Functional Significance of the Kainate Receptor GluR6(M836I) Mutation that is Linked to Autism

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Key Words

Kainate receptor • Glutamate receptor • GLUR₆ • Rab11 • Rab5 • Recycling • Expression

Abstract

Previous studies revealed a linkage of the kainate receptor GluR6 with autism, a pervasive developmental disorder. Mutational screening in autistic patients disclosed the amino acid exchange M836I in a highly conserved domain of the cytoplasmic C-terminal region of GluR6. Here, we show that this mutation leads to GluR6 gain-of-function. By using the two-electrode voltage clamp technique we observed a significant increase of current amplitudes of mutant GluR6 compared to wild type GluR6. Western blotting of oocytes injected with mutant or wild type GluR6 cRNA and transfection of EGFP-tagged GluR6 receptors into COS-7 cells revealed an enhanced plasma membrane expression of GluR6(M836I) compared to wild type GluR6. Membrane expression of GluR6(M836I) but not of wild type GluR6 seems to be regulated by Rab11 as indicated by our finding that GluR6(M836I) but not wild type GluR6 showed increased current amplitudes and protein expression when coexpressed with

Rab11. Furthermore, injection of GTP plus Rab11A protein into oocytes increased current amplitudes in GluR6(M836I) but not in wild type GluR6. By contrast, Rab5 downregulated the currents in oocytes expressing wild type GluR6 but had only little, statistically not significant effects on currents in oocytes expressing GluR6(M836I). Our data on altered functional properties of GluR6(M836I) provide a functional basis for the postulated linkage of GluR6 to autism. Furthermore, we identified new mechanisms determining the plasma membrane abundance of wild type GluR6 and GluR6(M836I).

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Introduction

Autism is a pervasive developmental disorder. It is characterized by impaired reciprocal social interaction and communication as well as restricted and stereotyped patterns of interests and activities [1].

Glutamate is an excitatory neurotransmitter in the central nervous system (CNS), which acts on two different groups of receptors: metabotropic (mGluR) and ionotropic (iGluR) receptors. The mGluRs are G protein-

coupled and modify neural and glial excitability. The iGluRs are cation permeable ion channels. Glutamate and its receptors are directly involved in cognitive functions such as learning and memory consolidation [2] and are candidates for several neurological and psychiatric diseases [3]. There are three different families within the iGluR-group: NMDA, AMPA, and kainate receptors [4, 5]. The kainate receptors (KARs) contribute to the excitatory postsynaptic potential in response to glutamate and modulate the release of neurotransmitters (GABA and glutamate) through presynaptic mechanisms [6-8]. KARs are regulated during brain development and by neuronal activity [9]. The mammalian KA receptor gene family consists of five subunits: the low affinity KA-receptors GluR5, 6, and 7 (or GLU_{K5} - GLU_{K7} , according to the recently proposed IUPHAR nomenclature), and the high affinity KA-receptors KA1 and KA2. GluR6 is abundantly expressed in brain regions involved in learning and memory, such as the hippocampus, as well as in cerebral structures serving motoric and motivational aspects of behaviour, such as basal ganglia and cerebellum [10]. Recently, it has been shown that postsynaptic KA receptors contribute to the excitatory postsynaptic current (EPSC) at different types of synapses [11, 12]. The KAR-mediated EPSCs have small amplitudes and show mostly slow kinetics compared to AMPA-mediated EPSCs. Genetic deletion of GluR6 [10] has revealed important and distinct roles for the GluR6 subunit in synaptic transmission and plasticity in the hippocampus [7, 13-15]. Little is known about regulation of GluR6. Earlier studies pointed to modulation of GluR6 activity by protein phosphorylation [16]. A recent study by Jamain et al. suggests significant association between GluR6 and autism [17]. Mutational screening revealed several nucleotide polymorphisms (SNPs). One polymorphism showed an amino acid change (M836I) in a highly conserved domain of the cytoplasmic C-terminal region of GluR6. We set out to investigate if the mutation M836I in GluR6 affects channel function. By using the two-electrode voltage clamp technique in *Xenopus* oocytes we compared current amplitudes of wild type and mutant GluR6. We also analyzed membrane expression by Western blotting of receptor proteins expressed in *Xenopus* oocytes and by transfection of fluorescently labeled wildtype and mutant cDNAs into COS-7 cells. We further investigated the molecular mechanism of membrane insertion of GluR6(M836I) compared to wildtype GluR6. As a result, we disclosed a novel regulatory mechanism of kainate receptors that presumably contributes to the development of autism.

Materials and Methods

Mutagenesis

GluR6-EGFP/pEGFP-N1 was done by replacing the stop codon of GluR6 DNA by an Age I restriction site (TGAAGC to CCGGTC) for subsequent subcloning of GluR6 cDNA into the pEGFP-N1 vector. The cloning sites for subcloning used were NheI and AgeI. In the original paper on GluR6 linkage to autism [17], the authors refer to mutant GluR6(M867I). We renumbered this mutant GluR6(M836I) because our numbering starts with the first amino acid of the mature protein and does not include the 31 amino acids of the signal peptide. The mutation M836I was introduced into rat GluR6 by using the QuikChange II Site-Directed Mutagenesis Kit (for GluR6(M836I)/pSGEM), or the QuikChange XL Site-Directed Mutagenesis Kit (for GluR6(M836I)-EGFP/pEGFP-N1) from Stratagene. The primers used for mutagenesis were the following: gta gcg caa tgg tgg aag agc tga gaa tat ccc tga ag (sense), ctt cag gga tat tct cag ctc ttc cac cat tgc gct ac (antisense) with a silent deletion of the restriction site Eco47III for analytical purposes. The generation of Rab cDNAs has been described previously [18-20].

cRNA synthesis

Template cDNA was linearized with NheI (for GluR6 and Rab 11 cDNAs) or with NarI (for Rab 5 cDNAs). cRNA was synthesized from 1 µg of linearized DNA using an in vitro transcription kit (mMessage mMachine T7 kit, Ambion). cRNA concentrations were evaluated by photospectrometry, and transcript quality was checked by agarose gel electrophoresis.

Electrophysiological measurements in *Xenopus* oocyte

Oocytes of stages V-VI were surgically removed from the ovaries of *Xenopus laevis* as described elsewhere [21]. Female *X. laevis* frogs were anaesthetized with 0.1 % tricaine (Sigma, Germany) and pieces of ovary were surgically removed. The incisions were sutured and the animals allowed to recover. Frogs were humanely killed after the final collection [22]. The experimental procedures were approved by the Regierungspräsidium Tuebingen, Germany. Oocytes of similar size were injected with 5 ng of GluR6 cRNA with or without 10 ng of either Rab cRNA using a Nanoliter 2000 injector (WPI inc., Florida, USA). Standard two-electrode voltage clamp recordings were performed 5 days after cRNA injection with a TurboTec 03 amplifier (NPI, Tamm, Germany) and an interface DIGIDATA 1322A from Axon Instruments. Data were recorded at a 500 Hz sampling rate and filtered at 100 Hz. Data analyses were done with pClamp 9.0/clampfit 9.0 software (Axon inc., CT, USA), and Origin 6.0 software (Microcal). Agonist solutions were prepared in ND-96 buffer (in mM, NaCl, 96; CaCl₂, 1.8; KCl, 2.0; MgCl₂, 1.0 and HEPES-NaOH, 5, pH 7.2 with NaOH). Current and voltage electrodes were filled with 3 M KCl and had resistances of 0.5-1.5 MΩ. The oocytes were held at -70 mV and agonist (300 µM glutamate, Sigma, Munich, Germany) was applied by superfusion for 10-15 s at a flow rate of 10-14 ml/min. Prior to agonist application, the oocytes were incubated for 6 min in concanavalin A (ConA) (100µM) (Sigma-Aldrich, Munich, Germany), which minimized desensitization.

Labelling of cell surface proteins using biotinylated ConA

To identify the fraction of receptor protein inserted in the plasma membrane, surface proteins were tagged with biotinylated concanavalin A and isolated by streptavidin/sepharose-mediated precipitation of the biotinyl-ConA/protein complex as described elsewhere [23]. Briefly, intact oocytes were incubated in 10 μ M biotinyl-ConA (Sigma, Munich, Germany) for 30 min at room temperature. At this step ConA binds to glycosylated plasma membrane proteins, e.g. glutamate receptors. Since intact oocytes were used, only plasma membrane proteins but not intracellular membrane proteins are labelled. After five 10 min washes in ND-96 buffer to remove excess ConA, 20 intact oocytes were homogenized with a Teflon pestle in H-buffer (20 μ l/oocyte; 100 mM NaCl, 20 mM Tris-HCl, pH 7.4, 1 % Triton X-100, plus a mixture of proteinase inhibitors (Complete; Boehringer Mannheim, Mannheim, Germany)) and were kept at 4 °C for 1 hr on a rotating rod. A 20 μ l aliquot was kept as a Total protein sample (T). After centrifugation of the remaining homogenate for 1 min at 16,000 x g, the supernatants were supplemented with 20 μ l of washed streptavidin-sepharose beads (Sigma, Munich, Germany) and incubated at 4 °C for 3 hrs on a rotating rod. During this step the streptavidin beads bound to the biotinylated ConA-plasma membrane receptor complex. The streptavidin-sepharose beads were then pelleted by a 2 min spin at 16,000 x g and washed three times in H-buffer. 20 μ l of the supernatant was kept as supernatant protein sample (SN). The final pellets (P) containing plasma membrane receptors were boiled in 20 μ l of SDS-PAGE loading buffer (0.8 M β -mercaptoethanol, 6 % SDS, 20 % glycerol, 25 mM Tris-HCl, pH 6.8, and 0.1% bromophenol blue).

Gel electrophoresis and Western blotting

Proteins from homogenized oocytes were separated by SDS electrophoresis and transferred to nitrocellulose filters. Blots were blocked in 1x PBS containing 5% milk powder for at least 1 hour at room temperature. For the detection of GluR6, primary rabbit immunoaffinity-purified anti-GluR6 antibody (1 μ g/ μ l, Upstate/Biomol, Hamburg, Germany) or primary rabbit monoclonal anti-GluR6 antibody (clone NL9, 1:2000 dilution, Upstate/Biomol, Hamburg, Germany) and secondary horseradish peroxidase-conjugated donkey anti-rabbit antibody (1:1000 dilution, Amersham Bioscience, Freiburg, Germany) were used. For verification of protein levels, Ponceau Red staining was performed.

GluR6 transfection and microscopic analysis

COS-7 cells were transfected with wild type GluR6-EGFP or mutant GluR6(M836I)-EGFP according to manufacturer's instructions (FuGENE 6 transfection kit, Roche Diagnostics, Mannheim, Germany). Cells were grown on small glass coverslips and fixed in 4% paraformaldehyde for 15 min. After rinsing in PBS the coverslips were mounted in Mowiol, and analyzed on a confocal laserscanning microscope (Zeiss LSM 510 META). Confocal images from the center of transfected cells were acquired using an argon laser excitation (488nm) with appropriate filter sets, and a 40x oil immersion lens (N.A.=1.3). Thus, optical section thickness of >0.8 μ m allowed

Fig. 1. Enhanced current amplitudes of GluR6(M836I) versus wild type GluR6. A, Current amplitudes of *Xenopus* oocytes expressing wild type GluR6 or GluR6(M836I) were measured and are shown normalized to the GluR6 currents. The significant difference to current amplitudes in oocytes expressing wild type GluR6 is indicated by *, $p=0.008$; $n=15-16$. The mutation (M836I) is located in the intracellular C-terminal region of the receptor. B, Representative current traces measured in *Xenopus* oocytes in response to superfusion with 300 μ M glutamate. All currents were measured at -70 mV and after pretreatment of oocytes with ConA to minimize desensitization.

the distinction between membrane-associated versus cytoplasmic fluorescence.

Statistical analysis

For the immunoblotting studies, representative immunoblots are shown, and a quantitative assessment of plasma membrane abundance was carried out by densitometric analysis (Scion Image software) of immunoblots from similar experiments. Before pooling the results from different blots, the result from each blot was expressed as a percentage of the control value (relative abundance). The combined results from all blots were then expressed as the mean $\pm$ SEM. Statistical analysis of the data was performed by Origin 6.0. Student's t-test was applied for unpaired data and $p<0.05$ was considered statistically significant. Oocyte experiments were analysed by student's t-test or ANOVA, as applicable. Electrophysiological data are presented as means $\pm$ SEM (n = number of cells). Statistical significance of differences between means was defined by ANOVA.

Fig. 2. Western blot and fluorescence labeling demonstrating an increased amount of membrane protein of GluR6 (M836I) compared to wild type GluR6 in *Xenopus* oocytes (A) and COS cells (B). A, Glycosylated plasma membrane proteins expressed in *Xenopus* oocytes were labelled with biotinylated ConA. Oocytes were homogenized and plasma membrane proteins streptavidin-precipitated. Samples including controls from uninjected oocytes were separated on a SDS gel, Western-blotted and probed with a rabbit immunoaffinity-purified antibody directed against a 16 amino acid fragment of the C-terminus of GluR6 (Upstate/Biomol, Hamburg, Germany; antibody not available anymore) or with a rabbit monoclonal antibody directed against a 14 amino acid fragment of the C-terminus of GluR6 (Upstate/Biomol, Hamburg, Germany). The double band might represent different glycosylation forms of the GluR6 protein. The GluR6 protein has an apparent molecular weight of ~115 kDa. Bar graph showing relative abundance of GluR6 plasma membrane protein. The band intensity was quantified by arithmetic analysis using the software Scion image. The values of three different blots from different batches were used for statistical analysis. * indicates significant ($p = 0.05$) difference to staining intensity after expression of GluR6 alone. Due to the saturating conditions of the Western Blot the analysis represents only an estimate of relative abundance of GluR6 plasma membrane protein. B, COS-7 cells transfected with wild type GluR6-EGFP or mutant GluR6(M836I)-EGFP. GluR6(M836I)-EGFP is located predominantly at the plasma membrane of the COS-7 cells, whereas for GluR6-EGFP no preference for membrane expression could be identified.

Results

The mutation M836I located in the C-terminus of GluR6 (Fig. 1A) was suggested to be linked to autism [17]. Therefore, we tested the functional effects of the mutation. We performed site-directed mutagenesis to introduce this mutation into rat GluR6/pSGEM and injected cRNA of wild type GluR6 or GluR6(M836I) into *Xenopus* oocytes. After 5 days of expression we used the two-electrode voltage clamp method to compare current amplitudes of wild type versus mutant GluR6. After application of the agonist glutamate we recorded significantly increased current amplitudes for GluR6(M836I) compared to wild type GluR6. The relative current amplitude for the GluR6 mutant was almost doubled compared to wild type GluR6 (Fig. 1), with mean absolute currents of $33.4 \pm 3.6 \mu\text{A}$ ($n=15$) for mutant and $18.8 \pm 3.6 \mu\text{A}$ ($n=16$) for wild type GluR6, respectively.

We then set out to investigate if the increase in current amplitude of GluR6(M836I) is a result of enhanced plasma membrane abundance. As illustrated in Fig. 2A,

the plasma membrane abundance of GluR6 is increased in *Xenopus* oocytes expressing GluR6(M836I) as compared to oocytes expressing wild type GluR6. Additionally, we transfected COS-7 cells with wild type or mutant GluR6-EGFP and compared plasma membrane expression of both. As depicted in Fig. 2B, GluR6(M836I) but not wild type GluR6 is expressed predominantly in the plasma membrane.

Mammalian cells and *Xenopus laevis* oocytes have been shown to possess and utilize highly conserved Rab-dependent trafficking pathways [24, 25]. To test whether Rab dependent transport from recycling endosomes to the plasma membrane was required for localization of GluR6(M836I) at the plasma membrane we took advantage of a mutant version of a constitutively inactive guanosine diphosphate-bound form of the small G-protein (GTPase) Rab11 (Rab11-S25N) (Fig. 3A). This mutant selectively impairs endosomal recycling by preventing Rab 11-mediated trafficking from recycling endosomes to the plasma membrane [26]. Oocytes injected with wild type GluR6 plus wild type Rab11 cRNAs showed no significant

Fig. 3. Constitutively expressed Rab11 increases GluR6 (M836I) membrane insertion in *Xenopus* oocytes. **A**, Current amplitudes of oocytes expressing wild type GluR6 or GluR6(M836I), either alone or plus Rab11 or Rab11 (S25N) were measured and are shown normalized to wild type GluR6 or GluR6(M836I) currents, as appropriate. Significant differences to current amplitudes in oocytes expressing GluR6 (M836I) alone are indicated by *, $p=0.04$; $n=10-18$. **B**, Western blot showing that Rab11 increases GluR6(M836I) membrane insertion. Western blotting was performed as described in the legend of Fig. 2A. GluR6 protein has an apparent molecular weight of ~115 kDa. Bar graph showing relative abundance of GluR6 plasma membrane protein. The band intensity was quantified by arithmetic analysis using the software Scion image. The values of four different blots from different batches were used for statistical analysis. * indicates significant ($p = 0.05$) difference to staining intensity after expression of GluR6 alone.

change in current amplitudes compared to wild type GluR6 injected with water or Rab11(S25N) cRNA (Fig. 3A), suggesting that Rab11 does not play an important role in endosomal recycling of wild type GluR6. However, coexpression of Rab11 with the mutant GluR6(M836I) resulted in a significant increase of current amplitudes of approximately 1.8-fold compared to GluR6(M836I) expressed alone, indicating that Rab11 is involved in the endosomal recycling of GluR6(M836I). In line with this interpretation, GluR6(M836I) coexpressed with Rab11(S25N) showed no significant difference in current amplitudes compared to GluR6(M836I) expressed alone (Fig. 3A).

If Rab11 regulates endosomal recycling of GluR6(M836I), as suggested by our voltage clamp experiments, we would expect increased plasma membrane insertion of GluR6(M836I). To explore this possibility, we performed Western blot experiments. Plasma membrane insertion was indeed increased for GluR6(M836I) when coexpressed with Rab11 compared to GluR6(M836I) or GluR6(M836I) plus Rab11(S25N) (Fig. 3B).

To further investigate the involvement of Rab11 in

the recycling of GluR6 receptors, we coinjected GTP and Rab11A protein into oocytes that expressed either wild type GluR6 or GluR6(M836I) and, over a time scale of 18 minutes, compared current amplitudes to control oocytes that were only injected with GTP or GTP-free Rab11A. Every 60-90 seconds we applied the agonist glutamate and recorded the resulting current amplitudes. For analysis, current amplitudes were normalized to currents before injection of GTP. As shown in Figure 4, GTP activated Rab11 which in turn increased mutant GluR6 (Fig. 4B) but not wild type GluR6 currents (Fig. 4A). Injection of GTP alone or GTP-free Rab11A protein into oocytes expressing wild type GluR6 receptors did not exert any activating effect on current amplitudes (Fig. 4A), indicating that GTP acts specifically via Rab11 and not directly on mutant GluR6 receptors. Interestingly, acute application of GTP-activated Rab11A protein seemed to decrease wild type GluR6 currents (Fig. 4A), an effect not seen when Rab11 was constitutively coexpressed with wild type GluR6 (Fig. 3A).

We further investigated the underlying mechanisms of recycling of wt GluR6. One molecule regulating the internalization of membrane proteins via early endosomes

Fig. 4. GTP-activated Rab11A stimulates GluR6 (M836I) but not wild type GluR6 after injection of protein into oocytes. A, B, GTP and Rab11A protein were coinjected into oocytes that expressed either wild type GluR6 (A) or GluR6(M836I) (B). Current amplitudes were compared to control oocytes that were only injected with GTP or GTP-free Rab11A. Every 60–90 seconds glutamate was applied. Current amplitudes were normalized to currents before injection of GTP. Statistically significant differences are indicated by * ($p < 0.05$).

is the small G-protein (GTPase) Rab5 [27]. Oocytes injected with wild type GluR6 plus wild type rab5 showed a significant decrease in current amplitudes compared to wild type GluR6 injected with water or the dominant negative mutant rab5(N133I) (Fig. 5A). Thus Rab5 presumably plays an important role in the internalization of wild type GluR6 membrane protein. However, coexpression of Rab 5 with the mutant GluR6(M836I) resulted in no significant change of current amplitudes compared to GluR6(M836I) expressed alone or coexpressed with Rab5(N133I) (Fig. 5A). In line with this we observed decreased plasma membrane expression of GluR6 in oocytes when coexpressed with Rab5 (Fig. 5B).

Discussion

Based on their mutation screening, Jamain et al. suggested a significant association between GluR6 and autism [17]. In their screening, they found several SNPs in the human GluR6 gene, one of which was exonic with the amino acid change M836I, three were exonic with conservative amino acid changes, and three SNPs were intronic. The methionine 836 is a highly conserved amino acid that is located in the C-terminus of GluR6 next to a putative protein phosphorylation site. The C-terminus plays an important role in the organisation and electrophysiological properties of the receptor. When the C-terminal region of GluR6 is deleted, the protein is not associated with the synapse-associated protein PSD95/SAP90, which might result in diminished targeting of the

receptor to the synapse. Also, interaction of the C-terminus of GluR6 with PSD95/SAP90 alters GluR6 function by reducing desensitization [28, 29].

Recently, Martin and Henley demonstrated that in cultured hippocampal neurons the surface expression of GluR6 is dynamically regulated [30]. Internalized GluR6 can enter different recycling or retention pathways depending on the endocytotic stimulus.

Rab proteins have been shown to regulate the endosomal recycling of membrane proteins. Here, we concentrate on the two Rab proteins Rab5 and Rab11. The small GTPase Rab5 regulates the internalization of membrane proteins via early endosomes [27] (Fig. 6), whereas Rab11 mediates trafficking from recycling endosomes to the plasma membrane [26] (Fig. 6).

Park et al. showed that Rab11A(S25N) impairs endosomal recycling of GluR1 but not of NMDAR1 [31]. The effect of Rab11A(S25N) on kainate receptors was not tested. We now show that the mutant Rab11A(S25N) impairs and wild type Rab11A facilitates endosomal recycling of GluR6(M836I), but not of wild type GluR6 (Fig. 6). The increase in membrane abundance of GluR6(M836I) compared to wild type GluR6 could therefore be explained by increased endosomal recycling by Rab11, a mechanism that does not appear to act on wild type GluR6. Furthermore, Rab5 seems to internalize wild type GluR6 but not mutant GluR6 (Fig. 6) which could similarly contribute to increased membrane abundance of GluR6(M836I). There remains the possibility that molecules other than Rab5 regulate the internalization of GluR6(M836I).

As illustrated in Figure 4, GTP-activated Rab11 pro-

Fig 5. Constitutively expressed Rab5 decreases wild type GluR6 currents and membrane expression in *Xenopus* oocytes. A, Current amplitudes of oocytes expressing wild type GluR6 or GluR6 (M836I), either alone or plus Rab5 or Rab5 (N133I) were measured and are shown normalized to wild type GluR6 or GluR6(M836I) currents, as appropriate. Significant differences to current amplitudes in oocytes expressing wild type GluR6 alone are indicated by *, $p=0.007$; $n=5-18$. B, Western blot showing that Rab5 decreases GluR6 wild type membrane insertion. Western blotting was performed as described in figure legend 2A. GluR6 protein has an apparent molecular weight of ~115 kDa. Bar graph showing relative abundance of GluR6 plasma membrane protein. The band intensity was quantified by arithmetic analysis using the software Scion image. The values of three different blots from different batches were used for statistical analysis. * indicates significant ($p < 0.05$) difference to staining intensity after expression of GluR6 alone.

Fig. 6. Schematic diagram of endosomal trafficking of GluR6. Schematic diagram showing a pathway of endosomal trafficking of protein between the plasma membrane and recycling endosomes. Regulatory proteins and the transport steps they control are indicated.

tein injected into oocytes expressing either wild type or mutant GluR6 increases mutant GluR6. However, it decreases wild type GluR6 currents, a result not observed when current amplitudes of oocytes expressing GluR6 from cRNA were recorded (Fig. 3A). Possibly, there is a relative lack of GTP-activated Rab11 under conditions of constitutive coexpression, and more GTP-activated Rab11 is required for the reduction effect in wild type

than for the current increase in mutant GluR6, thus explaining the different results.

In conclusion, the present observations disclose a novel mechanism determining the membrane abundance of wild type GluR6 and GluR6(M836I) and a novel function of the Rab proteins 5 and 11. Moreover they provide a functional basis for the postulated linkage of GluR6 to autism.

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