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Regulation of TRKB Surface Expression by Brain-derived Neurotrophic Factor and Truncated TRKB Isoforms*

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Brain-derived neurotrophic factor (BDNF) signaling through its receptor TRKB modulates survival, differentiation, and activity of neurons. BDNF activates TRKB on the cell surface, which leads to the initiation of intracellular signaling cascades and different biological responses in neurons. Neuronal activity has been shown to regulate TRKB levels on the plasma membrane of neurons, but little is known about other factors affecting TRKB surface expression levels. We report here that BDNF regulates the cell surface levels of transfected or endogenously expressed full-length TRKB, depending on the exposure time in neuroblastoma cells and primary hippocampal neurons. BDNF rapidly increases TRKB surface expression levels in seconds, whereas treatment of cells with BDNF for a longer time (minutes to hours) leads to decreased TRKB surface levels. Coexpression of the full-length TRKB together with the truncated TRKB.T1 isoform results in decreased levels of full-length TRKB on the cell surface. This effect is specific to the T1 isoform, because coexpression of a kinase-dead TRKB mutant or another kinase domain-lacking TRKB form, truncated T-Shc, leads to increased TRKB surface levels. Our results suggest that regulation of TRKB surface expression levels by different factors is tightly controlled by complex mechanisms in active neurons.

Neurotrophin brain-derived neurotrophic factor (BDNF)[1] is a key regulator of survival and differentiation of specific neuronal populations in the central nervous system (1, 2). In addition, increasing evidence indicates that BDNF plays important roles in regulating neuronal activity and synaptic events related to plasticity (for reviews, see Refs. 3–5). BDNF is released in an activity-dependent manner (6–10), and it regulates neurotransmitter release and synaptic transmission of neurons (11–17).

The biological effects of BDNF are mediated by TRKB transmembrane tyrosine kinase receptors (18–23). The high affinity full-length TRKB.TK+ (TK+) receptors are exclusively expressed in neurons in the central nervous system (24). Binding of BDNF to TRKB.TK+ activates it by inducing dimerization and autophosphorylation at specific tyrosine residues in the cytoplasmic kinase domains (25). The phosphorylated tyrosines serve as docking sites to cytoplasmic effector molecules, which activate different signaling pathways that eventually lead to changes in gene expression and different biological responses in neurons (26).

BDNF signaling through the TK+ receptors can be modulated by the low affinity p75 neurotrophin receptor (27–29) but also by truncated TRKB isoforms (TK−; see Refs. 20 and 24).

Different TRKB isoforms are generated by alternative splicing resulting in the TK+ form and three TK− splice variants: T1, T2, and T-Shc (20, 24, 30). Truncated T1 lacks the kinase domain but contains short isoform-specific cytoplasmic domain in its place (20, 24). Even though T1 is mostly expressed in non-neuronal cells in the central nervous system, it has been shown to colocalize with TK+ in a subpopulation of hippocampal as well as motor neurons (24, 31–33). TK− forms inhibit TK+ signaling by sequestering BDNF when expressed in non-neuronal cells (32–36) or functioning as dominant negative receptors by heterodimerization when coexpressed together with TK+ in the same cells (37–40). The recently cloned truncated T-Shc contains an Shc-binding site in the juxtamembrane domain similar to TK+, but it lacks the kinase domain and has a unique truncated C terminus (30). T-Shc is predominantly expressed in the brain, but its role is so far mostly unknown.

TK+ and T1 are localized to both somatodendritic and axonal compartments in neurons (41). Although the cell surface is the primary site where TK+ activation takes place and subsequent intracellular signaling is initiated, in unstimulated cells the majority of TK+ is located intracellularly, and only a small amount of TK+ can be detected on the neuronal surface (42, 43). Activation of neurons has been shown to increase the cell surface expression levels of TK+ in both retinal ganglion cells (42) and hippocampal neurons (43), but little is known about other factors that regulate TK+ levels on the cell surface.

In this study we have investigated the cell surface expression levels of transfected and endogenous TRKB isoforms in N2a neuroblastoma cells and primary hippocampal neuron cultures. We report that the cell surface levels of TK+ can be differentially regulated both by the ligand BDNF and coexpression of different truncated TRKB isoforms. These results may provide evidence of how BDNF signaling can be affected by regulating TK+ surface levels in active neurons *in vivo*.

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[1] The abbreviations used are: BDNF, brain-derived neurotrophic factor; TK+, full-length TRKB.TK+; T1, truncated TRKB.T1; T2, truncated TRKB.T2; T-Shc, truncated TRKB.T-Shc; ΔATP, kinase-dead mutant form of TRKB; GFP, green fluorescent protein; GFP-F, farnesylated GFP; DIV, days *in vitro*; VDCC, voltage-dependent Ca^{2+} channels; HVA, high voltage-activated; LVA, low voltage-activated; PBS, phosphate-buffered saline.

EXPERIMENTAL PROCEDURES

Plasmids and Cloning of ΔATP Mutant Construct by Overlap Extension PCR Method—Plasmid DNA encoding fusion proteins of full-length TRKB.TK+ and/or truncated TRKB.T1 and N-terminal GFP or FLAG tag were used for transfections (pGFP-TK+, pGFP-T1, pFLAG-TK+, pFLAG-T1 (39, 44)). T-Shc construct (pGFP-T-Shc) contains a 5′ GFP tag and encodes for GFP-T-Shc fusion protein (30). pGFP or pEGFP-F (GFP-F, Clontech, Palo Alto, CA) cDNAs were used as controls.

N-terminally FLAG-tagged ΔATP mutant construct (pFLAG-ΔATP) was cloned by using pFLAG-TK+ as a template by overlap extension PCR method. An A→T point mutation resulting in amino acid change from Lys^{560} to methionine at the ATP-binding site was introduced into pFLAG-TK+ cDNA (sequence numbering according to rat TRKB.TK+ sequence, GenBank accession number M55291 (20)). Two mutagenizing primers (*mut1* and *mut2*) with overlapping sequence and two outside primers (*out1* and *out2*) flanking the mutated sequence with following sequences were designed as follows: *mut1* (nucleotides 2366–2395) 5′-**GTGGCCGTGAT*GACGCTGAA**GGACGCCAG-3′; *mut2* (nucleotides 2355–2385) 5′-**TTCAGCGTCA*TCACGGCCAC**CAGGATCTTA-3′; *out1* (nucleotides 1466–1485) 5′-gcagaaaacctcgtcggaga-3′; *out2* (nucleotides 3282–3303) 5′-AGAAGCGAGTCGATACTGTCT-3′. Boldface letters in sequence indicate overlapping sequence between the two primers, and * indicates the A→T point mutation. First, two separate reactions (one with *out1* and *mut2* primers and the other with *out2* and *mut1* primers) were performed by using pFLAG-TK+ cDNA as a template. In the reactions 100 ng of template DNA, 20 pmol of each primer, 0.25 mM dNTP mixture, and 1 unit of Dynazyme EXT DNA polymerase (Finnzymes, Finland) were used. The PCR program used is as follows: initial denaturation 2 min at 94 °C, followed by 20 cycles (30 s, 94 °C; 90 s, 60 °C; 90 s, 72 °C). The next PCR was performed by using *out1* and *out2* primers and 0.5 μl of purified PCR products from each previous reaction (919 and 937 bp, respectively) as template. The 1837-bp PCR product and parental pFLAG-TK+ were digested with *Nde*I and *Hpa*I resulting in 1505- and 6595-bp DNA fragments, respectively. The fragments were ligated (Rapid DNA Ligation kit, Roche Molecular Biochemicals) and transformed to DHα-competent *Escherichia coli* cells. The correct clone was verified by automated sequencing. Receptor autophosphorylation assay in transfected N2a cells (39) showed that ΔATP mutant protein does not autophosphorylate after BDNF treatment, indicating that pFLAG-ΔATP encodes for a kinase-dead TRKB receptor (data not shown).

Cell Culture and Transfections—N2a mouse neuroblastoma cells were cultured and transfected as described elsewhere (39, 44). N2a cells used in electrophysiological recordings were harvested from a confluent monolayer, plated 1:4 unto sterile coverslips 22 × 22 mm (Warner Instruments) coated with poly-L-lysine (0.1 mg/ml), and grown for 3–4 days. Embryonic E17 hippocampal cultures were prepared as described (45) and transfected by calcium phosphate coprecipitation method as described by Xia *et al.* (46). Hippocampal neurons were used for experiments at 8–12 days *in vitro* (DIV). All cell culture reagents were from Invitrogen.

Surface Biotinylation Assay and Western Blotting—The cells were treated with 50 ng/ml BDNF (PeproTech, London, UK) for 5 min, 1 or 24 h, or with 200 nM (1 h) K252a (Calbiochem) at +37 °C in a cell culture incubator. Fifteen-second BDNF treatment was performed with the cell plate on ice with BDNF diluted to 50 ng/ml in cell culture medium pre-equilibrated to +21 °C. The cell culture plates were quickly rinsed twice in ice-cold PBS, pH 7.4, containing 1 mM $CaCl_2$ and 0.1 mM $MgCl_2$ (PBS-Ca-Mg) and kept on ice to stop protein trafficking immediately after the treatments. Cell surface proteins were biotinylated for 30 min with 0.25 mg/ml biotin (Sulfo-NHS-LC-Biotin, Pierce) diluted in PBS-Ca-Mg. Unbound biotin was quenched by washing cells twice and then again for 20 min at +4 °C with PBS-Ca-Mg containing 0.1 M glycine. Total protein fractions were extracted as described previously (39). Three hundred (N2a cells) or 150 μg (hippocampal neurons) of total protein were precipitated with 25 μl of streptavidin-agarose beads (Pierce) in a total volume of 200 μl overnight at +4 °C by rotation. The beads were washed three times in PBS, 1% Nonidet P-40, pH 7.4, two times in PBS, 1% Nonidet P-40, 0.5 M NaCl, pH 7.4, once in 50 mM Tris-HCl, pH 7.5, and then for 5 min in 50 mM Tris-HCl, 25 mM dithiothreitol, pH 7.5. Biotinylated proteins were eluted from the beads by boiling in 2× Laemmli sample buffer, separated on 6% SDS-PAGE, and blotted onto nitrocellulose filters as described previously (39). TRKB bands were detected with α-$TRKB_{out}$ antibody (1:5000, a gift from Dr. David Kaplan, Montreal Neurological Institute, Canada). SuperSignal West Pico chemiluminescent substrate (Pierce) was used for ECL detection. Streptavidin beads specifically precipitated only biotinylated proteins, since precipitation of unbiotinylated cells resulted in no signal on the nitrocellulose filter, which was stained for total protein in Ponceau S. The nontransfected biotinylated cells also did not give any signal after α-$TRKB_{out}$ antibody and ECL detection (data not shown). The intensities of TRKB bands on the films were quantified from digitized images by using MCID/M4 image analysis program, version 3 (Imaging Research Inc., St. Catharines, Ontario, Canada).

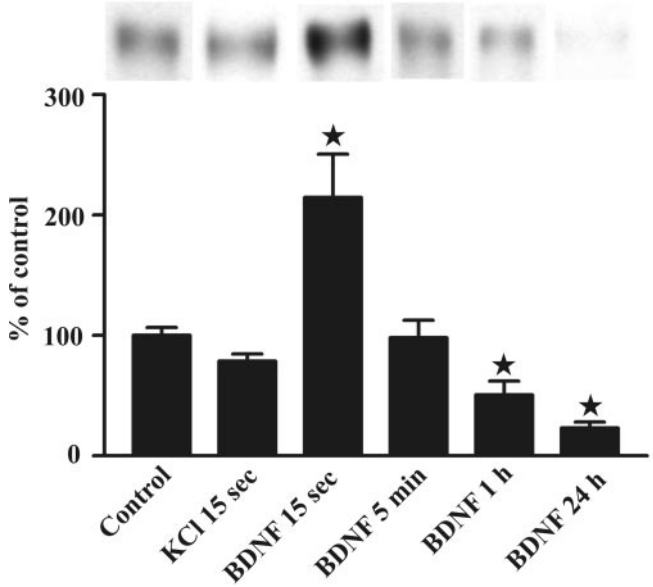

FIG. 1. **Regulation of full-length GFP-TK+ surface expression levels by BDNF in transfected N2a cells.** Cells were treated with 50 ng/ml BDNF for the indicated times. KCl was used at a concentration of 50 mM. Nontreated GFP-TK+-transfected cells were used as a control. Surface receptors were biotinylated and precipitated with streptavidin beads, separated by SDS-PAGE, and blotted onto nitrocellulose filters. The TRKB bands were detected by using α-$TRKB_{out}$ antibody. The *upper panel* shows representative images of biotinylated GFP-TK+ bands after BDNF and KCl treatments. The *bars* show quantified data from at least six independent treatments. The results are shown as percent of nontreated GFP-TK+-transfected control cells and are expressed as mean intensity of quantified TK+ bands ± S.E. *Star* indicates statistically significant difference in comparison to control cells (Student's *t* test, $p < 0.05$).

Western blotting of the total TRKB pool was performed by loading 40 μg of total protein from the biotinylated samples on 6% SDS-PAGE gels. Electroblotting, antibody detection, and quantification of the TRKB bands were performed as described above.

Confocal Microscopy—The transfected hippocampal neurons and N2a cells were fixed and stained with α-FLAG antibody (1:1000, Sigma) as described (44). The coverslips were mounted with Gel/Mount antifading mounting media (Biomeda, Foster City, CA). Confocal images were obtained at ×100 magnification with Nikon Eclipse TE 300 fluorescence microscope connected to Ultra View confocal scanner (Life Science Resources) and UltraPix CCD camera. The excitation wavelengths were 488 nm for GFP and 568 nm for α-FLAG/α-mouse Texas Red (Molecular Probes, Leiden, Netherlands)-stained samples.

Electrophysiological Recordings of N2a Cells—Voltage-dependent Ca^{2+} currents were studied in voltage-clamp mode at room temperature (+23 °C) using the standard whole-cell configuration (47). The extracellular bath solution consisted of the following (in mM): TEA-Cl 125, HEPES 10, $BaCl_2$ 15, $MgCl_2$ 1, glucose 10, tetrodotoxin 0.1; pH was adjusted to 7.3 with TEA-OH. The involvement of Ca^{2+} channels was verified with 100 μM Cd^{2+}. In some experiments, extracellular $BaCl_2$ was exchanged with $CaCl_2$ in order to identify low voltage-activated T-type Ca^{2+} currents; the relative conductance for Ba^{2+} and Ca^{2+} ions is in general similar to LVA T-type-like currents, whereas $Ba^{2+} > Ca^{2+}$ for HVA currents (48). The intracellular pipette solution consisted of the following (in mM): CsCl 100, HEPES 40, 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid 10, $CaCl_2$ 2.5, ATP (magnesium salt) 4.2, GTP (sodium salt) 0.6; pH was adjusted to 7.15 with CsOH. Cover glasses with N2a cells, grown 50–70% confluent, were placed on the bottom of an RC-24 40-μl fast exchange chamber (Warner Instruments), and round cells without processes were selected for investigation. The extracellular bath solution was perfused 0.75–1 ml/min via a

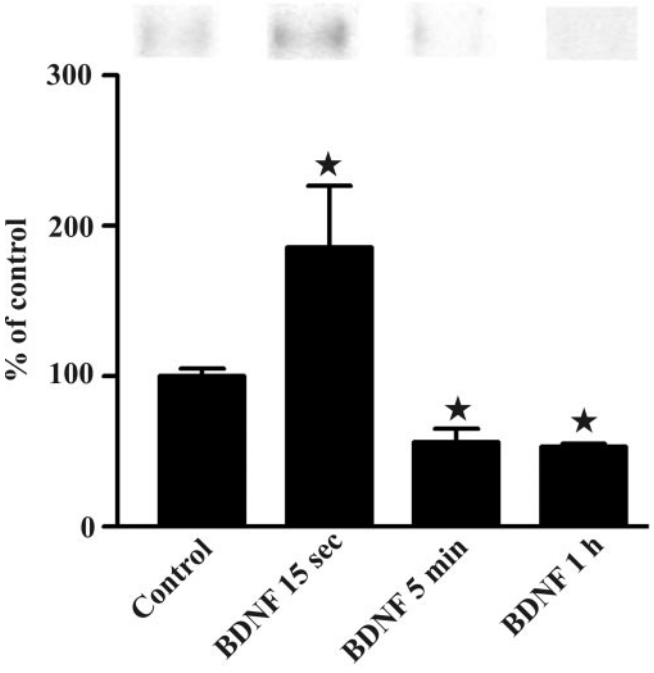

FIG. 2. **Regulation of endogenously expressed full-length TK+ surface expression levels by BDNF in hippocampal cultures.** The cells were treated at 8–10 DIV with 50 ng/ml BDNF for the indicated times. Nontreated cells were used as a control. Surface receptors were biotinylated and precipitated with streptavidin beads, separated by SDS-PAGE, and blotted onto nitrocellulose filters. The TRKB bands were detected by using α-$TRKB_{out}$ antibody. The *upper panel* shows representative images of biotinylated TK+ bands after treatments. The *bars* show quantified data from at least six independent treatments. The results are shown as percent of nontreated control cells and are expressed as mean intensity of quantified TK+ bands ± S.E. *Star* indicates statistically significant difference in comparison to control cells (Student's t test, $p < 0.05$).

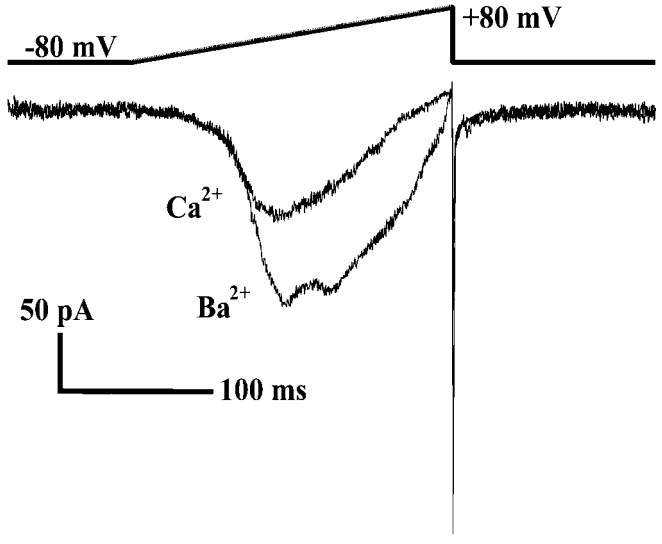

FIG. 3. **Ramp protocol showing voltage-dependent Ca^{2+} currents in N2a cells**. From a holding potential of −80 mV, currents were evoked every 5 s with a ramp protocol of −80 to +80 mV of 200-ms duration (*upper trace*). Cells were perfused with 20 mM Ba^{2+} (*trace* Ba^{2+}) following exchange to 20 mM Ca^{2+} (*trace* Ca^{2+}). Traces are average of three sweeps each. *Bars* indicate time (ms) and current (pA), respectively.

manually gravidity controlled application system consisting of a series of reservoirs attached to a perfusion manifold (Warner Instruments) positioned just before the chamber inlet. Patch clamp pipettes (PG150T, Harvard Apparatus, UK) were prepared with a PC-10 puller and fire-polished with a micro-forge MF-900 (Narishige, UK) to a resistance of

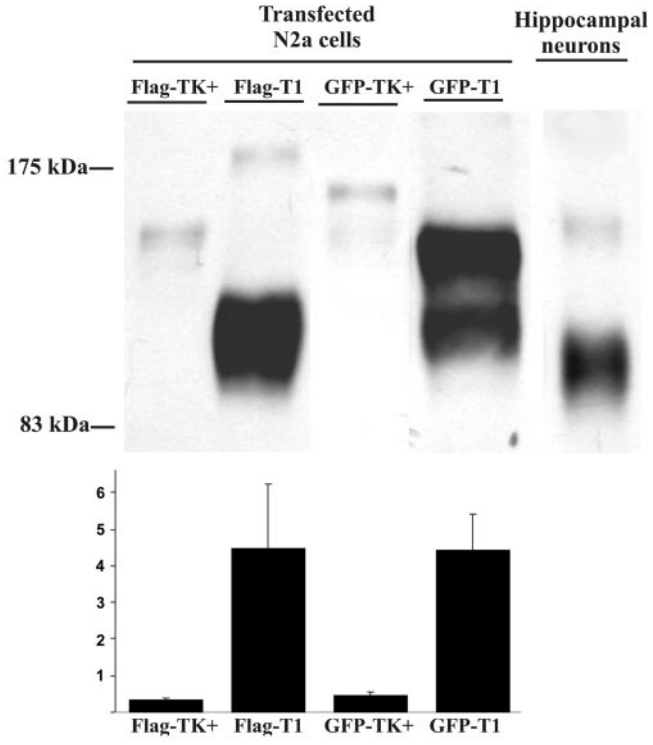

FIG. 4. **Cell surface expression levels of TK+ and T1 in N2a cells and hippocampal neurons.** *Upper panel,* N2a cells were transfected with FLAG- and GFP-tagged TK+ or T1. The cell surface receptors in transfected N2a cells and hippocampal cultures were biotinylated and precipitated with streptavidin beads, separated by SDS-PAGE, and blotted onto nitrocellulose filters. TRKB bands were detected by using α-$TRKB_{out}$ antibody, which specifically recognizes both full-length and truncated TRKB forms. FLAG-TK+ and GFP-TK+ migrate on the gels at ~145 and ~ 175 kDa, respectively, and FLAG-T1 and GFP-T1 at ~95 and ~125 kDa, respectively. Size differences of FLAG- and GFP-tagged TK+ and T1 can be explained by different sizes of the N-terminal tags: FLAG tag is 8 amino acids in length, whereas the GFP tag is ~30 kDa. Endogenous TK+ is detected at ~145 kDa and T1 at ~95 kDa in hippocampal cultures. *Lower panel,* quantitation of the cell surface-expressed FLAG or GFP-tagged T1 or TK+ in N2a cells. Means ± S.E. (in arbitrary units), from three independent experiments, n = 5–7 per lane.

3.0–3.5 megohms measured in the extracellular bath solution. The perfusion chamber was grounded using a 2% agar bridge with 0.9% NaCl connected to an Ag/AgCl wire. Voltage protocols and data acquisition were controlled with pClamp 8.1 (Axon Instruments). An Osborne 800 MHz PIII computer was used to control the patch clamp amplifier Axopatch 200A via a Digidata 1320E SCSI PCI interface (Axon Instruments). Recordings were digitally sampled at 5 kHz and filtered at 2 kHz using the analog low pass bessel filter on the recording amplifier. Series resistance was compensated to 70–75%. Leak current was automatically subtracted using a P/4 protocol in pClamp 8.1. From a holding potential of −80 mV, ramp protocols (−80 to +80 mV; 200 ms) were introduced. Recordings were imported into Microcal Origin™ 6.0 for analysis and graphic visualization.

RESULTS

Full-length TRKB Cell Surface Expression Levels Are Regulated by BDNF—We have investigated how BDNF affects the cell surface expression levels of full-length TRKB.TK+ by treating pGFP-TK+-transfected N2a cells and primary hippocampal neurons with 50 ng/ml BDNF for different times. Surface biotinylation assay indicated that BDNF differentially regulates cell surface levels of both transfected and endogenous TK+ depending on the exposure time. A brief 15-s BDNF treatment of TK+-transfected N2a cells at +21 °C resulted in an over 2-fold increase in the surface expression levels of TK+ when compared with the nontreated control cells (Fig. 1). BDNF similarly induced a rapid increase in cell surface levels

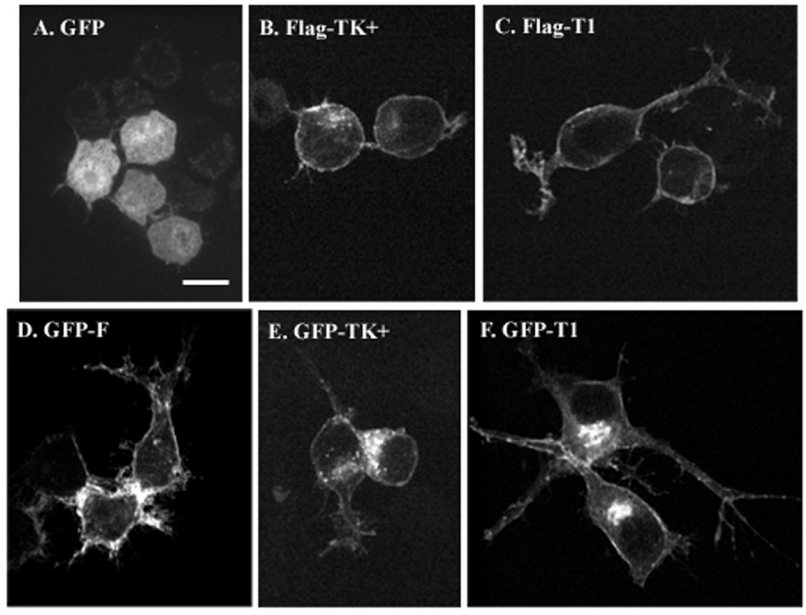

FIG. 5. **Subcellular localization of full-length TK+ and truncated T1 in N2a cells.** Confocal microscope images of N2a cells transfected with TK+ and T1 containing different N-terminal tags. *A,* conventional GFP-transfected control cells showing cytoplasmic expression of GFP. *B,* FLAG-tagged TK+-transfected cells, in which FLAG-TK+ expression is observed partially on the plasma membrane but predominantly in the cytoplasm. *C,* FLAG-tagged T1 transfected cells showing that FLAG-T1 is mainly expressed on the cell surface highlighting strongly the filopodia and processes. *D,* farnesylated GFP-F-transfected control cells showing that GFP-F expression is targeted to the cell surface. *E,* GFP-tagged TK+-transfected cells showing the expression on plasma membrane but more strongly in the cytoplasm. *F,* GFP-tagged T1-transfected cells, in which GFP-T1 expression is shown on the plasma membrane, even though some expression is found also in the cytoplasm. Expression of FLAG-tagged TK+ and T1 were visualized by α-FLAG-antibody staining under permeabilizing conditions. *Bar* in *A* represents 10 μm for all images. The images were taken with ×100 magnification.

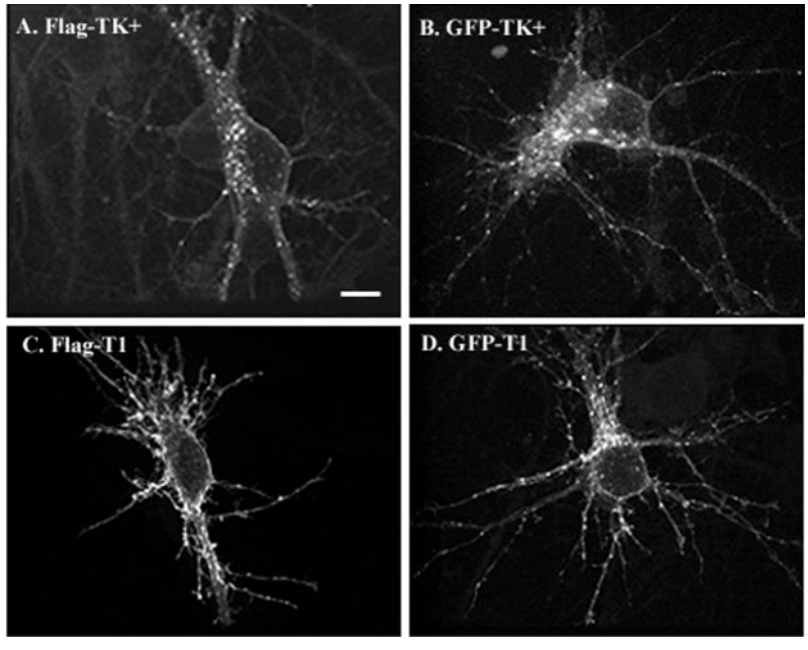

FIG. 6. **Subcellular localization of full-length TK+ and truncated T1 in hippocampal neurons.** Confocal microscope images of hippocampal neurons at 10–12 DIV transfected with TK+ and T1 containing different N-terminal tags. FLAG-TK+-transfected (*A*) and GFP-TK+-transfected neuron (*B*), showing that the expression pattern of FLAG- and GFP-tagged TK+ is mostly cytoplasmic and granular in the soma and proximal processes, even though some expression can be also observed in the soma plasma membrane. FLAG-T1-transfected (*C*) and GFP-T1-transfected neuron (*D*), showing that in contrast to TK+, FLAG- and GFP-T1 are mainly localized on the plasma membrane in the soma region and proximal processes. Furthermore, T1 expression can be clearly detected far along the processes. Expression of α-FLAG-tagged TK+ and T1 were visualized by FLAG antibody staining under permeabilizing conditions. *Bar* in *A* represents 10 μm for all images. The images were taken with ×100 magnification.

of endogenously expressed TK+ in hippocampal neuron cultures (Fig. 2). A 5-min BDNF treatment, which robustly induces TRKB autophosphorylation in our system (39), did not increase the surface levels of TK+ in transfected N2a cells or hippocampal neuronal cultures (Figs. 1 and 2). Rather, it resulted in decreased surface levels of endogenously expressed TK+ in neurons (Fig. 2). After 1 h, the TK+ surface levels were significantly decreased in both transfected N2a cells and hippocampal cultures (Figs. 1 and 2), and after 24 h the TK+ signal was barely detectable on the surface of transfected N2a cells (Fig. 1). The total protein levels of TK+ were not significantly altered in transfected N2a cells, even after 24 h of BDNF treatment when compared with the nontreated control cells as indicated by Western blotting (data not shown). This suggests that the changes observed in biotinylation assay represent events taking place on the cell surface only.

The mechanism by which BDNF increases the rapid TK+ surface expression may involve membrane depolarization and activation of voltage-dependent Ca^{2+} channels (VDCC). A rapid translocation of the GLYT2 glycine transporter to the plasma membrane has been shown to occur in response to depolarization in a Ca^{2+}-dependent manner (49). We therefore tested the effect of depolarization of TK+-transfected N2a cells with 50 mM KCl on the TK+ surface expression. Exposure of

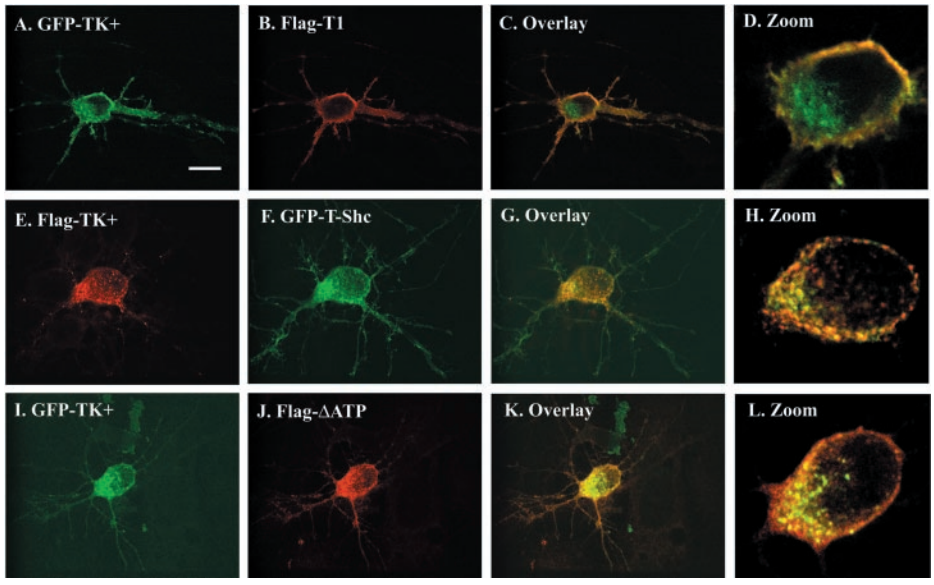

FIG. 7. **Subcellular localization of transfected TRKB forms in hippocampal neurons.** Confocal microscope images of cotransfected hippocampal neurons. *A–D* show a neuron cotransfected with GFP-TK+ and FLAG-T1. TK+ shows mostly granular intracellular localization in the soma and proximal processes (*A*), whereas T1 is clearly expressed on the plasma membrane and also can be detected further along the processes than TK+ (*B*). *C* shows colocalization of the two receptor isoforms on the cell surface of soma and proximal processes (*yellow*). Colocalization on the plasma membrane surrounding the soma is shown in more detail as *yellow* in *D*. *E–H* show FLAG-TK+ and GFP-T-Shc-cotransfected neuron. FLAG-TK+ is mostly localized intracellularly in soma and proximal processes (*E*). GFP-T-Shc is expressed on the plasma membrane but also partially in cytoplasm in the soma. The expression is strong also in the processes more distally from the soma (*B*). TK+ and T-Shc are partially colocalized on and close to the plasma membrane in the soma in granular structures (*G*). Colocalization in the soma area is shown in more detail as *yellow* in the zoomed image in (*H*). *I–L* show a neuron cotransfected with GFP-TK+ and FLAG-ΔATP. GFP-TK+ localization is shown in *I*. FLAG-ΔATP expression is highly similar to that of TK+ and can be detected as granular in the soma surface and cytoplasm and punctated in the processes (*J*). Colocalization of TK+ and ΔATP is shown as *yellow* granules in *K* and at close-up in *L*. FLAG-tagged receptors were visualized by α-FLAG antibody staining in permeabilizing conditions. *Bar* in *A* represents 10 μm for all other images, except for *D, H,* and *L*.

cells to KCl for 15 s did not lead to increased surface levels of TK+ compared with the control cells under the same conditions, whereas BDNF did induce a significant increase (Fig. 1).

To verify that this lack of response was not due to failure of N2a cells to express VDCCs, we used the whole-cell patch clamp technique in voltage mode (47) to investigate the functional expression in N2a cells. VDCC currents were evoked by ramp protocols using 20 mM Ba^{2+} as the current carrier. In 3/6 cells, we observed two components peaking at around −5 and +20 mV, respectively (Fig. 3). The currents were abolished by the introduction of 100 μM Cd^{2+} demonstrating the involvement of Ca^{2+} currents (data not shown). Substitution of Ba^{2+} with Ca^{2+} abolished the peak at +20 mV, while only slightly affecting the peak at −5 mV, suggesting the presence LVA and HVA currents (Fig. 3). In other 3/6 cells, we only observed a peak at +20 mV, suggesting the presence of solely HVA currents (data not shown). Thus, N2a cells express VDCC currents.

Subcellular Localizations of the Full-length and the Truncated TRKB Isoforms Are Different—Biotinylation assay was also used to compare the surface expression levels of transfected and endogenous full-length TK+ and truncated T1 in N2a cells and hippocampal cultures. It was observed that the levels of T1 expressed on the cell surface are much greater than those of TK+ (Fig. 4). In transfected N2a cells the surface levels of FLAG- and GFP-tagged TK+ (~145 and ~175 kDa, respectively) are clearly lower than those of FLAG- or GFP-tagged T1 (~95 and ~125 kDa, respectively; Fig. 4). There were no apparent differences between the surface expression levels of differently tagged (FLAG tag, eight amino acids; GFP tag, ~30 kDa) TK+ or T1 when compared with each other, even though the differently tagged TRKBs migrate at different molecular weights (Fig. 4). However, GFP-T1 migrated as a double band, which may reflect incomplete glycosylation after transient transfection of this construct. In hippocampal neuron cultures, the endogenously expressed TRKB isoforms exhibited similar distribution: the levels of TK+ (~145 kDa) were lower than those of T1 (~95 kDa) on the cell surface (Fig. 4). This indicates that overexpression of the receptors in N2a cells after transfection does not affect the expressional distribution of the two TRKB isoforms.

Confocal microscopy showed that in transfected N2a cells, small amounts of FLAG- and GFP-tagged TK+ were localized to the plasma membrane, but a substantial amount of the protein was located in the cytoplasm around the nucleus in granular structures (Fig. 5, *B* and *E*), which is consistent with the small amounts of surface TK+ detected in biotinylation assay. In contrast, FLAG-T1 was clearly predominantly localized to the plasma membrane in the soma and processes (Fig. 5*C*). GFP-T1 was also strongly expressed on the plasma membrane, but some cytoplasmic expression could be observed (Fig. 5*F*). This may reflect a pool of incompletely glycosylated T1 also observed in Fig. 4 as a lower molecular weight band in transfected N2a cells. Control transfections with soluble GFP (Fig. 5*A*) and farnesylated GFP-F (Fig. 5*D*) showed that GFP is clearly cytoplasmic, whereas GFP-F is mostly targeted onto the cell surface highlighting the filopodia and processes, as expected.

The subcellular localization of transfected TK+ and T1 in hippocampal neurons closely resembles that detected in N2a cells. Both FLAG-TK+ and GFP-TK+ were mostly localized intracellularly in the soma region and proximal processes, where they were found in granular structures resembling endoplasmic reticulum/Golgi apparatus. However, a small amount could be observed on the cell surface of the soma (Fig. 6, *A* and *B*). Further down in distal processes, TK+ was expressed as bright punctations scattered along otherwise hardly detectable processes (Fig. 6, *A* and *B*). In contrast, FLAG- and GFP-tagged T1 were mostly localized to the cell surface and could be clearly observed on the plasma membrane surround-

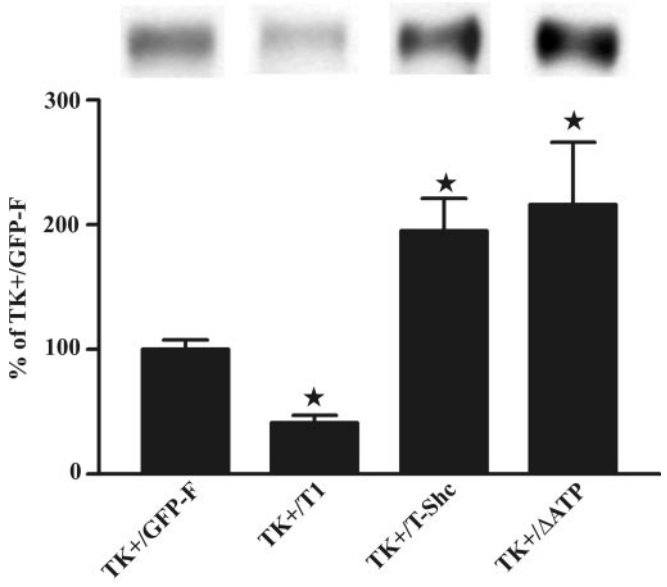

FIG. 8. **Regulation of full-length GFP-TK+ surface expression levels by coexpression of truncated GFP-T1 and GFP-T-Shc and kinase-dead FLAG-ΔATP in N2a cells.** Cells cotransfected with GFP-TK+ and GFP-F were used as a control. Surface receptors were biotinylated and precipitated with streptavidin beads, separated by SDS-PAGE, and blotted onto nitrocellulose filters. The TRKB bands were detected by using α-TRKB$_{out}$ antibody. *Upper panel* shows representative images of biotinylated GFP-TK+ in the cotransfected cells. The *bars* show quantified data from at least six independent transfections. The results are shown as percent of GFP-TK+ and GFP-F-cotransfected control cells and are expressed as mean intensity of quantified TK+ bands ± S.E. *Star* indicates statistically significant difference in comparison to control cells (Student's *t* test, $p < 0.05$).

ing the soma and proximal processes (Fig. 6, *C* and *D*). Furthermore, T1 was expressed very strongly, even in the most distal processes.

Also, when coexpressed in the same neuron, TK+ and T1 displayed mostly different subcellular localizations (Fig. 7, *A–D*), with T1 being localized to the surface (Fig. 7*B*) and TK+ mostly to the cytoplasm (Fig. 7*A*). Interestingly, however, the two TRKB isoforms were observed to be partially colocalized on the surface of neurons, especially on the plasma membrane surrounding cell soma (Fig. 7, *C* and *D, yellow*).

Truncated TRKB Isoforms Differentially Regulate Subcellular Localization of Full-length TRKB —-T1 is known to be able to interact with TK+ and inhibit its action (39). We investigated whether coexpression of TK+ and T1 in the same neuron also influences their subcellular localization. Biotinylation assay indicated that coexpression of T1 with TK+ in N2a cells resulted in a significant decrease in TK+ surface expression levels when compared with the control cells transfected with TK+ alone (Fig. 8). To investigate if the reason T1 reduces TK+ on the cell surface is because it lacks tyrosine kinase activity, we cotransfected cells together with TK+ and ΔATP, a kinase-dead mutant form of TRKB. Confocal microscopy showed that TK+ and ΔATP shared a highly similar expression pattern in hippocampal neurons (Fig. 7, *I* and *J*), and the expression of both appeared granular on the plasma membrane and cytoplasm of the soma where they were also partially colocalized (Fig. 7, *K* and *L, yellow*). The expression of TK+ and ΔATP appeared punctated in the processes. Surface biotinylation assay showed that coexpression of ΔATP together with TK+ resulted in a significantly increased surface expression of TK+ when compared with the cells transfected with TK+ alone (Fig. 8), indicating that ΔATP has an opposite effect on TK+ surface levels, as does T1. Supporting this observation, inhibition of TK+ kinase activity by the tyrosine kinase inhibitor K252a (200 nM, 1 h (50)) also leads to an increase of TK+ levels on the cell surface (data not shown).

Coexpression of T-Shc, the other kinase domain-lacking truncated TRKB form, with TK+ leads to an almost 2-fold increase in TK+ levels on the cell surface as shown by biotinylation assay (Fig. 8), indicating that T-Shc also has an opposite effect on TK+ surface levels compared with T1. Confocal microscopy showed that expression of TK+ (Fig. 7*E*) and T-Shc (Fig. 7*F*) partially overlapped especially on the plasma membrane and in part in the cytoplasm of the cell soma. The coexpression pattern appeared granular (Fig. 7, *G* and *H, yellow*). Similarly to T1, T-Shc was observed to be more strongly expressed in the processes than TK+ (Fig. 7*F*). Together these results suggest that coexpression of T1 with TK+ leads to decreased TK+ surface expression levels but that the decrease is not due to the lack of a functional kinase domain in T1.

DISCUSSION

Effects of BDNF on the Surface Expression Levels of TK+— The cell surface is the primary site where the full-length TRKB.TK+ is activated by BDNF and subsequent intracellular signaling is initiated. However, the majority of TK+ is located intracellularly in vesicles. TK+ levels on the cell surface are increased in response to neuronal activity (42, 43), but regulation of TK+ surface levels by other factors has not been extensively studied. We have investigated here rapid effects of BDNF on TK+ surface expression. To achieve this, we studied the surface expression levels of TK+ at a lowered temperature (+21 °C) after 15 s of BDNF exposure, for the shortest time that the experiments could be performed reproducibly. Very interestingly, in 15 s BDNF increased TK+ levels on the cell surface by over 2-fold. Similar fast Ca^{2+}-dependent translocation has recently been shown for glycine transporter 2 (GLYT2), which moves to the membrane in seconds after induction of glycine release (49). A 5-min treatment with BDNF had no effect on TK+ surface levels in transfected N2a cells and decreased TK+ on the cell surface of neurons, even though TK+ autophosphorylation was greatly enhanced at this time (39). These results suggest that BDNF first activates TK+ located on the cell surface, and this serves as a signal to recruit more TK+ from the cytoplasm to the plasma membrane within seconds. Our results, together with previously published data (42, 43), imply that there is an intracellular pool of TK+ in the cytoplasm that can be very rapidly recruited onto the cell surface upon an activation signal. This sort of activable reserve pool has been suggested to also exist for BDNF (9, 51).

It has been suggested that BDNF preferentially acts on active neurons and that BDNF itself can induce fast depolarization of neurons (14, 43). One possible mechanism by which BDNF could rapidly induce TK+ translocation to the cell surface could take place by membrane depolarization. However, treating TK+-transfected cells with depolarizing KCl for 15 s did not lead to increased levels of TK+ on the cell surface, suggesting that the mechanism for the fast translocation of TK+ is not by BDNF-induced depolarization. This is consistent with the observation that KCl-induced depolarization was ineffective in inducing TRKB surface expression when applied for 60 min, whereas patterned electrical stimulation, a treatment that has been shown to increase BDNF release in culture (8, 10), effectively increased surface-expressed TK+ (43).

When the transfected N2a cells or hippocampal neurons were treated with BDNF for a longer time (1 or 24 h), the cell surface levels of TK+ decreased dramatically. It has been reported previously (52–56) that prolonged BDNF treatment leads to TRKB down-regulation by internalization from the plasma membrane. Our results are in accordance with those

findings and corroborate that prolonged BDNF exposure of TK+-expressing cells leads to the removal of the receptor from the cell surface. Similar to Carter *et al.* (53), we did not observe a significant decrease in the total amount of expressed TK+ protein, even after a 24-h BDNF treatment in transfected N2a cells. However, in another study (56) it has been reported that down-regulation leads to degradation of the TRKB receptor protein.

Our data suggest that BDNF rapidly increases the surface expression of its signaling receptor TRKB.TK+. It has been shown recently (57, 58) that BDNF induces BDNF release in neurons. Furthermore, we have recently observed that inhibition of TRKB activation *in vivo* reduces the induction of BDNF mRNA in response to kainic acid administration, suggesting that TRKB signaling, presumably through BDNF release, is at least partially responsible for the activity-dependent regulation of BDNF mRNA in neurons (59). Taken together, these observations suggest that BDNF release potentiates its own signal transduction through multiple positive feedback systems. In contrast, continuous long term exposure to BDNF induces a negative feedback loop by severely depleting the functional TRKB receptors on the neuronal surface.

Different Subcellular Localization of Full-length and Truncated TRKB Isoforms—Even though TK+ and T1 are sorted to both somatodendritic and axonal compartments in neurons (41), their subcellular localization within these compartments was observed to be dramatically different in both transfected N2a cells and primary hippocampal neurons, which agrees with the results obtained by electron microscopy in hippocampal slices (60). In agreement with the surface biotinylation experiments, confocal microscopy revealed that TK+ was mainly localized in intracellular granules and in granular punctations along processes, whereas T1 was on the plasma membrane highlighting filopodia and processes in N2a cells and the entire neuritic arborization in neurons.

The differential localization of TK+ and TK− isoforms apparently reflects structural differences in their intracellular domains. Even though the extracellular and transmembrane domains of TK+, T1, and T-Shc isoforms are exactly identical, and all contain a signal sequence for plasma membrane targeting in their N termini, the isoforms differ considerably in their intracellular structures (20, 24, 61). The fact that the ΔATP mutant resembled TK+ in its subcellular localization supports the idea that the structure of the intracellular domain predominantly dictates the intracellular localization and demonstrates that the ability of the TK+ domain to autophosphorylate is not required for the differential localization. Furthermore, the observation that the localization of TK+, ΔATP, and T-Shc resembled one another, whereas that of T1 was clearly different, suggests that specific interactions of the short intracellular tail of the T1 specifically influence its intracellular distribution.

The control construct GFP-F displayed a similar expression pattern to T1, because the farnesylation signal drives its expression to the membrane. In contrast, soluble GFP, the other control construct, was cytoplasmic and did not highlight filopodia or processes. We have reported previously (44) that transfected T1 induces the outgrowth of filopodia and processes in N2a cells, whereas TK+-transfected cells appear mostly round and devoid of those morphological structures and are similar to the GFP-transfected control cells. We have subsequently observed that when control cells are transfected with GFP-F, instead of soluble GFP, to highlight the processes, the cells appear structurally very similar to the T1-transfected N2a cells. Therefore, it seems likely that the previously observed differences in the morphologies of T1- and TK+-transfected N2a cells reflect the differential localization of the two TRKB isoforms, rather than a direct regulation of N2a cell morphology by T1. However, it has been reported recently (62) that TK+ and T1 can differentially regulate dendritic morphology in visual cortical neurons, which suggests that, at least under certain conditions, T1 can induce morphological alterations independent of TK+.

Regulation of Full-length TRKB Surface Expression Levels by the Truncated TRKB Isoforms—Different truncated TRKB isoforms were observed to differentially regulate TK+ surface levels. Coexpression of the truncated T1 led to a significant decrease of TK+ on the cell surface. Even though the two isoforms were mostly differentially localized, they were partially colocalized on the cell surface of neurons, implying an interaction. We have shown previously (39) that coexpression of T1 in TK+-expressing PC12 cells inhibits BDNF-mediated cell survival by preventing TK+ autophosphorylation in a dominant negative mechanism. Our results suggest that, in addition to inhibiting autophosphorylation, T1 can affect TK+ signaling by regulating the cell surface levels of the latter isoform. It is possible that in cells coexpressing TK+ and T1, interaction of the two isoforms may lead to increased internalization of the receptor complex from the cell surface.

To study whether the effect of T1 on TK+ surface levels was due to the lack of a functional kinase domain, we cotransfected cells with the kinase-dead TRKB.ΔATP and TK+. The expression pattern of ΔATP resembled that of TK+, and it was also mostly localized intracellularly and appeared punctated. Contrary to T1, coexpression of ΔATP led to increased surface levels of TK+. Accordingly, inhibition of TK+ activity pharmacologically by the kinase inhibitor K252a also resulted in an increased cell surface level of TK+, further implying that the lack of kinase domain does not explain why T1 decreases TK+ levels on the cell surface, and that there have to be yet other elements involved. Furthermore, these results suggest that if TK+ kinase activity is prevented, more TK+ is recruited onto the cell surface, possibly to compensate for inhibited TK+ signaling.

The other truncated TRKB form, T-Shc, was observed to be expressed on the cell surface, even though some expression could also be detected in the cytoplasm. Similar to T1, T-Shc was localized further along the neurites when compared with TK+. Coexpression of T-Shc with TK+ increased the levels of TK+ on the cell surface, again suggesting that the lack of kinase domain cannot explain the effects of T1 on TK+ surface expression levels. Colocalization of T-Shc and TK+ in neurons suggests also that T-Shc and TK+ may interact, which could lead to the transport of more TK+ onto the cell surface. T-Shc has a longer juxtamembrane region than T1, and it contains an Shc-binding site similar to TK+ (30). Therefore, interaction of TK+ and T-Shc may play a yet unrecognized role in cellular signaling.

Taken together, our results suggest that regulation of BDNF signaling by controlling TK+ surface expression levels is highly complex and tightly regulated. TK+ surface levels can be differentially affected by the ligand, depending on the exposure time, neuronal activity, and coexpression of different truncated TRKB isoforms, or a combination of these factors. Our results may provide evidence of how BDNF signaling may be regulated in neurons also *in vivo*.

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