A Novel Endocytic Recycling Signal Distinguishes Biological Responses of Trk Neurotrophin Receptors^D

Zhe-Yu Chen,^{*†} Alessandro Ieraci,^{*} Michael Tanowitz,[‡] and Francis S. Lee^{*§}

Departments of ^{*}Psychiatry and [§]Pharmacology, Weill Medical College of Cornell University, New York, NY 10021; [†]The Key Laboratory of Experimental Teratology, Ministry of Education, Shandong University, Jinan, Shandong 250012, People's Republic of China; and [‡]Departments of Psychiatry and Cellular and Molecular Pharmacology, University of California, San Francisco, San Francisco, CA 94143

Submitted July 19, 2005; Revised September 11, 2005; Accepted September 22, 2005
Monitoring Editor: Richard Assoian

Endocytic trafficking of signaling receptors to alternate intracellular pathways has been shown to lead to diverse biological consequences. In this study, we report that two neurotrophin receptors (tropomyosin-related kinase TrkA and TrkB) traverse divergent endocytic pathways after binding to their respective ligands (nerve growth factor and brain-derived neurotrophic factor). We provide evidence that TrkA receptors in neurosecretory cells and neurons predominantly recycle back to the cell surface in a ligand-dependent manner. We have identified a specific sequence in the TrkA juxtamembrane region, which is distinct from that in TrkB receptors, and is both necessary and sufficient for rapid recycling of internalized receptors. Conversely, TrkB receptors are predominantly sorted to the degradative pathway. Transplantation of the TrkA recycling sequence into TrkB receptors reroutes the TrkB receptor to the recycling pathway. Finally, we link these divergent trafficking pathways to alternate biological responses. On prolonged neurotrophin treatment, TrkA receptors produce prolonged activation of phosphatidylinositol 3-kinase/Akt signaling as well as survival responses, compared with TrkB receptors. These results indicate that TrkA receptors, which predominantly recycle in signal-dependent manner, have unique biological properties dictated by its specific endocytic trafficking itinerary.

INTRODUCTION

Neurotrophins, such as brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF), are a family of growth factors that have been established to play critical roles in vertebrate nervous system and cardiovascular development and function (Tessarollo, 1998; Huang and Reichardt, 2001; Chao, 2003). The actions of neurotrophins are dictated by two classes of cell surface receptors, the tropomyosin-related kinase (Trk) receptor tyrosine kinase and the p75 neurotrophin receptor. It has been established that upon binding to neurotrophins, Trk receptors are rapidly endocytosed in a clathrin-dependent manner (Grimes *et al.*, 1997). Postendocytic sorting of Trk receptors to diverse pathways after ligand binding has significant impact on the physiological responses to neurotrophins because they also determine the strength and duration of intracellular signaling cascades initiated by activated Trk receptors (Heerssen and Segal, 2002). One of the most extensively studied postendocytic pathways is retrograde trafficking of Trk recep-

tors in neurons, which has been shown to be required for trophic responses (Ginty and Segal, 2002). However, it has been demonstrated that only a minor fraction of Trk receptors are retrogradely transported. In compartmentalized cultures of sympathetic neurons, only a small proportion (~2%) of the activated Trk receptors in the distal compartment were found in the cell body after 8 h (Ure and Campenot, 1997; Tsui-Pierchala and Ginty, 1999).

Two alternate endocytic pathways that Trk receptors can follow is trafficking to lysosomes or recycling to the plasma membrane. The degradative pathway to lysosomes is characterized by down-regulation of the total number of receptors at the cell surface and decreased responsiveness to ligand. It has been shown for both TrkA and TrkB that a proportion of these receptors can be sorted to the degradative pathway after ligand binding to the receptor (Knusel *et al.*, 1997; Sommerfeld *et al.*, 2000; Jullien *et al.*, 2002; Saxena *et al.*, 2005). In addition, in clinical trials, systemic BDNF treatment of amyotrophic lateral sclerosis (ALS) patients has led to desensitization or a limitation of the supportive actions of BDNF (Vejsada *et al.*, 1994), which was likely because of a down-regulation of TrkB receptors (Thoenen, 2001; Thoenen and Sendtner, 2002).

Conversely, less has been established as to whether Trk receptors traffic to the recycling pathway. In general, recycling of receptors back to the plasma membrane can lead to functional resensitization and prolongation of cell surface-specific signaling events. It has been shown that many cell surface receptors, such as the transferrin receptor, recycle to the plasma membrane by "default" via membrane bulk flow (Mayor *et al.*, 1993; Gruenberg, 2001). Alternatively, for certain receptors, such as G protein-coupled receptors, specific "recycling" sequences and sorting molecules have been

This article was published online ahead of print in *MBC in Press* (<http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05-07-0651>) on October 5, 2005.

^D The online version of this article contains supplemental material at *MBC Online* (<http://www.molbiolcell.org>).

Address correspondence to: Zhe-Yu Chen (zhc2003@med.cornell.edu) or Francis S. Lee (fslee@med.cornell.edu).

Abbreviations used: BDNF, brain-derived neurotrophic factor; EGFR, epidermal growth factor receptor; GPCR, G protein-coupled receptor; LAMP1, lysosome-associated membrane protein 1; NGF, nerve growth factor; Trk, tropomyosin-related kinase.

identified that are required for ligand-dependent targeting of the endocytosed receptors to the recycling pathway (Cao *et al.*, 1999; Cong *et al.*, 2001; Gage *et al.*, 2001; Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004). In Trk receptors, one study, suggesting possible Trk receptor recycling, used iodinated nerve growth factor (NGF) to demonstrate that the endocytosed NGF was released into the media (Zapf-Colby and Olefsky, 1998).

Endocytic trafficking of Trk receptors to degradative or recycling pathways is a complex, highly regulated process, the mechanisms of which remain unclear. In this context, three fundamental questions are raised regarding trafficking fates for this physiologically important receptor tyrosine kinase. First, do Trk receptors traffic to the recycling pathway? And if so, are there structural determinants such as a "recycling signal" mediating this sorting decision between recycling and degradative pathways? Second, are there differences in the postendocytic fates of different Trk receptors? Previous studies have indicated that TrkA and TrkB receptors have potentially differential signaling and biological responses (Carter *et al.*, 1995; Sommerfeld *et al.*, 2000), especially in the context of prolonged treatment with their respective ligand (NGF or BDNF). In particular, through domain swapping between TrkA and TrkB receptors, certain intracellular regions in the TrkB receptor were identified to be required for optimal TrkB receptor degradation (Sommerfeld *et al.*, 2000). Third, if there are differences in the postendocytic fates for TrkA and TrkB, does alternate endocytic trafficking result in subsequent differences in biological responses?

To address these questions, we conducted an analysis of the postendocytic fates (degradation and recycling) of TrkA and TrkB in neurosecretory cells and neurons. We have determined, that, unlike TrkB, TrkA efficiently recycles in a ligand-dependent manner and trafficking to the recycling pathway requires a specific sequence in the juxtamembrane region, which is absent in TrkB, that recycles less efficiently. These observations provide evidence for how differential biological responsiveness between these two neurotrophin receptors is determined by postendocytic sorting to different membrane pathways.

MATERIALS AND METHODS

Reagents and Antibodies

Murine NGF was obtained from Harlan Bioproducts for Science (Indianapolis, IN), and human recombinant BDNF was obtained from PeproTech (Rocky Hill, NJ). Anti-FLAG antibodies were obtained from Sigma-Aldrich (St. Louis, MO) (monoclonal, M1, and polyclonal), and anti-Trk antibodies (B-3) were from Santa Cruz Biotechnology (Santa Cruz, CA). Lysosome-associated membrane protein 1 (LAMP1) monoclonal antibody was obtained from the Developmental Studies Hybridoma Data Bank (University of Iowa, Iowa City, IA). Anti-Alexa-488- and fluoresceinated transferrin were from Invitrogen (Carlsbad, CA). Fluorescent secondary antibodies were from Jackson ImmunoResearch Laboratories (West Grove, PA). The restriction enzymes were purchased from MBI Fermentas (Hanover, MD), and *Pfu* Turbo DNA was from Stratagene (La Jolla, CA). All other compounds were from Sigma-Aldrich.

Plasmid Constructs

Rat TrkA and TrkB cDNAs were subcloned into pCDNA3.1neoexpression vector (Invitrogen) by using *EcoRI* and *EcoRV* sites. Rat epidermal growth factor receptor (EGFR) cDNA, provided by Dr. Jeffrey Segall (Albert Einstein College of Medicine, Bronx, NY), was subcloned into pCDNA3.1 plasmid by using *HindIII* and *XhoI* sites. The amino terminal FLAG epitope tag was added to the 5' end of the receptor tyrosine kinases by PCR. TrkA mutants in Shc binding site, FLAG-TrkA-N487A, FLAG-TrkA-Y490A, and the kinase-dead mutant (FLAG-TrkA-K538A) were made by site-directed mutagenesis. All the TrkA deletion constructs and TrkA, TrkB chimeric constructs were generated by means of two-step PCR. All of the constructs were confirmed by DNA sequence to exclude potential PCR-introduced mutations.

Analysis of Trk Receptor Recycling by Using Fluorescence Ratio Microscopy

To quantify the extent of recycling observed after ligand removal in individual cells, a modified version of previous receptor recycling methods (Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004) was devised. Transiently transfected cells or cells stably expressing Trk or epidermal growth factor (EGF) receptors were grown on glass coverslips and incubated with Alexa-488-conjugated M1 anti-FLAG antibody (prepared by standard methods using Alexa-Fluor-488 *N*-hydroxysuccinimide ester; Invitrogen) to selectively label FLAG-tagged receptors present in the plasma membrane at the beginning of the experiment. Cells were then incubated (at 37°C for 30 min) in the presence of NGF, BDNF, or EGF to drive internalization. At the end of this incubation, cells were quickly washed three times in phosphate-buffered saline (PBS) lacking Ca^{2+} or Mg^{2+} and supplemented with 1 mM EDTA to dissociate FLAG antibody bound to residual surface receptors remaining in the plasma membrane, thereby leaving antibody bound only to the internalized pool of receptors. EDTA-stripped cells were then incubated (at 37°C for 45 min) in the presence of Cy3-anti-mouse to label all receptors that returned to the cell surface, and then cells were fixed with 4% paraformaldehyde. In each experiment, and for each receptor construct examined, two parallel control coverslips were included, one in which cells were fixed after a 30-min incubation in the absence of ligand and without an EDTA stripping step (100% surface receptor control), and one in which cells were fixed immediately after the EDTA-mediated stripping step (0% recycled control). Cells were examined by epifluorescence microscopy by using appropriate filter sets to selectively detect Alexa-488 or Cy3, and staining intensities of each fluor in individual cells were integrated using MetaMorph software (Molecular Devices, Sunnyvale, CA). This analysis indicated that the efficiency of the EDTA strip (reduction of Cy3 staining intensity in the 0% recycled control relative to the 100% surface receptor control) was >95%, consistent with previous measures using fluorescence flow cytometry. The percentage of receptors recycled in individual cells after ligand washout was then calculated from the red/green ratios determined from the control conditions according to the following formula: $(E - Z)/(C - Z) \times 100$, where *E* is the mean ratio for the experimental coverslip, *Z* is the mean ratio for the zero surface control, and *C* is the mean ratio for the 100% surface control. Twenty to 30 cells/construct/condition were analyzed at random in this manner for each experiment, and average values reported under *Results* represent mean recycling percentages derived from five independent experiments.

Fluorescence Flow Cytometry

Internalization and recycling of epitope-tagged receptors were estimated using fluorescence flow cytometry of stably transfected cells to measure changes in the relative amount of FLAG-tagged receptors present in the plasma membrane after surface labeling with Alexa-488-conjugated M1 antibody as described previously (Gage *et al.*, 2001). Fluorescence flow cytometry was performed using a FACScan instrument (BD Biosciences, San Jose, CA). Cells (20,000) were collected for each sample. Triplicate samples were analyzed for each condition in each experiment. The mean fluorescence values for each experiment (*n* = 5 experiments) were averaged, and the SEM was calculated across all experiments.

Surface Biotinylation and Assays of Receptor Proteolysis

Cell surface biotinylation was used to specifically detect receptors present in the plasma membrane and to measure their proteolysis, as described previously (Rajagopal *et al.*, 2004). Briefly, stably transfected PC12 cells expressing the indicated FLAG-tagged receptors were washed twice with ice-cold PBS, and incubated with 300 $\mu\text{g}/\text{ml}$ sulfo-*N*-hydroxysuccinimide-biotin (Pierce Chemical, Rockford, IL) in PBS for 30 min at 4°C to biotinylate surface proteins. Unreacted biotin was quenched and removed with Tris-buffered saline. Biotinylated cells were then transferred to prewarmed medium containing ligand for the indicated times, and then cells were immediately chilled on ice and lysed in extraction buffer [1.0% (vol/vol) Triton X-100, 10 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM KCl, 1 $\mu\text{g}/\text{ml}$ leupeptin, 1 $\mu\text{g}/\text{ml}$ pepstatin, 2 $\mu\text{g}/\text{ml}$ aprotinin, and 0.5 mM phenylmethylsulfonyl fluoride]. Extracts were clarified by centrifugation ($12,000 \times g$ for 20 min), and then biotinylated proteins were isolated from cell extracts by immobilization on streptavidin-conjugated Sepharose beads (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) and in PBS containing 0.5% Triton X-100. Washed beads were eluted with SDS sample buffer, and eluted proteins were resolved by SDS-PAGE.

PC12 Cell Culture and Immunoprecipitation and Immunoblotting

PC12 cells stably overexpressing FLAG-TrkA or FLAG-TrkB receptors were maintained in DMEM (Invitrogen) containing 10% fetal bovine serum (Invitrogen), 5% horse serum (Invitrogen), supplemented with 100 U/ml penicillin-streptomycin (Invitrogen), and 2 mM glutamine plus 200 $\mu\text{g}/\text{ml}$ hygromycin (Invitrogen). Lysates or condition medium was collected and analyzed by Western blot as described previously (Lee and Chao, 2001). For densito-

Figure 1. Trk receptors exhibit differential ligand-induced trafficking to degradative pathways. PC12 cells stably expressing FLAG-tagged TrkA (A) or TrkB (B) were surface biotinylated and incubated at 37°C for the indicated times in the absence or presence of 50 ng/ml ligand (NGF for A and BDNF for B). Surface-labeled receptors were detected by streptavidin pull-down followed by anti-Trk immunoblotting. Representative anti-Trk immunoblots are shown for TrkA- and TrkB-expressing cells. (C) Colocalization of TrkA and TrkB receptors relative to LAMP1, visualized by confocal microscopy in PC12 cells fixed after 75-min incubation at 37°C in the presence of ligand.

metric analysis, immunoreactive bands were scanned, and intensity was quantitated using NIH Image (Scion, Frederick, MD).

Cortical Cell Cultures

Dissociated primary cultures of cortical neurons from embryonic day 18 rats were prepared from timed-pregnant Harlan Sprague Dawley rats as described previously (Chen *et al.*, 2004). Fetuses were removed under sterile conditions and kept in PBS on ice for microscopic dissection of the cerebral cortex. The meninges were removed, and the tissue was placed in Neurobasal media (Invitrogen). The tissue was briefly minced with fine forceps and then triturated with a fire-polished Pasteur pipette. Cells were counted and plated on culture wells coated with 0.01 mg/ml poly-D-lysine overnight in a Neurobasal media containing B27 supplement (Invitrogen) and L-glutamine (0.5 mM) (Invitrogen). Primary cortical neurons were transfected with FLAG-Trk receptor constructs by electroporation (Amaxa, Cologne, Germany) as described previously (Gresch *et al.*, 2004), and experiments were conducted 4 d after plating.

Immunocytochemical Staining and Fluorescence Microscopy

PC12 cells or cortical neurons were grown on glass coverslips (Corning Glassworks, Corning, NY), coated with poly-L-lysine (Sigma-Aldrich), and transfected with epitope-tagged neurotrophin constructs. Forty-eight hours after transfection, cells were fixed with a 4% paraformaldehyde solution in PBS and permeabilized with brief treatment with cold ethanol (5 min), followed by washing with PBS three times. Cells were blocked with 5% goat serum in PBS for 30 min. Specimens were incubated with primary antibodies and then incubated with subtype-specific fluorescinated secondary antibodies. Stained PC12 specimens were examined by epifluorescence microscopy by using a CoolSNAP charge-coupled device camera (Photometrics, Huntington Beach, CA) mounted on a Nikon (Tokyo, Japan) TE2000 microscope equipped with a motorized Z drive and either a 100×, 1.4 numerical aperture objective or 60×, 1.4 numerical aperture objective and standard fluorescein isothiocyanate-Texas Red dichroic filter sets. Confocal fluorescence microscopy was performed on cortical neuron specimens by using an LSM510 microscope (Carl Zeiss, Oberkochen, Germany) fitted with a Zeiss 63×, 1.4 numerical aperture objective with standard filter sets and standard (1 Airy disk) pinhole.

RESULTS

Because previous studies have suggested that TrkA and TrkB receptors have potentially differential signaling and biological responses (Carter *et al.*, 1995; Sommerfeld *et al.*, 2000; Thoenen, 2001), especially in the context of prolonged treatment with their respective ligand (NGF or BDNF), we sought to determine whether there were differences in the

trafficking fate of the receptors after ligand treatment. First, it has been previously established that both TrkA and TrkB receptors are degraded via lysosomes in a ligand-dependent manner, in cell lines such as PC12 cells as well as in primary neuronal cultures (Knusel *et al.*, 1997; Sommerfeld *et al.*, 2000; Jullien *et al.*, 2002, 2003). Although altered kinetics of degradation has been postulated previously to explain differential signaling responses (Knusel *et al.*, 1997; Sommerfeld *et al.*, 2000; Carter *et al.*, 2002), it has not been determined directly. To examine whether these two receptors are differentially degraded, we directly compared the rates of ligand-dependent degradation of the Trk receptors in parallel sets of PC12 cells stably overexpressing FLAG-tagged TrkA or TrkB. In particular, PC12 cells stably overexpressing FLAG-TrkA or FLAG-TrkB receptors were used that had equivalent levels of surface FLAG-Trk receptors (Figure 1, A and B). We performed the degradation assays based on biotinylation assays that have used previously to monitor degradation of a variety of signaling receptors (Tsao and von Zastrow, 2000; Jullien *et al.*, 2002; Tanowitz and von Zastrow, 2002). After biotinylation, PC12 cells were treated with ligand (NGF or BDNF) for various times periods (1, 2, 3, 4, or 5 h), lysed, pulled down with streptavidin beads, and immunoblotted for Trk antibodies, which allowed for assessment of proteolysis of the endocytosed Trk receptors. Immunoblotting of with Trk antibodies revealed that TrkA degraded at significantly slower rate than TrkB (Figure 1, A and B). The rate of TrkA degradation (>1 h for more than one-half of the biotinylated pool to be degraded) is similar in magnitude to what has been reported previously in studies of PC12 stably overexpressing TrkA (Jullien *et al.*, 2002, 2003). To independently examine whether the decreased degradation rate of TrkA, compared with TrkB, represented decreased targeting of TrkA to lysosomes for degradation, we used confocal microscopy to visualize the localization of internalized Trk receptors relative to the late endosomes/lysosomal marker LAMP1 (Figure 1C). TrkB was localized more extensively with LAMP1 within 75 min after BDNF treatment, compared with TrkA after NGF treatment (Figure 1C). These results are consistent with the biochemical deg-

Figure 2. Trk receptors exhibit similar ligand-induced internalization rates. (A) Schematic of internalization assay. PC12 cells stably expressing FLAG-tagged TrkA or TrkB were incubated at 37°C for 0, 5, 10, 20, 30, or 60 min in the presence or absence of 50 ng/ml ligand (NGF for TrkA and BDNF for TrkB) and then incubated at 4°C for 1 h with fluorescently labeled anti-FLAG antibodies. The cells were then assayed for ligand-dependent (B) and ligand-independent (C) internalization of receptors by flow cytometric determination of surface immunofluorescence. Squares, TrkA; circles, TrkB.

radiation studies (Figure 1, A and B) that indicate greater TrkB degradation than TrkA degradation after 1 h of ligand addition and suggest that the more rapid TrkB degradation is due to increased targeting to late endosomes/lysosomes.

We hypothesized that the differences in degradation rates may be due to alterations in TrkA and TrkB receptor trafficking at certain steps after ligand addition, namely, 1) at the initial internalization step and/or 2) at a postendocytic step in which Trk receptors can be sorted to either degradative or recycling pathways. To address these questions related to the Trk endocytic pathways, we took advantage of novel features of the amino-terminal FLAG epitope-tagged versions of TrkA and TrkB receptors, in which calcium-sensitive fluorescent anti-FLAG antibodies can be rapidly dissociated with PBS/EDTA. This dissociable FLAG system has been extensively used to study G protein-coupled receptor (GPCR) endocytic trafficking (Gage *et al.*, 2001; Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004), in particular, internalization rates as well as recycling and degradation of these receptors. Because these antibodies bind to the extracellular regions of the Trk receptors, we first determined that addition of antibodies by itself did not induce autoactivation of the Trk receptors by Western blot analysis (Supplemental Figure 1) or induce internalization of the receptor in the absence of ligand by immunofluorescence microscopy (Figure 4).

To determine whether the initial rates of TrkA and TrkB receptor internalization differed, fluorescence flow cytometry was performed on PC12 cells stably expressing the respective FLAG-Trk receptors in the presence and absence of ligand, as described previously (Keith *et al.*, 1998). Unlike the previous biotinylation assay in which the receptors were labeled before ligand addition (Figure 1), cells were initially treated with ligand (NGF or BDNF) for various periods of time at 37°C and then chilled to 4°C, and Alexa-488-conjugated FLAG antibodies was added for 1 h. The cells were then subjected to quantitation of fluorescence by flow cytometry. In this manner, unlike the previous biotinylation assay, which monitored the degradation fate of a set of labeled receptors upon ligand treatment (Figure 1), this assay specifically is a measure of the internalization of receptors. Both TrkA and TrkB were rapidly internalized in the presence of ligand (Figure 2B), and half-lives for internalization were calculated (TrkA $t_{1/2} = 5 \pm 1$ min and TrkB $t_{1/2} = 5 \pm 1$ min). No significant differences were observed in the initial rates of TrkA and TrkB internalization (Figure 2B).

To determine the initial rates of Trk receptor recycling, we then used a live cell assay that was previously used to determine recycling to the plasma membrane for TGN38 (Ghosh *et al.*, 1998) and the mannose 6-phosphate receptor

(Lin *et al.*, 2004). Cells stably expressing FLAG-TrkA or FLAG-TrkB were incubated with Alexa-488-conjugated FLAG antibodies for 10 min at 37°C, and then ligand (NGF or BDNF) was added for 30 min at 37°C, followed by stripping of the residual surface FLAG antibodies with a 30-s incubation with 1 mM EDTA. Cells were then fixed or chased for 5–60 min with anti-Alexa-488 antibodies, which has been established to quench with an ~92% efficiency (Lin *et al.*, 2004), in the chase media. If the fluoresced internalized receptor were recycled, upon return to the plasma membrane, it would be quenched by the anti-Alexa-488 antibody. We observed rapid loss of cell-associated fluorescence during the chase (Figure 3A). After a 2-min chase, the fluoresced Trk receptors are visible in endocytic structures. After a 20-min chase, however, the cell-associated fluorescence was diminished significantly (Figure 3, A and B). The rate of loss of fluorescence was used as a measure of the rate of receptor recycling. From these experiments, we calculated that both TrkA and TrkB initially recycled with half-lives ~7 min. We, however, noted that at steady-state conditions, such as after 45 min, that there was more TrkB fluorescence compared with TrkA, suggesting that although the initial rate of TrkB recycling is similar to TrkA, at steady-state, TrkB may be sorted to an alternate endocytic pathway, namely, the degradative pathway. Consistent with this finding is that at these steady-state time points, TrkA colocalizes significantly more with the transferrin receptor compared with TrkB, as determined by confocal microscopy (Figure 3C). Together, these results suggest that TrkA is predominantly sorted to the recycling pathway, consistent with the decreased rate of its ligand-dependent degradation (Figure 1A).

To investigate further the potential differences in steady-state TrkA and TrkB postendocytic recycling, we modified a live cell ratiometric fluorescence-based assay, previously established to quantitate GPCR recycling (Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004). This assay is a variation of the above-mentioned live cell assay (Figure 3) in which the internalized pool of receptors is specifically labeled with fluoresced anti-FLAG monoclonal antibodies, and recycling of the internal receptor pool is detected by surface accessibility to a fluoresced anti-mouse secondary antibody (Figure 4A). The 45-min time point was chosen based on the recycling time course (Figure 3) as a measure of steady-state recycling and is consistent with previous versions of this assay for GPCR recycling (Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004). The main variation from the previous ratiometric recycling assay is that the secondary antibody (Cy3-anti-mouse) is in the media throughout the recycling phase of the assay (Figure 4A).

Figure 3. Determination of ligand-dependent Trk receptor recycling rates. (A) PC12 cells stably expressing FLAG-tagged TrkA or TrkB were incubated at 37°C for 15 min with fluoresceinated (Alexa-488) anti-FLAG antibodies (2 μ g/ml) and then incubated at 37°C with 50 ng/ml ligand (NGF for TrkA and BDNF for TrkB) for 30 min. Afterward, the noninternalized fluoresceinated anti-FLAG antibodies were stripped with EDTA, and cells incubated in the presence of 10 μ g/ml anti-Alexa-488 antibodies for the indicated times before fixation. To obtain the zero-minute time point, cells were fixed after the EDTA strip step. Unquenched fluorescence was detected by wide-field microscopy. Relative fluorescence intensities per cell are plotted as a function of time of incubation with anti-Alexa-488. Dotted lines represent steady-state levels of immunofluorescence intensity/cell. Squares, TrkA; circles, TrkB. (B) Representative images from such an experiment, showing Alexa-488 fluorescence after 0 min or 45 min with anti-Alexa-488, or 45-min chase without anti-Alexa-488. (C) Colocalization of internalized TrkA and TrkB receptors relative to transferrin receptor visualized by confocal microscopy. PC12 cells stably expressing FLAG-tagged TrkA or TrkB were incubated at 37°C for 15 min with fluoresceinated (Alexa-488) anti-FLAG antibodies and then incubated at 37°C with 50 ng/ml ligand (NGF for TrkA, BDNF for TrkB) and fluoresceinated transferrin (rhodamine-transferrin) for 15 min. The noninternalized fluoresceinated anti-FLAG antibodies were stripped with PBS/EDTA. Cells were reincubated at 37°C in the presence of rhodamine-transferrin, fixed for 30 min, and imaged by confocal microscopy. Representative micrographs are shown. The proportion of colocalization between internalized Trk and transferrin receptors is presented as a mean $\pm$ SEM determined from analysis of four independent experiments (* represents $p < 0.01$, Student's t test).

This modification ensures that even if the Trk receptor reinternalizes again after one round of recycling, it will still be measured as having “recycled.” The ratiometric assay was validated by comparing Trk receptor recycling with that of the EGFR, a receptor tyrosine kinase that has been established not to recycle efficiently after ligand-dependent activation and to be primarily sorted to the degradative pathway (Sorkin and von Zastrow, 2002; Marmor and Yarden, 2004). Consistent with previous studies, minimal EGFR recycling was observed after 45 min, as indicated by weak cell-associated fluorescence (Figure 4B, bottom). In contrast, under the same conditions, a strong signal was observed for TrkA (Figure 4B, top). Surprisingly, the fluorescent signal for TrkB was significantly weaker than TrkA (Figure 4B, middle). TrkB-associated fluorescence, however, was still greater than that associated with EGFR. Quantification of these results by ratiometric analyses (see *Materials and Methods*) confirmed that TrkA recycled in a ligand-dependent

manner significantly ($71 \pm 1\%$), whereas TrkB recycled to a reduced degree ($39 \pm 1\%$) (Figure 4C). In addition, as predicted, EGFR recycled to a much reduced degree ($22 \pm 1\%$) (Figure 4C), consistent with receptors primarily sorted to the degradative pathway (Tanowitz and von Zastrow, 2003).

To determine whether this unexpected difference in TrkA and TrkB receptor endocytic trafficking was observed in primary neurons, we assessed Trk recycling in cortical neurons expressing FLAG-tagged versions of these receptors. We chose cortical neurons as they have been established to express both TrkA (albeit low levels) and TrkB receptors endogenously (Pizzorusso *et al.*, 1999; Pitts and Miller, 2000; Huang and Reichardt, 2001; Rossi *et al.*, 2002). We determined that like in PC12 cells, TrkA exhibits significant ligand-dependent recycling ($61 \pm 2\%$) in cortical neurons (Figure 5, A and C). Conversely, TrkB receptors recycled minimally after ligand addition ($28 \pm 3\%$), to an extent that was even more reduced than observed in PC12 cells (Figure

Figure 4. Determination of ligand-dependent Trk receptor recycling in PC12 cells. (A) Cells expressing FLAG-tagged TrkA or TrkB were incubated at 37°C for 15 min with fluorescenated (Alexa-488) anti-FLAG antibodies and then incubated at 37°C with 50 ng/ml ligand (NGF for TrkA, BDNF for TrkB, and EGF for EGFR) for 30 min. Afterward, the noninternalized fluorescenated anti-FLAG antibodies were stripped with EDTA, and cells were incubated in the presence of Cy3 anti-mouse at 37°C for 45 min before fixation. (B) Representative images from such an experiment with PC12 cells. The control condition was a parallel coverslip incubated for 30 min in the absence of ligand and without an EDTA stripping step. The strip condition was a parallel coverslip in which cells were fixed immediately after the EDTA-mediated stripping step. (C) The recycling behavior was quantitated as described in *Materials And Methods*, and the error bars represent the SEM of five independent experiments.

5, A and C). Thus, these observations provide strong evidence that the TrkA and TrkB traverse different endocytic pathways after ligand addition not only in cell lines but also in primary neurons that naturally express both forms of Trk receptors.

The ratiometric recycling assay was then used to define potential regions in the TrkA receptor that is required for the enhanced ligand-dependent recycling to the plasma membrane compared with TrkB. We generated chimeras in which different TrkA domains (extracellular, juxtamembrane, kinase, and C terminal) were substituted for the corresponding TrkB domains (Figure 5A). We hypothesized that monitoring for an increase in recycling behavior from these chimeras would allow us to identify a domain that is *sufficient* to function as an autonomous endocytic recycling signal. In PC12 cells, we observed that substitution of only the TrkA juxtamembrane region into TrkB (TrkB^{JM}) led to a significant increase in recycling to levels comparable with

wild-type (WT) TrkA ($65 \pm 2\%$) (Figure 6A). Swapping in other TrkA domains had no effect on TrkB chimeras' recycling behavior. These observations indicate that the 80-aa TrkA juxtamembrane region contains structural elements that are sufficient for optimal ligand-dependent Trk receptor recycling, by virtue of being able to be transplanted to another Trk receptor to increase recycling.

We next sought to determine whether the juxtamembrane domain was *necessary* for ligand-dependent TrkA recycling. To address this question, various domains including the juxtamembrane region were deleted from TrkA, and recycling was assessed. Only TrkA mutants lacking the juxtamembrane domain (TrkA Δ JM) showed a significant reduction in recycling as compared with wild-type receptor (control, $70 \pm 2\%$; TrkA Δ JM, $25 \pm 3\%$) (Figure 6B). To define more precisely the region in the juxtamembrane required for efficient TrkA recycling, we divided the 80-aa JM domain into three boxes: Box1 (aa 444–472), Box2 (aa 473–493), and

Figure 5. Determination of ligand-dependent Trk receptor recycling in cortical neurons. (A) Representative images from recycling experiment described in Figure 4 with primary cortical neurons expressing FLAG-tagged TrkA receptors. (B) Representative images from such an experiment with primary cortical neurons expressing FLAG-tagged TrkB receptors. (C) Quantitation of recycling in A and B as described in *Materials and Methods*, and the error bars represent the SEM of five independent experiments.

Box3 (aa 502–512). Amino acids 494–501 were retained in all mutants, because there is a critical set of residues therein (N496, Y499) required for Shc binding and Trk signaling (Lamiet *et al.*, 1996). Deletion of Box1 had no effect on TrkA recycling (Figure 6B). Conversely, deletion of Box2 caused an inhibition of TrkA recycling similar to that observed with TrkA Δ JM (TrkA Δ Box2, $28 \pm 2\%$) (Figure 6B). In addition, deletion of Box3 caused an inhibition of TrkA recycling to a lesser degree than deletion of Box 2 (TrkA Δ Box3, $44 \pm 3\%$) (Figure 6B). An alternate TrkA deletion of Box 3 that included aa 494–501 (TrkA Δ Box3') produced similar recycling levels as TrkA Δ Box3 (our unpublished data). The results from the TrkA Δ Box3 studies suggest that this region (aa 502–512) contributes, albeit to a lesser degree than Box2 (aa 473–493), to optimal recycling of TrkA receptors. For all these TrkA juxtamembrane mutants, there was no alteration in surface targeting of the receptor or alterations in initial endocytosis rate (our unpublished data). These observations indicate that the JM region of TrkA is necessary for ligand-dependent recycling, and a discrete region therein (aa 473–493) is an essential element for TrkA recycling after endocytosis.

To assess more directly whether the residues required for Shc binding (N496 and Y499) and Trk signaling (Lamiet *et al.*, 1996) affected Trk recycling, point mutations of each of these residues to alanines were made. Both point mutants

(N496A and Y499A) did not alter recycling compared with TrkA WT controls (Figure 6C). This result is consistent with the subsequent finding that kinase activity, assessed by two mutant TrkA receptors lacking either the entire kinase domain (TrkA Δ TK) or a point mutation in the kinase domain established to abolish kinase activity (K546A), did not reduce TrkA recycling after ligand-induced endocytosis (Figure 6, B and C). These results point out the unexpected observation that TrkA kinase activity is not required for the recycling operation of the receptor after endocytosis by ligand addition. It should be noted that in the absence of ligand there is minimal endocytosis (Figure 4), and minimal detectable TrkA recycling (Figure 4A), suggesting that TrkA recycling is ligand dependent but kinase independent.

To further investigate whether the recycling signal in TrkA functions to specify trafficking to the recycling rather than degradative pathways, we assessed by surface biotinylation experiments the kinetics of ligand-induced degradation of the TrkA mutant lacking Box2 (TrkA Δ Box2). We observed substantially enhanced receptor degradation of Δ Box2 with a half-life similar to TrkB (Figure 7A). We next examined whether transplanting this TrkA JM domain into analogous region in TrkB affects lysosomal trafficking of TrkB. We had previously determined that this TrkB mutant (TrkB^{JM}) had enhanced ligand-dependent recycling compared with WT TrkB (Figure 6A). This current experiment

Figure 6. Identification of a sequence in TrkA responsible for ligand-dependent receptor recycling. (A) PC12 cells were transfected with TrkB chimeras containing corresponding regions of TrkA. (TrkB^{ecA}, TrkA extracellular; TrkB^{tmA}, TrkA transmembrane; TrkB^{jmA}, TrkA juxtamembrane; TrkB^{tkA}, TrkA tyrosine kinase; and TrkB^{ctA}, TrkA carboxy terminal). TrkA and TrkB was also transfected as controls. Steady-state ligand-dependent recycling was measured in these cells as described in Figure 4. (B) PC12 cells were transfected with TrkA constructs lacking the following intracellular regions: carboxy terminus, TrkAΔCT; tyrosine kinase, TrkAΔTK; juxtamembrane, TrkAΔJM, as well as smaller subregions of the juxtamembrane region (TrkAΔBox1–3), and steady-state ligand-dependent recycling was measured in these cells as described in Figure 4. (C) PC12 cells were transfected with TrkA constructs containing point mutations in the tyrosine kinase region (K546A), and the juxtamembrane region (N496A and Y499A), and steady-state ligand-dependent recycling was measured in these cells as described in Figure 4. In all experiments, error bars represent the SEM of five independent experiments. (* represents $p < 0.01$, Student's *t* test).

was a more stringent test of whether the TrkA JM regulates Trk endocytic sorting activity because it requires the receptors to recycle efficiently through multiple rounds of endocytosis and in the continuous presence of ligand, as opposed to the recycling assay in which the ligand was washed out during the recycling phase. In our surface biotinylation degradation assay, as observed previously, TrkB was significantly proteolysed after 1.5 h (Figure 7B). In contrast, ligand-induced proteolysis of the TrkB^{jmA} was significantly inhibited (Figure 7B). The rate of degradation for this TrkB mutant was now similar to that of wild-type TrkA. These observations suggest that the TrkA JM signal is sufficient to promote rapid ligand-dependent recycling of Trk receptors and to strongly inhibit postendocytic degradation. Thus, this recycling signal functions as a major determinant distinguishing the endocytic trafficking of neurotrophin receptor subtypes TrkA and TrkB.

To determine whether this “recycling” signal affects the biological responses for the Trk receptors, we assessed both intracellular signaling and cell survival. Both NGF stimula-

tion of TrkA and BDNF stimulation of TrkB leads to enhanced survival in various cell lines and primary neuronal cultures through initiation of signaling cascades, including phosphatidylinositol 3-kinase (PI3 kinase) (Kaplan and Miller, 2000; Chao, 2003). Conversely, it has also been shown that prolonged treatment of these two neurotrophins can lead to divergent signaling responses (Carter *et al.*, 1995; Knusel *et al.*, 1997; Sommerfeld *et al.*, 2000). To address whether the differences in Trk receptor endocytic trafficking fates (recycling or degradative) observed in our studies also leads to divergent biological responses, we performed a series of experiments to address whether the enhanced recycling behavior TrkA led to enhanced signaling and subsequent survival in PC12 cells. We used an established paradigm of prolonged serum withdrawal (8 h) followed by prolonged neurotrophin treatment (24 h) to assess neurotrophin-dependent survival in PC12 cells stably expressing TrkA or TrkB trafficking mutants. We performed three sets of experiments. First, we determined the levels of surface Trk receptors by surface biotinylation and avidin pull-down

Figure 7. Trk receptors mutants exhibit differential ligand-induced trafficking to degradative pathways. PC12 cells stably expressing TrkA (A) and TrkB (B) mutants were surfaced biotinylated and incubated at 37°C for the indicated times in the absence or presence of 50 ng/ml ligand (NGF for A and BDNF for B). Surface-labeled receptors were detected by streptavidin pull-down followed by anti-Trk immunoblotting. Representative anti-Trk immunoblots are shown for TrkA- and TrkB-expressing cells.

before and after prolonged treatment with the respective neurotrophin (NGF or BDNF) (Figure 8A). We observed before ligand treatment, all the forms of Trk expressed equally at the cell surface (Figure 8A). Then, we observed that we could still detect significant cell surface wild-type TrkA after 24-h treatment with NGF but could not detect any cell surface wild-type TrkB after 24-h treatment with BDNF (Figure 8B). Cell surface levels of TrkA mutants lacking a portion of the JM region necessary for endocytic recycling (TrkA Δ Box2) (Figure 8B) were not detectable after 24-h treatment with NGF. Conversely, cell surface TrkB mutants containing the TrkA JM (TrkB^{JM}), which enhanced TrkB recycling (Figure 6A), were detected after 24-h treatment with BDNF (Figure 8B). Together, these observations are consistent with the previous observations that in the continuous presence of NGF, TrkA Δ Box2 degraded more rapidly than wild-type TrkA (Figure 7A), and that in the continuous presence of BDNF, TrkB^{JM} degraded less significantly than wild-type TrkB (Figure 7B).

Second, having demonstrated enhanced cell surface levels for Trk receptors with enhanced ligand-dependent recycling and diminished degradation, we wanted to determine whether this led to altered survival signaling. A primary survival signaling pathway activated by Trk receptors is PI3-kinase/Akt (Kaplan and Miller, 1997; Chao, 2003). After 24-h treatment with neurotrophins, PC12 cells stably expressing Trk or Trk mutants were assessed for PI3 kinase/Akt signaling as detected by a phospho-specific Akt antibody. After prolonged neurotrophin treatment, wild-type TrkA-expressing cells still had significant levels of phospho-Akt, whereas none was detected in wild-type TrkB-expressing cells (Figure 8C). In addition, cells expressing TrkA Δ Box2 did not have any detectable phospho-Akt, whereas cells expressing TrkB^{JM} had phospho-Akt levels similar to wild-type TrkA (Figure 8C). Together, these observations are consistent with the previous findings that those Trk receptors that are localized at the cell surface after prolonged neurotrophin treatment (Figure 8B) will be able to continue to elicit survival signaling.

Third, to assess the functional consequences of differential localization of TrkA and TrkB after ligand treatment, we performed cell survival assays in these same sets of PC12 cells containing Trk receptors in which cells were incubated for 8 h in serum-free media followed by 24-h neurotrophin

treatment. In the absence of any neurotrophin, native PC12 cells (WT) maintained in serum-free media in this time frame undergo significant cell death after as assessed by a terminal deoxynucleotidyl transferase dUTP nick-end labeling (TUNEL) assay (Figure 8, D and E). NGF treatment led to significant cell survival in native PC12 cells as well as TrkA-overexpressing cells. In contrast, BDNF treatment was unable to maintain cell survival in TrkB-overexpressing cells to the same level as NGF. There was a significant increase in TUNEL-positive cells in TrkB-expressing PC12 cells compared with TrkA-expressing PC12 cells (Figure 8, D and E). This observation is consistent with the finding that after prolonged BDNF treatment, there is a significant reduction in cell surface TrkB, and PI3 kinase/Akt signaling. In PC12 cells expressing the mutant TrkA (TrkA Δ Box2) with impaired recycling (Figure 6B), a significant increase in TUNEL-positive cells was observed compared with wild-type TrkA cells. Conversely, cells containing mutant TrkB (TrkB^{JM}) with enhanced recycling (Figure 6A) exhibited minimal cell death with prolonged BDNF treatment, comparable with those observed in cells expressing wild-type TrkA (Figure 8E). Together, these observations provide further evidence that the endocytic fate of Trk receptors not only has relevance to subsequent downstream signaling events but also to biological responses such as cell survival.

DISCUSSION

Endocytic trafficking of signaling receptors to alternate intracellular pathways has been shown to lead to diverse biological consequences. In this study, we have demonstrated that two neurotrophin receptors (TrkA and TrkB) traverse divergent endocytic pathways after binding to their respective ligands (NGF and BDNF). We provide direct evidence that TrkA predominantly recycles back to the cell surface after ligand treatment, whereas TrkB predominantly is sorted to the degradative pathway, which contribute to altered Trk-mediated biological responses.

Our results provide several new insights into the mechanism of endocytic trafficking of Trk receptors. Previous TrkA trafficking studies in compartmentalized sympathetic neuronal cultures using iodinated NGF (¹²⁵I-NGF) noted aspects of ¹²⁵I-NGF trafficking that suggested TrkA receptors may be trafficked to the recycling pathway (Ure and Campenot,

Figure 8. Effect of prolonged neurotrophin treatment on Trk-dependent survival. PC12 cells stably expressing TrkA or TrkB mutants were serum starved for 8 h and then surfaced biotinylated (A and B) and incubated at 37°C with 50 ng/ml ligand (NGF for TrkA and BDNF for TrkB) for 24 h. Surface-labeled receptors were detected by streptavidin pull-down followed by anti-Trk immunoblotting. Total receptors were detected by Trk immunoprecipitation followed by anti-Trk immunoblotting. Representative anti-Trk immunoblots are shown for TrkA- and TrkB-expressing cells. (C) Native PC12 cells (WT) or PC12 cells stably expressing TrkA or TrkB mutants were serum starved for 8 h and then incubated at 37°C with or without 50 ng/ml ligand (NGF for TrkA and BDNF for TrkB) for 5 min or 24 h. Levels of phospho-Trk, Trk, phospho-Akt, and Akt were determined by immunoblotting. (D) Native PC12 cells (WT) or PC12 cells stably expressing TrkA or TrkB mutants were serum starved for 8 h and then incubated at 37°C with 50 ng/ml ligand (NGF for TrkA and BDNF for TrkB) for 24 h, fixed, and processed for TUNEL analysis. The proportion of TUNEL-positive cells was scored for each culture condition, compared with WT PC12 cells (–NGF). Error bars indicate SEM from three independently conducted experiments.

1997; Tsui-Pierchala and Ginty, 1999). There was a kinetic delay in retrograde transport of ^{125}I -NGF as well as small percentage (~2%) of the ^{125}I -NGF-TrkA complex that was finally retrogradely transported, which suggested that most of ^{125}I -NGF-TrkA complexes were trafficked to alternate endocytic pathways. Previous studies directly examining TrkA trafficking with cleavable biotinylation assays to assess TrkA receptor recycling behavior noted minimal TrkA recycling (Saxena *et al.*, 2005). However, in these biotinylation assays, TrkA receptors that recycle to the cell surface and reinternalize are not detected. In our study, the use of a live cell fluorescence-based recycling assay allowed us to quantitate the proportion of Trk receptors sorted in a ligand-dependent manner to the recycling pathway. The level of ligand-dependent recycling for TrkA (~70%) is equivalent to the previously established signaling receptors such as the

β -adrenergic and μ -opioid receptors that recycle in a ligand-dependent manner (Cao *et al.*, 1999; Tanowitz and von Zastrow, 2003). Thus, with our ratiometric recycling assay, the presence of fluorescenated anti-mouse antibodies in the media allowed for its greater sensitivity in detection of TrkA recycling events and allowed for the novel finding that TrkA receptors predominantly recycle after ligand treatment. In addition, in this same study, minimal colocalization of TrkA with transferrin receptor was noted (Saxena *et al.*, 2005), in contrast with our observation of significant overlap endocytosed TrkA with transferrin receptor (Figure 3C). The main differences in these two studies were that the previous study (Saxena *et al.*, 2005) assessed colocalization of total TrkA receptors with transferrin receptor, whereas in this study, the endocytosed TrkA receptors were selectively visualized

by cell surface immunofluorescent labeling of the FLAG epitope.

In addition, with this assay, we were able to determine that efficient recycling of TrkA receptors to the plasma membrane requires a unique sequence (aa 473–493) in the TrkA cytoplasmic juxtamembrane region that is not found in the corresponding region in TrkB or recycling signals identified in recycling GPCRs (Cao *et al.*, 1999; Tanowitz and von Zastrow, 2003; Vargas and von Zastrow, 2004). This TrkA recycling signal is both necessary for efficient recycling of TrkA (Figure 6B) and sufficient to promote rapid recycling of the predominantly degradative TrkB (Figure 6A). In this context, a previous study had transplanted the TrkA juxtamembrane region into TrkB receptors and assessed TrkB receptor degradation after 24-h treatment with BDNF in HN10 cells (Sommerfeld *et al.*, 2000). In densitometric analyses of their degradation assay, the study also observed a >50% decrease in TrkB chimeric receptor degradation containing the TrkA juxtamembrane region (Sommerfeld *et al.*, 2000). This previous finding is consistent with our observation that TrkB^{imA} receptor degradation is delayed significantly in a 5-h degradation assay (Figure 7B).

Second, previous trafficking studies of receptor tyrosine kinases, namely, the EGF receptor, have focused on covalent modification, such as ubiquitylation, as the sorting signal to direct trafficking to lysosomes (Marmor and Yarden, 2004). Conversely, trafficking of EGF receptors to recycling pathways occurs presumably by default when these sorting signals are disrupted (Babst *et al.*, 2000; Bishop *et al.*, 2002) or in the absence of ligand (Felder *et al.*, 1990). In contrast, we present evidence that activated TrkA receptors that are sorted to the recycling pathway require a specific recycling signal in the juxtamembrane region, and removal of this signal leads to enhanced degradation of TrkA receptors (Figure 7A). Thus, TrkA receptors represent an example of a receptor tyrosine kinase that predominantly recycles in signal-dependent manner and may represent a unique property for regulating postendocytic trafficking of neurotrophin receptors.

Third, by demonstrating that TrkA and TrkB are sorted to different endocytic pathways, we provide one plausible mechanism to explain the differential responses to prolonged NGF or BDNF treatments (Carter *et al.*, 1995; Sommerfeld *et al.*, 2000). Consistent with previous studies, prolonged NGF treatment in neurons and cell lines led to persistent TrkA-dependent signaling, which is consistent with NGF activating a receptor that can undergo many rounds of recycling (Figure 8). In contrast, prolonged BDNF exposure led to decreased responsiveness of TrkB receptors, consistent with a receptor predominantly sorted to the degradative pathway and displaying decreased responsiveness with prolonged ligand treatment (Carter *et al.*, 1995; Sommerfeld *et al.*, 2000). We extended the links between Trk trafficking fate to biological consequences by providing evidence that Trk receptors that recycle predominantly (TrkA or TrkB^{imA}) are capable also of enhanced trophic responses, compared with Trk receptors that are predominantly sorted to the degradative pathway (TrkB and TrkAΔBox2) (Figure 8). These findings suggest that endocytic mechanisms that regulate maintenance of Trk receptors in the recycling pathway will have significant effects on biological consequences such as Trk-dependent trophic responses in neurons. In this regard, it is striking that the TrkA “recycling” signal can also route TrkB to the recycling pathway and alter its biological signaling and function.

In this context, these findings may have implications for clinical efforts to use NGF and BDNF as therapeutic agents.

In previous clinical trials, prolonged systemic BDNF treatment of ALS patients was shown to lead to limitation of the supportive actions of BDNF possibly due to a down-regulation of TrkB receptors (Thoenen, 2001; Thoenen and Sendtner, 2002). Recently, a cell therapy clinical trial involving prolonged NGF treatment into basal forebrains of patients with Alzheimer disease demonstrated therapeutic potential (Tuszynski *et al.*, 2005). Although a major area of focus for these therapeutic efforts has mainly involved in pharmacokinetic and delivery issues of the neurotrophic ligands, it is possible that understanding the molecular mechanisms regulating the postendocytic fate of the Trk receptors will also be an important determinant of clinical benefits of these treatment strategies.

ACKNOWLEDGMENTS

We thank Mark von Zastrow, Frederick Maxfield, and Pilar Perez for helpful discussions. This work was supported by National Institutes of Health Grant MH-068850 (to F.S.L.), National Alliance for Research on Schizophrenia and Depression (to F.S.L. and Z.-Y.C.), Foundation for the Author of National Excellent Doctoral Dissertation of People's Republic of China (No. 200229) (to Z.-Y.C.), Shanghai Rising-Star Program 05QM1401 (to Z.-Y.C.), and National Natural Science Foundation of China 30000048 (to Z.-Y.C.).

REFERENCES

- Babst, M., Odorizzi, G., Estepa, E., and Emr, S. (2000). Mammalian tumor susceptibility gene 101 (TSG101) and the yeast homologue, Vps23p, both function in late endosomal trafficking. *Traffic* 1, 248–258.
- Bishop, N., Horman, A., and Woodman, P. (2002). Mammalian class E vps proteins recognize ubiquitin and act in the removal of endosomal protein-ubiquitin conjugates. *J. Cell Biol.* 157, 91–101.
- Cao, T., Deacon, H., Reczek, D., Bretscher, A., and von Zastrow, M. (1999). A kinase-regulated PDZ-domain interaction controls endocytic sorting of the β_2 -adrenergic receptor. *Nature* 401, 286–290.
- Carter, A., Chen, C., Schwartz, P., and Segal, R. (2002). Brain-derived neurotrophic factor modulates cerebellar plasticity and synaptic ultrastructure. *J. Neurosci.* 22, 1316–1327.
- Carter, B. D., Zirrgebel, U., and Barde, Y. A. (1995). Differential regulation of p21ras activation in neurons by nerve growth factor and brain-derived neurotrophic factor. *J. Biol. Chem.* 270, 21751–21757.
- Chao, M. V. (2003). Neurotrophins and their receptors: a convergence point for many signalling pathways. *Nat. Rev. Neurosci.* 4, 299–309.
- Chen, Z. Y., Patel, P. D., Sant, G., Meng, C. X., Teng, K. K., Hempstead, B. L., and Lee, F. S. (2004). Variant brain-derived neurotrophic factor (BDNF) (Met66) alters the intracellular trafficking and activity-dependent secretion of wild-type BDNF in neurosecretory cells and cortical neurons. *J. Neurosci.* 24, 4401–4411.
- Cong, M., Perry, S. J., Hu, L. A., Hanson, P. I., Claing, A., and Lefkowitz, R. J. (2001). Binding of the β_2 adrenergic receptor to N-ethylmaleimide-sensitive factor regulates receptor recycling. *J. Biol. Chem.* 276, 45145–45152.
- Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C. R. (1990). Kinase activity controls the sorting of the epidermal growth factor receptor within the multivesicular body. *Cell* 61, 623–634.
- Gage, R. M., Kim, K. A., Cao, T. T., and von Zastrow, M. (2001). A transplantable sorting signal that is sufficient to mediate rapid recycling of G protein-coupled receptors. *J. Biol. Chem.* 276, 44712–44720.
- Ghosh, R., Mallet, W., Soe, T., McGraw, T., and Maxfield, F. (1998). An endocytosed TGN38 chimeric protein is delivered to the TGN after trafficking through the endocytic recycling compartment in Chinese hamster ovary cells. *J. Cell Biol.* 142, 923–936.
- Ginty, D. D., and Segal, R. A. (2002). Retrograde neurotrophin signaling: Trk-ing along the axon. *Curr. Opin. Neurobiol.* 12, 268–274.
- Gresch, O., *et al.* (2004). New non-viral method for gene transfer into primary cells. *Methods* 33, 151–163.
- Grimes, M., Beattie, E., and Mobley, W. (1997). A signaling organelle containing the nerve growth factor-activated receptor tyrosine kinase, TrkA. *Proc. Natl. Acad. Sci. USA* 94, 9909–9914.
- Gruenberg, J. (2001). The endocytic pathway: a mosaic of domains. *Nat. Rev. Mol. Cell. Biol.* 2, 721–730.

- Heerssen, H. M., and Segal, R. A. (2002). Location, location, location: a spatial view of neurotrophin signal transduction. *Trends Neurosci.* 25, 160–165.
- Huang, E., and Reichardt, L. (2001). Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* 24, 677–736.
- Jullien, J., Guili, V., Derrington, E. A., Darlix, J. L., Reichardt, L. F., and Rudkin, B. B. (2003). Trafficking of TrkA-green fluorescent protein chimerae during nerve growth factor-induced differentiation. *J. Biol. Chem.* 278, 8706–8716.
- Jullien, J., Guili, V., Reichardt, L. F., and Rudkin, B. B. (2002). Molecular kinetics of nerve growth factor receptor trafficking and activation. *J. Biol. Chem.* 277, 38700–38708.
- Kaplan, D. R., and Miller, F. D. (1997). Signal transduction by the neurotrophin receptors. *Curr. Opin. Cell Biol.* 9, 213–221.
- Kaplan, D. R., and Miller, F. D. (2000). Neurotrophin signal transduction in the nervous system. *Curr. Opin. Neurobiol.* 10, 381–391.
- Keith, D. E., Anton, B., Murray, S. R., Zaki, P. A., Chu, P. C., Lissin, D. V., Montelliet-Agius, G., Stewart, P. L., Evans, C. J., and von Zastrow, M. (1998). μ -Opioid receptor internalization: opiate drugs have differential effects on a conserved endocytic mechanism in vitro and in the mammalian brain. *Mol. Pharmacol.* 53, 377–384.
- Knusel, B., Gao, H., Okazaki, T., Yoshida, T., Mori, N., Hefti, F., and Kaplan, D. R. (1997). Ligand-induced down-regulation of Trk messenger RNA, protein and tyrosine phosphorylation in rat cortical neurons. *Neuroscience* 78, 851–862.
- Lamiet, A. A., Apell, G., Conroy, L., and Kavanaugh, W. M. (1996). Affinity, specificity, and kinetics of the interaction of the SHC phosphotyrosine binding domain with asparagine-X-X-phosphotyrosine motifs of growth factor receptors. *J. Biol. Chem.* 271, 264–269.
- Lee, F., and Chao, M. (2001). Activation of Trk neurotrophin receptors in the absence of neurotrophins. *Proc. Natl. Acad. Sci. USA* 92, 3555–3560.
- Lin, S. X., Mallet, W. G., Huang, A. Y., and Maxfield, F. R. (2004). Endocytosed cation-independent mannose 6-phosphate receptor traffics via the endocytic recycling compartment en route to the trans-Golgi network and a subpopulation of late endosomes. *Mol. Biol. Cell* 15, 721–733.
- Marmor, M. D., and Yarden, Y. (2004). Role of protein ubiquitylation in regulating endocytosis of receptor tyrosine kinases. *Oncogene* 23, 2057–2070.
- Mayor, S., Presley, J. F., and Maxfield, F. R. (1993). Sorting of membrane components from endosomes and subsequent recycling to the cell surface occurs by a bulk flow process. *J. Cell Biol.* 121, 1257–1269.
- Pitts, A. F., and Miller, M. W. (2000). Expression of nerve growth factor, brain-derived neurotrophic factor, and neurotrophin-3 in the somatosensory cortex of the mature rat: coexpression with high-affinity neurotrophin receptors. *J. Comp. Neurol.* 418, 241–254.
- Pizzorusso, T., Berardi, N., Rossi, F. M., Vieg, A., Venstrom, K., Reichardt, L. F., and Maffei, L. (1999). TrkA activation in the rat visual cortex by antirat trkA IgG prevents the effect of monocular deprivation. *Eur. J. Neurosci.* 11, 204–212.
- Rajagopal, R., Chen, Z. Y., Lee, F. S., and Chao, M. V. (2004). Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes. *J. Neurosci.* 24, 6650–6658.
- Rossi, F. M., Sala, R., and Maffei, L. (2002). Expression of the nerve growth factor receptors TrkA and p75NTR in the visual cortex of the rat: development and regulation by the cholinergic input. *J. Neurosci.* 22, 912–919.
- Saxena, S., Howe, C. L., Cosgaya, J. M., Steiner, P., Hirling, H., Chan, J. R., Weis, J., and Kruttgen, A. (2005). Differential endocytic sorting of p75NTR and TrkA in response to NGF: a role for late endosomes in TrkA trafficking. *Mol. Cell Neurosci.* 28, 571–587.
- Sommerfeld, M. T., Schweigreiter, R., Barde, Y. A., and Hoppe, E. (2000). Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an involvement of the proteasome and differential regulation of TrkA and TrkB. *J. Biol. Chem.* 275, 8982–8990.
- Sorkin, A., and von Zastrow, M. (2002). Signal transduction and endocytosis: close encounters of many kinds. *Nat. Rev. Mol. Cell Biol.* 3, 600–614.
- Tanowitz, M., and von Zastrow, M. (2002). Ubiquitination-independent trafficking of G protein-coupled receptors to lysosomes. *J. Biol. Chem.* 277, 50219–50222.
- Tanowitz, M., and von Zastrow, M. (2003). A novel endocytic recycling signal that distinguishes the membrane trafficking of naturally occurring opioid receptors. *J. Biol. Chem.* 278, 45978–45986.
- Tessarollo, L. (1998). Pleiotropic functions of neurotrophins in development. *Cytokine Growth Factor Rev.* 9, 125–137.
- Thoenen, H. (2001). Treatment of Degenerative Disorders of the Nervous System: From Helpless Descriptive Categorization to Rational Therapeutic Approaches, New York: Marcel Dekker.
- Thoenen, H., and Sendtner, M. (2002). Neurotrophins: from enthusiastic expectations through sobering experiences to rational therapeutic approaches. *Nat. Neurosci.* 5 (suppl) 1046–1050.
- Tsao, P., and von Zastrow, M. (2000). Type-specific sorting of G protein-coupled receptors after endocytosis. *J. Biol. Chem.* 275, 11130–11140.
- Tsui-Pierchala, B., and Ginty, D. (1999). Characterization of an NGF-P-TrkA retrograde-signaling complex and age-dependent regulation of TrkA phosphorylation in sympathetic neurons. *J. Neurosci.* 19, 8207–8218.
- Tuszynski, M. H., *et al.* (2005). A phase 1 clinical trial of nerve growth factor gene therapy for Alzheimer disease. *Nat. Med.* 11, 551–555.
- Ure, D. R., and Campenot, R. B. (1997). Retrograde transport and steady-state distribution of 125I-nerve growth factor in rat sympathetic neurons in compartmented cultures. *J. Neurosci.* 17, 1282–1290.
- Vargas, G. A., and von Zastrow, M. (2004). Identification of a novel endocytic recycling signal in the D1 dopamine receptor. *J. Biol. Chem.* 279, 37461–37469.
- Vejsada, R., Sagot, Y., and Kato, A. (1994). BDNF-mediated rescue of axotomized motor neurones decreases with increasing dose. *Neuroreport* 5, 1889–1892.
- Zapf-Colby, A., and Olefsky, J. (1998). Nerve growth factor processing and trafficking events following TrkA-mediated endocytosis. *Endocrinology* 139, 3232–3240.