi, M. and Hirokawa, N. (2003). Protein 4.1 G localizes in rodent microglia. J. Cell Sci. 116, 371-379.