Tau Tubulin Kinase TTBK2 Sensitivity of Glutamate Receptor GluK2

Kristina Nieding a Veronika Matschke b Sven G. Meuth c Florian Lang d Guiscard Seebohm a Nathalie Strutz-Seebohm a

aDepartment of Cardiovascular Medicine, Institute for Genetics of Heart Diseases (IfGH), University Hospital Muenster, Muenster, bDepartment of Cytology, Institute of Anatomy, Ruhr University Bochum, Bochum, cDepartment of Neurology, Westfaelische Wilhelms - Universitaet Muenster, Muenster, dDepartments of Cardiology, Vascular Medicine and Physiology, University Tuebingen, Tuebingen, Germany

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Spinocerebellar ataxia type 11 • Neuroexcitotoxicity • Kainate receptor • Rab5

Abstract
Background/Aims: Inherited, autosomal dominant spinocerebellar ataxia type 11 (SCA11) is caused by loss of function mutations of TTBK2 (tau tubulin kinase 2). Mutations observed in patients with SCA11 include truncated TTBK2(450). The present study explored the possibility that TTBK2 influences the function of the glutamate receptor GluK2. Methods: GluK2 was expressed in Xenopus oocytes without and with additional expression of wild type TTBK2, the truncated mutant TTBK2(450), or the kinase dead mutants TTBK2(KD) and TTBK2(450/KD). GluK2 current was determined by dual electrode voltage clamp and GluK2 protein abundance in the cell membrane utilizing confocal microscopy. Results: Glutamate exposure of GluK2 expressing oocytes generated a current, which was significantly lower in oocytes expressing GluK2 together with TTBK2 wt or TTBK2(KD) than in oocytes expressing GluK2 alone or together with either TTBK2(450) or TTBK2(450/KD). According to confocal microscopy of EGFP-tagged GluK2, TTBK2 wt decreased the GluK2 protein abundance in the cell membrane. Overexpression of an inactive RAB5(N133I) mutant but not RAB5wt could reverse the TTBK2 effect on GluK2 suggesting that RAB5 function is required for the effect. Conclusions: TTBK2 down-regulates GluK2 activity by decreasing the receptor protein abundance in the cell membrane via RAB5-dependent endocytosis, an effect that may protect against neuroexcitotoxicity.

Introduction

The tau tubulin kinase 2 (TTBK2) is a serine/threonine kinase [1] underlying the pathophysiology of the rare [2], inherited, autosomal dominant spinocerebellar ataxia type 11 (SCA11) [3, 4], a neurodegenerative movement disorder affecting primarily cerebellar neurons [5].

G. Seebohm and N. Strutz-Seebohm contributed equally.
TTBK2 belongs to the CK1 (casein kinase 1) superfamily and is closely related to the kinase TTBK1 which is suspected to play an important role in the pathophysiology of Morbus Alzheimer [6]. While TTBK1 is expressed specifically in the central nervous system, TTBK2 can be found in various types of tissues such as heart, liver, skeletal muscle, pancreas but also in the brain [7]. High expression levels could be detected in Purkinje cells and granular cell layer of the cerebellum, hippocampus, midbrain and substantia nigra while the protein is expressed in lower amounts in the cortex [8]. SCA11 truncating mutations [9] result in decreased TTBK2 kinase activity and enhanced nuclear localization [3] eventually leading to spinocerebellar degeneration [8]. Homozygous SCA11 mutations lead to lethality at embryonic day 10 [3], whereas patients with a heterozygote genotype have a normal life expectancy but develop a severe movement disorder showing primarily a progressive cerebellar ataxia and abnormal ocular motor signs, occasionally complicated by pyramidal features, peripheral neuropathy and dystonia. The age of onset ranges from the early teens to the mid of 20s [4].

TTBK2 has further been found to play a role in the initiation of ciliogenesis [7], to affect the sensitivity of tumor cells against antiangiogenic treatment [10], to regulate membrane transporters like the GABA transporter BGT1 [11] or the Na-coupled glucose transporter SGLT1 [12], and to phosphorylate TDP43, a DNA/RNA binding protein that is suspected to be of relevance in connection with the neurodegenerative diseases amyotrophic lateral sclerosis (ALS) and frontotemporal lobar degeneration (FTLD) [7].

Causes of neurodegeneration include excessive activity of glutamate receptors such as kainate and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors [13]. Kainate receptors contribute to the excitatory postsynaptic potential [14-16] and are subject to developmental and activity-dependent regulation [17]. The kainate receptor subunit GluK2 is particularly expressed in hippocampus, basal ganglia and cerebellum [18, 19]. GluK2 plays an important and distinct role in synaptic transmission and plasticity [14, 20-22]. GluK2 activity is modified by protein phosphorylation [23]. Kinases involved in the regulation of GluK2 include the serum and glucocorticoid inducible kinase SGK1 [24].

The present study explored the influence of TTBK2 on GluK2 activity. cRNA encoding GluK2 was injected in Xenopus oocytes with or without cRNA encoding TTBK2, channel activity determined with dual electrode voltage clamp and GluK2 protein abundance in the cell membrane determined by confocal microscopy and an EGFP-tagged GluK2.

Materials and Methods

Constructs and cRNA synthesis

For generation of cRNA, constructs encoding wild-type GluK2 [24], wild type human TTBK2, defective TTBK2(450) and kinase dead mutants TTBK2(KD) and TTBK2(450/KD) (kindly provided by Dario Alessi) were used. In the kinase inactive mutant TTBK2(KD) an aspartic acid at position 163 was replaced by alanine [TTBK2(D163A)]. The defective mutant TTBK2(450) is a truncated form of TTBK containing the first 450 residues of the kinase. TTBK2(450/KD) is a truncated mutant including the mutation D163A. Both mutations were reported in SCA11 patients [3, 4]. The generation of Rab cDNAs has been described previously [25]. 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

cRNA (GluK2 4 ng, TTBK2-variants 10 ng; if not otherwise stated, Rab5 4 ng) was injected into stage V-VI Xenopus oocytes, provided by Ecocyte Bioscience (Castrop-Rauxel, Germany). All measurements were performed 6 days after injection of cRNA. Oocytes were incubated at 17-18°C. Oocytes of similar size were injected with cRNA using a Nanoliter 2000 injector (WPI inc., Florida, USA).

Standard two-electrode voltage clamp (TEVC) recordings were performed 6 days after cRNA injection with a TurboTec 03 amplifier (NPI, Tamm, Germany) and an interface Digidata 1322A from Axon Instruments
(Molecular Devices Com., CA, USA). 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; CaCl$_2$, 1.8; KCl, 2.0; MgCl$_2$, 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 20 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) (Sigma-Aldrich, Munich, Germany), which minimized desensitization.

Detection of GluK2 cell surface expression

EGFP-tagged ion channels expressed in Xenopus oocytes were imaged using a TCS SP2 AOBS confocal microscope (Leica Microsystems, Wetzlar, Germany). EGFP was excited at 488 nm, and fluorescence was detected between 500 and 600 nm in combination with 10x objective (HC PL APO Ibd.BL 20x/0.7 water). The data were analyzed with Imagej, plug-in “straighten” (U.S. National Institutes of Health, Bethesda, MD, USA).

Statistical analysis

Data are provided as means ± SEM, n represents the number of oocytes investigated. All experiments were repeated with two or three batches of oocytes; in all repetitions qualitatively similar data were obtained. Data were tested for significance using Student’s t-test and ANOVA, and results with p < 0.05 were considered statistically significant.

Results

The present paper explored whether the tau tubulin kinase 2 (TTBK2) influences the function of the glutamate receptor GluK2. To this end, GluK2 was expressed in Xenopus oocytes with or without TTBK2 and glutamate-induced current determined by dual electrode voltage clamp. Non-injected Xenopus oocytes did not show a glutamate (300 µM) induced current after pretreatment of the oocyte with concanavalin A to block desensitization. Thus, Xenopus oocytes do not express appreciable endogenous GluK2 glutamate receptors. In contrast, 300 µM glutamate induced a strong current in Xenopus oocytes expressing GluK2.

Expression of wild type TTBK2 in addition to GluK2 significantly decreased the current amplitude in a concentration dependent manner (Fig. 1). When 10 or 30 ng TTBK2 wt were injected, a significant decrease of GluK2 currents could be observed, whereas injection of 1 ng TTBK2 wt resulted in no significant reduction of currents (Fig. 1). Therefore, subsequent experiments were performed with 10 ng injected TTBK2.

![Fig. 1](image-url)

**Fig. 1.** Co-expression of TTBK2 wt down-regulates glutamate-induced currents in GluK2-expressing Xenopus oocytes. A: Representative original tracings showing currents evoked by 300µM glutamate in Xenopus oocytes expressing GluK2 without or with additional co-expression of wild type TTBK2 at different concentrations. TTBK2 wt cRNAs injected were 1 ng, 10 ng, or 30 ng, respectively. All currents were measured at -70 mV and after pretreatment of oocytes with ConA to minimize desensitization. B: Dot bar showing arithmetic means ± SEM of currents evoked by 300µM glutamate in Xenopus oocytes expressing GluK2 alone or together with wild type TTBK2 in different concentrations (see above A). Currents shown are normalized to GluK2 currents. Statistical analysis was performed using ANOVA and significant differences are indicated by ***p<0.001and **p<0.01. n = 3-37.
The down-regulation of GluK2 activity following co-expression of TTBK2 wt could have resulted from a decrease of receptor protein abundance in the plasma membrane. Thus, an EGFP-tagged GluK2 and confocal microscopy was employed to quantify GluK2 protein abundance in the cell membrane of *Xenopus* oocytes expressing GluK2-EGFP. As shown in Fig. 2, the co-expression of TTBK2 wt in different concentrations (1 ng, 10 ng, or 30 ng injected) was followed by a significant decrease of GluK2-EGFP protein abundance within the *Xenopus* oocyte cell membrane (Fig. 2A,B). The decrease in glutamate evoked current is highly paralleled by a decrease in plasma membrane GluK2-EGFP (Fig. 2C) suggesting that TTBK2 exerts its effects by modulation of GluK2 cell surface abundance.

Co-expression of the truncated TTBK2(450) and truncated kinase dead TTBK2(450/KD) was followed by a slight but not significant decrease of GluK2 currents. However, co-expression of the kinase dead full length TTBK2(KD) showed a significant strong reducing effect on GluK2 currents (Fig. 3).

Again, down-regulation of GluK2 activity by TTBK2 wt or TTBK2(KD) could have resulted from a decrease of receptor protein abundance in the plasma membrane. Thus, GluK2-EGFP protein abundance was analyzed by confocal microscopy.

As shown in Fig. 4, the expression of GluK2-EGFP in the oocyte plasma membrane was reduced when TTBK2 wt or kinase dead TTBK2(KD) were co-expressed, whereas no significant effect on membrane expression was seen following co-expression of TTBK2(450) or truncated kinase dead TTBK2(450/KD).

Therefore, the data on expression pattern of GluK2 matches the data on GluK2 current amplitudes. This observation indicates that TTBK2 affects trafficking of GluK2.

A protein regulating the internalization of membrane proteins via early endosomes is the small G-protein (GTPase) Rab5 [26, 27]. Oocytes injected with GluK2 plus Rab5 wt showed a significant decrease in current amplitudes (Fig. 5) and plasma membrane expression (Fig. 6) compared to GluK2 expressed alone or GluK2 injected with the dominant negative mutant Rab5(N133I). Similar reductions in GluK2 current amplitudes and plasma membrane abundance were seen when GluK2 was co-expressed with TTBK2 or TTBK2 and RAB5 wt. However, GluK2 co-expressed with TTBK2 and RAB5(N133I) showed similar...
Fig. 3. The regulatory effect of TTBK2 depends on the C-terminal interaction domain. A: Schematic structure of TTBK2. The N-terminus of TTBK2 encodes a serine-threonine-tyrosine kinase domain whereas the C-terminus contains interaction domains. At amino acid position 163, an amino acid exchange of aspartic acid to alanine (D163A) leads to the kinase dead mutant [TTBK2(KD)]. Several mutations (insertion or deletion of base pairs) lead to truncation of the protein at position 450 [TTBK2(450)] [8]. The complete length of TTBK2 wt is 1244 amino acids. B: Representative original tracings showing currents evoked by 300µM glutamate in Xenopus oocytes expressing GluK2 alone (control) or GluK2 + TTBK2 wt, GluK2 + TTBK2(KD), GluK2 + TTBK2(450), or GluK2 + TTBK2(450/KD). All currents were measured at -70 mV and after pretreatment of oocytes with ConA to minimize desensitization. C: Bar graph showing arithmetic means ± SEM (n = 3-8) of currents evoked by 300µM glutamate in Xenopus oocytes expressing GluK2 alone (control) or GluK2 + TTBK2 wt [TTBK2 wt], GluK2 + TTBK2(KD) [TTBK2(KD)], GluK2 + TTBK2(450) [TTBK2(450)], or GluK2 + TTBK2(450/KD) [TTBK2(450/KD)]. Currents shown are normalized to GluK2 currents. Statistical analysis was performed using ANOVA and significant differences are indicated by ***p<0.001. n= 3-27.

Fig. 4. TTBK2 wt and TTBK2(KD) reduce surface GluK2-EGFP protein abundance in Xenopus oocytes. A: Confocal laser-scanning microscopy image of enhanced green fluorescent protein GluK2-EGFP expression in Xenopus oocytes without (control) or with additional co-expression of wild type TTBK2 at different constellations: GluK2-EGFP + TTBK2 wt [TTBK2 wt], GluK2-EGFP + TTBK2(KD) [TTBK2(KD)], GluK2-EGFP + TTBK2(450) [TTBK2(450)], or GluK2-EGFP + TTBK2(450/KD) [TTBK2(450/KD)]. Uninjected oocytes were used as negative control. Representative images show the GluK2-EGFP expression under indicated conditions at the optical slice approximately bisecting the oocytes. B: Bar graph showing normalized (to GluK2-EGFP alone) mean pixel intensities of GluK2-EGFP in oocytes at different constellations (see above A) of injected cRNA (n=8-49). The intensity was quantified by arithmetic analysis using the software ImageJ. Statistical analysis was performed using ANOVA and significant differences are indicated by ***p<0.001.

Current amplitudes and plasma membrane expression compared to GluK2 expressed alone or together with RAB5(N133I) suggesting that RAB5(N133I) could sufficiently counteract TTBK2 (Fig. 5 and 6). Thus, TTBK2 presumably stimulates internalization of GluK2 membrane protein and this scenario critically depends on RAB5 function.
The present observations disclose a novel negative regulator of the glutamate receptor GluK2 in heterologous expression. The tau tubulin kinase 2 (TTBK2) down-regulates the activity of the kainate receptor subunit GluK2. Using TEVC and confocal imaging, we show that TTBK2 is effective by decreasing the GluK2 protein abundance in the cell membrane, which in turn reduces the glutamate-induced current.

Plasma membrane density of GluK2 is determined by small GTPases RAB5 (endocytosis) and RAB11a (exocytosis) [28], a process modulated by the cell survival kinase SGK1.
kinase stimulates GluK2 exocytosis and thus increases plasma membrane abundance shown in heterologous expression systems and mice neurons [24, 28]. However, GluK2 is negatively regulated by NDRG2 phosphorylation in astrocytes [29].

Overexpression of an inactive RAB5-N133I mutant but not RAB5 wt could reverse the reducing TTBK2 effect on GluK2 suggesting that RAB5 function is required for the effect. Furthermore, RAB5 wt alone reduced GluK2 similarly as TTBK2 and overexpression of RAB5/TTBK2 showed no additive or synergistic effect suggesting that TTBK2 acts via RAB5-dependent endocytosis.

TTBK2 contains an N-terminal kinase domain and a C-terminal non-catalytic moiety, which is probably involved in kinase localization and substrate recruitment [7]. Overexpression of a kinase dead, but full-length TTBK2 was similarly effective to reduce GluK2 plasma membrane abundance and current amplitudes indicating that the TTBK2 kinase function is not required for its effects on GluK2. In contrast, deletion of the C-terminal domain at amino acid 450 completely abolished TTBK2 effects. Therefore, the C-terminal domain is required for full TTBK2 effects. Deletion of the C-terminus at amino acid 450 represents a rare spinocerebellar-ataxia-causing mutation in Spinocerebellar ataxia type 11 (SCA11) [8, 9]. The resulting truncated TTBK2(450) protein accumulates perinuclear and therefore its expression at the plasma membrane may be reduced [3]. Reduced TTBK2 expression at or close to the plasma membrane is consistent with reduced effects on GluK2, as TTBK2 effects are dose-dependent (Fig. 1c). Therefore, it cannot be concluded that the TTBK2 C-terminal domain structure is directly involved in GluK2 endocytosis.

Although these experiments presented here were performed in heterologous expression, it can be speculated that the effect of TTBK2 decreases the activity of kainate receptors in neurons and/or astrocytes as well. Excessive activity of several glutamate receptors including kainate receptors is known to foster neurodegeneration [13]. The present observations may thus provide an explanation for the impact of TTBK2 on cerebellar function. The truncated TTBK2 studied here has previously been found to be associated with spinocerebellar degeneration [8]. According to the present observations, a decreased activity of TTBK2 was expected to up-regulate GluK2, which would in turn enhance the risk of excitotoxicity. The clinical picture caused by TTBK2 deficiency presumably depends on the localization of TTBK2 and its impact on glutamate receptor regulation in the specific neural cells. GluK2 is upregulated by the serum and glucocorticoid inducible kinase SGK1 which stimulates GluK2 exocytosis [30]. SGK1- and TTBK2- stimulating GluK2 endocytosis could represent a functional antagonistic pair whose balance might be crucial to determine GluK2 plasma membrane density and function. The balance of these stimulating and inhibiting kinases determine the glutamate-induced current and may be highly relevant for kainate induced cerebellar neuronal cytotoxicity.

Recently, it was shown that TTBK2 directly binds to phosphatidylinositol 4-phosphates (PI(4)P) which determines TTBK2 localization at plasma membrane subdomains like the
centrosome/ciliary base [31]. Further, TTBK2 protein-interaction is determined by PI(4)P interaction. On the other side, GluK2 is regulated by SGK1 [24, 28]. SGK1 in turn determines phosphatidylinositol 3,5-phosphate (PIKfyve) abundance in recycling vesicles [32, 33]. Moreover, a closely related glutamate receptor directly interacts with phosphatidylinositol-4,5-phosphate (PI(4,5)P₂) and GluK2 [34]. Because endocytic membrane protein cycling is closely related to specific phosphatidylinositol phosphate species, it is tempting to speculate that SGK1 and TTBK2 may mediate adverse effects on phosphatidylinositol phosphate metabolism and thus GluK2 endocytic cycling (Fig. 7). Thus, TTBK2 regulates RAB5-dependent early endosome endocytosis involving PI(4,5)P₂. Clearly, further research is required to support this hypothesis.

In conclusion, TTBK2 down-regulates the glutamate receptor subunit GluK2 by decreasing the channel protein abundance in the cell membrane. The effect is independent of its kinase function and expected to decrease neuroexcitation and thus neuroexcitotoxicity.

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Disclosure Statement

The authors declare that they have no conflict of interest.

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