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Original Paper

Upregulation of Na^+,Cl^--Coupled Betaine/ γ-Amino-Butyric Acid Transporter BGT1 by Tau Tubulin Kinase 2

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Key Words
Osmolyte transporter • Neurodegeneration • Kinase • Electrophysiology

Abstract
Background/Aims: The serine/threonine kinase Tau-tubulin-kinase 2 (TTBK2) is expressed in various tissues including kidney, liver and brain. Loss of function mutations of TTBK2 lead to autosomal dominant spinocerebellar ataxia type 11 (SCA11). Cell survival is fostered by cellular accumulation of organic osmolytes. Carriers accomplishing cellular accumulation of organic osmolytes include the Na^+,Cl^--coupled betaine/ γ-amino-butyric acid transporter BGT1. The present study explored whether TTBK2 participates in the regulation of BGT1 activity. ***Methods:*** Electrogenic transport of GABA was determined in *Xenopus* oocytes expressing BGT1 with or without wild-type TTBK2, truncated TTBK2[1-450] or kinase inactive mutants TTBK2- KD and TTBK2[1-450]-KD. ***Results:*** Coexpression of wild-type TTBK2, but not of TTBK2[1- 450], TTBK2-KD or TTBK2[1-450]-KD, increased electrogenic GABA transport. Wild-type TTBK2 increased the maximal transport rate without significantly modifying affinity of the carrier. Coexpression of wild-type TTBK2 significantly delayed the decline of transport following inhibition of carrier insertion with brefeldin A, indicating that wild-type TTBK2 increased carrier stability in the cell membrane. ***Conclusion:*** Tau-tubulin-kinase 2 TTBK2 is a powerful stimulator of the osmolyte and GABA transporter BGT1.

Introduction

The Tau tubulin Kinase 2 (TTBK2), a serine/threonine kinase [1] expressed in various tissues including the brain [2-4], heart [5], kidney [5], intestine [5] and tumor cells [6], contributes to the maintenance of neuron survival [2]. Accordingly, loss of function mutations of TTBK2 lead to autosomal dominant spinocerebellar ataxia type 11 (SCA11) [7]. Moreover,

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TTBK2 may contribute to the resistance of kidney tumor cells and melanoma cells to therapy [6]. Gene targeted mice lacking functional TTBK2 die at embryonic day 10 [7].

The maintenance of cell survival is supported by cellular accumulation of organic osmolytes, which have been shown to counteract apoptosis [8]. Mechanisms contributing to cellular accumulation of organic osmolytes include Na^+ coupled osmolyte transporters, such as the betaine/γ-amino-butyric acid (GABA) transporter BGT1 (SLC6A12) [9, 10]. BGT1 is a member of the Na^+,Cl^- coupled transporter superfamily accomplishing the transport of neurotransmitters (e.g. dopamine, GABA, serotonin and norepinephrine), amino acids (e.g. glycine) [11], creatine [12], and the organic osmolytes betaine [13] and taurine [14]. BGT1 is expressed in a wide variety of tissues including brain [15, 16], liver [16-18], kidney [16] and airways [19]. BGT1 expression is stimulated by osmotic cell shrinkage [20-22]. Activity of the carrier is regulated by Ca^{2+} [23], protein kinase C [24], prostaglandin E_2 [25, 26], cytoskeleton [27, 28] and ATP [29]. The carrier is upregulated following epileptic seizures [30]. However, the BGT1 deficient mouse does not develop seizures [31].

At least in theory, the protective effect of TTBK2 on neurons could involve modification of transport systems. As a matter of fact, a previous study revealed that TTBK2 upregulates the Na^+ coupled glucose transporter SGLT1. Carriers with potentially protective effect include the BGT1. The present study thus explored, whether TTBK2 modifies the activity of BGT1. To this end, BGT1 was expressed in *Xenopus* oocytes with or without additional coexpression of wild-type TTBK2, truncated $TTBK2^{[1-450]}$, kinase inactive full-length TTBK2-KD or kinase inactive truncated $TTBK2\text{-}KD^{[1-450]}$. BGT1 activity was determined from γ-amino-butyric acid (GABA)-induced current utilizing two-electrode voltage clamp.

Materials and Methods

Constructs

For generation of cRNA, constructs were used encoding wild-type human BGT1 (SLC6A12) [32, 33], wild-type human full-length TTBK2 and truncated mutant $TTBK2^{[1-450]}$ containing the first 450 residues of the kinase. $TTBK2^{[1-450]}$ has been identified in SCA11 patients [34] and leads to decreased kinase activity [7]. Further constructs used were the kinase inactive mutants TTBK2-KD and $TTBK2^{[1-450]}$-KD in which an aspartic acid at position 163 was replaced by alanine [D163A]. Constructs encoding wild type and mutated TTBK2 have all been kindly provided by Dario Alessi, University of Dundee. The cRNA was generated as described previously [35, 36].

Voltage clamp

For voltage clamp analysis, *Xenopus* oocytes were prepared as previously described [37]. On the day following oocyte preparation, *Xenopus* oocytes were injected with water, 15 ng of cRNA encoding BGT1 and 10 ng of cRNA encoding $TTBK2^{WT}$, TTBK2-KD , $TTBK2\text{-}^{[1-450]}$ or $TTBK2^{[1-450]}$-KD. The oocytes were maintained at 17°C in ND96 solution containing 88.5 mM NaCl, 2mM KCl, 1mM $MgC1_2$, 1.8mM $CaC1_2$, 5mM HEPES. Tretracycline (Sigma, 0.11mM), Ciprofloxacin (Sigma, 4μM), Gentamycin (Refobacin © 0.2mM) and Theophylin (Euphylong ©, 0.5mM) as well as sodium pyruvate (Sigma, 5mM) were added to ND96, pH was adjusted to 7.5 by addition of NaOH. The two-electrode voltage clamp recordings were performed at room temperature 4 days after the injection [38] at a holding potential of -70 mV. The data were filtered at 10 Hz and recorded with a DigidataA/D-D/A converter and ClampexV 9.2 software for data acquisition and analysis (Axon Instruments, Union City, CA, USA) [39, 40]. The control superfusate was ND96 containing 96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$,1 mM $MgCl_2$ and 5 mM HEPES, pH was adjusted to 7.4 by addition of NaOH. The substrate γ-amino-butyric acid (GABA) was added to the solutions at the indicated concentrations. The flow rate of the superfusion was approximately 20 ml/min, and a complete exchange of the bath solution was reached within about 10 sec [41]. Where indicated, brefeldin A (5μM) was added to the solutions in order to test for alterations of BGT1 protein stability in the cell membrane. Brefeldin disrupts the organization of the microtubule and the actin network [42] and interferes with the function of Golgi-specific coat proteins involved in the regulation of membrane transport in the secretory pathway [43]. Treatment of *Xenopus*

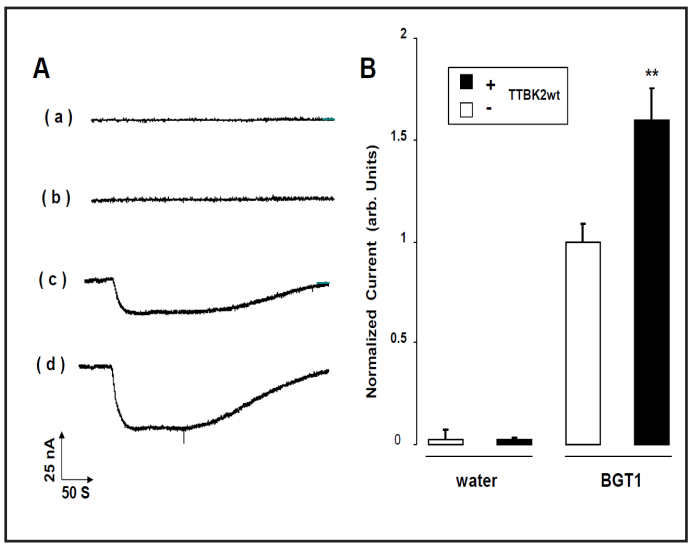

Fig. 1. GABA induced current in *Xenopus* oocytes expressing BGT1 with or without wild-type TTBK2. A. Original tracings of GABA (1 mM) induced currents in *Xenopus* oocytes injected with water (a), or with cRNA encoding wild-type TTBK2 alone (b), BGT1 alone (c) or BGT1 together with wild-type TTBK2 (d). B. Arithmetic means ± SEM (n = 5-18) of GABA (1 mM) induced current in *Xenopus* oocytes injected without (left bars) and with (right bars) BGT1, without (white bars) or with (black bars) cRNA encoding wild-type TTBK2. ** ($p<0.01$) indicates statistically significant difference from *Xenopus* oocytes expressing BGT1 alone.

oocytes with brefeldin A prevents insertion of new channel protein into the cell membrane and the decay of transporter activity could be taken as a measure of channel protein clearance from the cell membrane [44].

Statistical analysis

Data are provided as arithmetic means ± SEM; n represents the number of oocytes investigated. All oocyte experiments were repeated for at least three batches of oocytes and in all repetitions qualitatively similar data were obtained [45]. All data were tested for significance by using ANOVA or t-test, as appropriate. Results with $p< 0.05$ were considered statistically significant.

Results

In order to explore, whether the Tau tubulin Kinase 2 (TTBK2) influences the activity of the Na^+,Cl^--coupled betaine/GABA transporter BGT1, cRNA encoding BGT1 was injected into *Xenopus* oocytes without or with additional cRNA encoding TTBK2 and two-electrode voltage clamp experiments were performed to determine BGT1-mediated electrogenic GABA transport. As illustrated in Fig. 1, GABA (1 mM) did not induce an appreciable inward current in water injected *Xenopus* oocytes, indicating that *Xenopus* oocytes do not express appreciable endogenous electrogenic GABA transporters. Similarly, no significant GABA-induced current was observed in *Xenopus* oocytes expressing wild-type TTBK2 alone (Fig. 1). In *Xenopus* oocytes injected with cRNA encoding BGT1, however, addition of GABA (1 mM) was followed by an inward current, reflecting electrogenic entry of Na^+, Cl^- and GABA. The GABA induced current was significantly enhanced by additional coexpression of wild-type TTBK2 (Fig. 1).

In contrast to wild-type TTBK2, truncated TTBK2$^{[1-450]}$ did not significantly increase the GABA induced current in BGT1 expressing *Xenopus* oocytes. Moreover, neither coexpression of the full-length kinase inactive mutant TTBK2-KD nor coexpression of the truncated kinase inactive mutant TTBK2-KD$^{[1-450]}$ significantly modified the GABA induced current in BGT1 expressing *Xenopus* oocytes (Fig. 3). Thus, kinase activity was required for the effect of TTBK2 on BGT1-induced currents.

Kinetic analysis of the GABA-induced currents in BGT1-expressing *Xenopus* oocytes (Fig. 2) yielded a maximal current of 15.7 ± 1.3 nA (n = 7). Coexpression of wild-type TTBK2 significantly enhanced the maximal current to 35.0 ± 1.4 nA (n = 6). Calculation of the GABA concentration required for the half-maximal current (K_M) yielded a value of 416 ± 116 μM

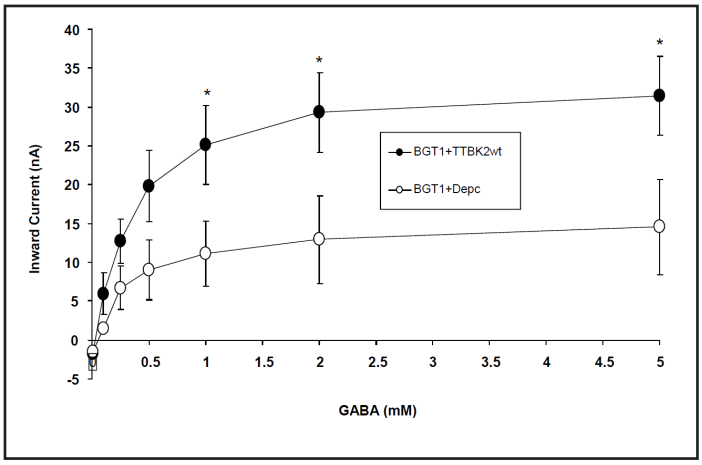

Fig. 2. Kinetics of GABA induced current in *Xenopus* oocytes expressing BGT1 with or without wild-type TTBK2. Arithmetic means ± SEM (n = 6 - 7) of GABA induced currents as a function of GABA concentration in *Xenopus* oocytes expressing BGT1 alone (open circles) or BGT1 together with wild-type TTBK2 (closed circles). * ($p<0.05$) indicates statistically significant difference from *Xenopus* oocytes expressing BGT1 alone.

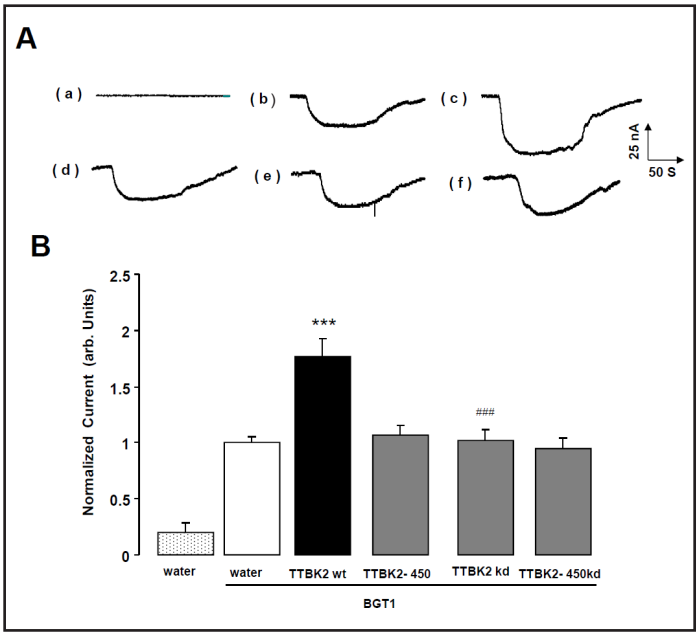

Fig. 3. GABA induced current in *Xenopus* oocytes expressing BGT1 with or without wild type TTBK2, truncated TTBK2$^{[1-450]}$ mutant, kinase inactive mutant TTBK2-KD or truncated kinase inactive mutant TTBK2$^{[1-450]}$-KD. A.Original tracings of GABA (1 mM) induced currents measured in *Xenopus* oocytes injected with water (a), or expressing BGT1 without (b), or with (c) additional coexpression of wild-type TTBK2^{WT}, truncated wild-type TTBK2$^{[1-450]}$ (d), full-length kinase inactive mutant TTBK2-KD (e) or truncated kinase inactive mutant TTBK2$^{[1-450]}$-KD (f). B. Arithmetic means ± SEM (n = 11-15) of GABA-induced current (1 mM) in *Xenopus* oocytes injected with water (dotted bar), expressing BGT1 without (white bar) or with additional coexpression of wild-type TTBK2 (black bar), truncated wild-type TTBK2$^{[1-450]}$ (1st grey bar), full-length kinase inactive mutant TTBK2-KD (2nd grey bar) or truncated kinase inactive mutant TTBK2$^{[1-450]}$-KD (3rd grey bar). *** ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes expressing BGT1 alone. ### ($p<0.001$) indicates statistically significant difference from *Xenopus* oocytes coexpressing BGT1 and wild-type TTBK2.

(n = 7) in the absence, and a value of 413 ± 58 μM (n = 6) in the presence of wild-type TTBK2. The K_M was not significantly different between *Xenopus* oocytes expressing BGT1 together with wild-type TTBK2 and *Xenopus* oocytes expressing BGT1 alone. Thus, wild-type TTBK2 did not significantly modify K_M but significantly increased the maximal current.

The enhanced BGT1 activity could have resulted from stimulation of carrier activity, from accelerated insertion of new carriers into the cell membrane, or from delayed clearance of carriers from the cell membrane. The effect on maximal transport rate without influence on substrate affinity pointed to increased protein abundance in the cell membrane. To discriminate between accelerated insertion and delayed clearance of carrier protein, the BGT1-expressing *Xenopus* oocytes were treated with 5 μM brefeldin A, which blocks the insertion of new carrier protein into the cell membrane. Following incubation with brefeldin A, the GABA induced current declined at a faster rate in oocytes expressing BGT1 alone

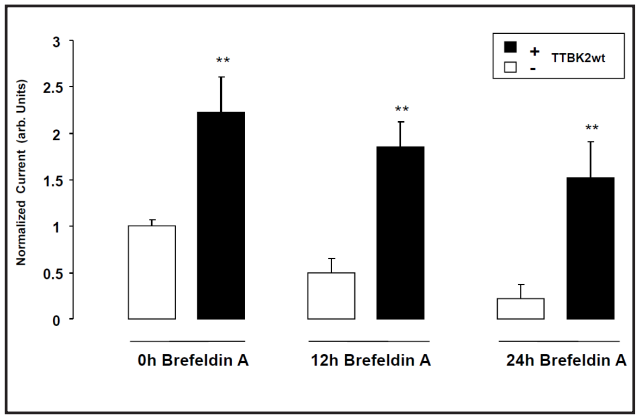

Fig. 4. Effects of Brefeldin A on GABA induced currents in *Xenopus* oocytes expressing BGT1 with or without wild-type TTBK2. Arithmetic means ± SEM (n = 9-12) of GABA (1 mM) induced current in *Xenopus* oocytes injected with cRNA enconding BGT1, without (white bars) and with (black bars) wild-type TTBK2 in the absence (left bars) and presence of 5 μM Brefeldin A for 12 hours (middle bars) or 24 hours (right bars) prior to the measurement. ** ($p<0.01$) indicates statistically significant difference from the absence of wild-type TTBK2.

than in oocytes expressing BGT1 together with wild-type TTBK2 (Fig. 4). The ratio of BGT1 induced current in oocytes expressing BGT1 together with wild-type TTBK2 over that of oocytes expressing BGT1 alone increased significantly from 223% ± 40% (n = 12) prior to treatment with brefeldin A to 370% ± 62% (n = 9-10) following a 12 hours treament with brefeldin A and to 680% ± 191% (n = 10-11) following a 24 hours treatment with brefeldin A. The observations suggest that wild-type TTBK2 delays the clearance of carrier protein from the cell membrane.

Discussion

The present observations reveal a completely novel regulator of the Na^+,Cl^--coupled betaine/GABA transporter BGT1. The tau-tubulin-kinase 2 (TTBK2) up-regulates BGT1 activity, an effect at least in part due to stabilization of carrier protein in the cell membrane. The effect of TTBK2 requires functional kinase activity and is disrupted by mutations truncating the C-terminus noncatalytic domain of TTBK2 protein ($TTBK2^{[1-450]}$). The truncation has previously been shown to cause spinocerebellar degeneration [2].

In theory, TTBK2 could be effective by modifying the function of the microtubular network, which does participate in the regulation of transport processes [46-48]. Moreover, TTBK2 is critically important for the formation of cilia [49], which in turn are known to participate in the deranged transport regulation of polycystic kidney disease [50]. Clearly, additional experimental effort is needed to elucidate the mechanisms linking TTBK2 activity and carrier protein abundance in the cell membrane.

The regulation of BGT1 or a related carrier could well participate in the protection against neurodegeneration by TTBK2. Osmolyte transporters counteract cell shrinkage, which is known to parallel and facilitate suicidal cell death [51-60]. BGT1 participates in the cellular accumulation of organic osmolytes [9, 10], which are well known to foster cell survival [8, 26]. Thus, betaine has previously been shown to counteract apoptosis [61-64].

Carriers related to BGT1 include the transporters for creatine CreaT [12] and taurine TauT [14]. Those carriers may similarly be candidates for regulation by TTBK2. Genetic defects affecting the creatine transporter CreaT result in mental retardation with seizures [65-82]. Defective cellular taurine uptake by TauT fosters apoptosis [83-91].

At least in theory, TTBK2 sensitive BGT1 activity could modify neuronal function and survival in addition by transport of GABA. BGT1 mediates the cellular uptake of GABA, a function which was expected to decrease extracellular GABA concentration and thus to enhance neuronal excitation. Other carriers belonging to the superfamily of Na^+,Cl^- coupled transporters include various transporters involved in the cellular uptake of further neurotransmitters, such as dopamine, serotonin, norepinephrine and amino

acids [11]. Additional experiments will be needed to explore whether TTBK2 influences neurotransmitter transport and thus neuronal function and survival by regulating one or more of those related transporters.

In conclusion, the present study discloses that TTBK2 is a powerful regulator of the Na^+,Cl^--coupled betaine/GABA transporter BGT1 and thus participates in the regulation of cellular betaine and GABA uptake. The effect may contribute to the cytoprotective role of this widely expressed kinase.

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