BMB
reports

Nerve growth factor-induced neurite outgrowth is potentiated by stabilization of TrkA receptors

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Exogenous stimuli such as nerve growth factor (NGF) exert their effects on neurite outgrowth via Trk neurotrophin receptors. TrkA receptors are known to be ubiquitinated via proteasome inhibition in the presence of NGF. However, the effect of proteasome inhibition on neurite outgrowth has not been studied extensively. To clarify these issues, we investigated signaling events in PC12 cells treated with NGF and the proteasome inhibitor MG132. We found that MG132 facilitated NGF-induced neurite outgrowth and potentiated the phosphorylation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways and TrkA receptors. MG132 stimulated internalization of surface TrkA receptor and stabilized intracellular TrkA receptor, and the Ub^{K63} chain was found to be essential for stability. These results indicate that the ubiquitin-proteasome system potentiated neurite formation by regulating the stability of TrkA receptors. [BMB reports 2011; 44(3): 182-186]

INTRODUCTION

The outgrowth of neurites is an essential process in the development of the nervous system and is mainly regulated by neurotrophins such as nerve growth factor (NGF). NGF induces neurite outgrowth through the activation of receptor tyrosine kinases, such as TrkA receptors (1-3), after which NGF-bound TrkA receptors are internalized and trafficked to signaling endosomes (4, 5). Activated TrkA receptors mediate signaling cascades, including the extracellular signal-regulated kinase/mitogen activated protein kinase (ERK/MAPK) and phosphatidylinositol-3-kinase (PI3K)/AKT pathways (6, 7).

In the ubiquitination pathway, free ubiquitin (Ub) is activated by Ub-activating enzyme (E1) through the formation of a thioester between E1 and the C terminus of Ub. The thioester is subsequently transferred to members of Ub-conjugating enzyme (E2). Ub-protein ligase (E3) provides substrate recognition and promotes ligation of Ub to specific substrates. These multiubiquitin-tagged substrates are then recognized and degraded by the 26S proteasome (8, 9). Although the role of the ubiquitin-proteasome pathway in protein degradation is well known, it has been implicated in a number of additional biological processes, including receptor endocytosis, protein sorting, subnuclear trafficking, gene expression, as well as neuronal differentiation (10, 11).

Recent reports have indicated that TrkA receptors are ubiquitinated by NGF and that this ubiquitination modulates TrkA internalization and TrkA-mediated signaling pathways (12-14). In addition, proteasomal inhibition alters the trafficking of TrkA receptors (5). NGF-induced neurite outgrowth coincides with elevated levels of endogenous ubiquitin and ubiquitinated proteins (15), whereas proteasome inhibitors, such as lactacystin, TMC-95A, and tyropeptin A have been shown to induce neurite outgrowth (16-18) and enhance oligodendroglial cell differentiation (19). However, the relationship between neurite outgrowth and proteasomal inhibition is not understood well.

In this study, we investigated the additional effects of proteasome inhibitors on NGF-stimulated cells. We found that the proteasome inhibitor MG132 facilitated the NGF-induced outgrowth of neurites and also potentiated the ERK and AKT pathways. MG132 appeared to stabilize NGF-stimulated TrkA receptors, and the UB^{K63} chain seemed to be required for the stability of these receptors. These results indicate that the ubiquitin-proteasome system controls neurite formation by regulating the stability of TrkA receptors.

RESULTS AND DISCUSSION

Proteasome inhibition enhances neurite outgrowth by NGF

To examine whether or not proteasome inhibitors influence NGF-induced neurite outgrowth, PC12 cells treated with MG 132 in the presence of 50 ng/ml of NGF for 24 h. As shown in Fig. 1, NGF induced neurite outgrowth in about 12% of the cells. Co-treatment of PC12 cells with 50 ng/ml of NGF and 0.5 μM MG132 enhanced neurite outgrowth in up to 18% of the cells, which was the highest percentage detected. However, NGF-induced outgrowth of neurites was decreased at

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DOI 10.5483/BMBRep.2011.44.3.182

Received 16 December 2010, Accepted 3 January 2011

Keywords: MG132, Neurite outgrowth, NGF, Proteasome inhibitor, TrkA receptor

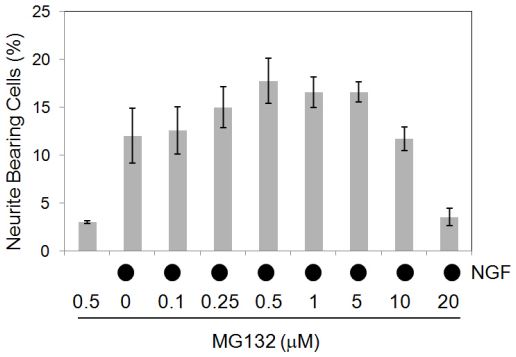

Fig. 1. Proteasome inhibition induces neurite outgrowth. PC12 cells grown on poly-L-lysine-coated culture dishes were treated with the indicated concentrations of MG132 and 50 ng/ml of NGF for 24 h. Neurite outgrowth was determined by counting the number of cells with neurites in images obtained of at least 1,000 cells. The values shown are mean ± S.D of three independent experiments (P value < 0.05).

MG132 concentrations greater than 10 μM.

To determine whether or not co-treatment of PC12 cells with NGF and MG132 was cytotoxic, we assessed cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Cellular viability decreased when the concentration of MG132 was greater than 10 μM (data not shown). Therefore, subsequent experiments were performed using 50 ng/ml of NGF and 0.5 μM MG132.

Proteasome inhibition prolongs NGF-induced TrkA signaling

The effect of proteasome inhibitors on the NGF-induced signaling pathway was analyzed by studying the degree of ERK and AKT phosphorylation in PC12 cells treated with NGF and MG132 individually or simultaneously. As reported previously (4, 20), NGF induced ERK and AKT phosphorylation (Fig. 2A). NGF and MG132 together also induced ERK and AKT phosphorylation to a degree stronger than that induced by NGF alone (Fig. 2A). In addition, we compared the time course of kinase activation by NGF to that by both NGF and MG132. NGF-induced phosphorylation was sustained only for 1 h after NGF treatment. Further, the degree of ERK and AKT phosphorylation induced by NGF and MG132 at the same time was slightly stronger than that induced by NGF alone and was sustained for a longer time (Fig. 2B).

NGF is known to induce phosphorylation of tyrosine 490 within the Shc-binding site of TrkA receptors. Once phosphorylated, TrkA receptors can mediate signaling through various pathways (3, 6). To investigate whether or not the activation of TrkA receptors is altered by the proteasome inhibitor MG132, we treated PC12 cells with NGF and/or MG132 and examined the degree of TrkA receptor phosphorylation by Western blot analysis. TrkA receptor was phosphorylated in the presence of NGF alone and NGF and MG132 at the same time (Fig. 2B). The phosphorylation of TrkA receptor was increased at 15 min and 30 min of NGF treatment, decreased after 1 h of treatment, and returned to pre-treatment levels thereafter. When PC12 cells were treated with NGF and MG132, TrkA receptor phosphorylation was sustained for 3 h and decreased thereafter. Taken together, these results suggest that prolonged phosphorylation of TrkA receptors by proteasome inhibition enhanced NGF-induced signaling pathways.

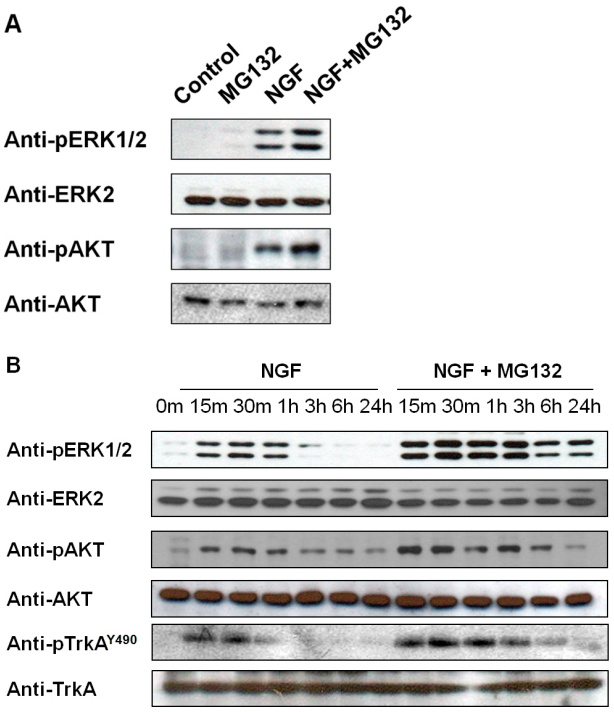

Fig. 2. The proteasome inhibitor MG132 stimulates NGF-induced activation of ERK, AKT and TrkA receptors. PC12 cells were treated with 50 ng/ml of NGF in the absence or presence of 0.5 μM MG 132 for 30 min (A) or for the indicated times (B). Cell lysates were separated by SDS-PAGE on 12% gel and immunoblotted with anti-phospho-ERK1/2$^{Thr202/Tyr204}$, anti-ERK2, anti-phospho-AKT^{T308}, anti-AKT antibodies, anti-phospho-TrkA^{Y490}, and anti-TrkA antibodies.

Proteasome inhibition stabilizes TrkA receptors

To examine whether or not the prolonged phosphorylation of TrkA receptors by proteasome inhibitor is caused by stabilization of TrkA receptors, PC12 cells were treated with cycloheximide to inhibit protein biosynthesis for 1 h and then treated with NGF alone or NGF and MG132 at the same time in the presence of cycloheximide. As shown in Fig. 3A, TrkA receptors were degraded after 1 h of NGF treatment. When PC12 cells were treated with NGF and MG132 together, TrkA receptor expression was strongly maintained for 1 h and then decreased after 3 h. These results show that proteasome inhibition increased the stability of TrkA receptors.

Next, we examined whether or not proteasome inhibition could alter the internalization of TrkA receptors. NGF-medi-

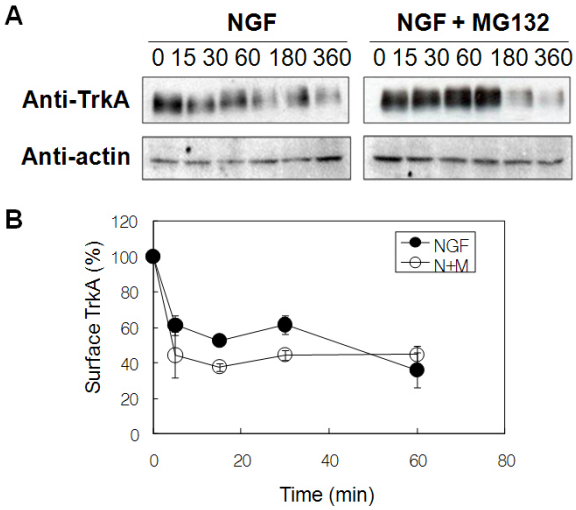

Fig. 3. Proteasome inhibition stabilizes TrkA receptors and stimulates internalization of surface TrkA receptors by NGF. (A) PC12 cells were treated with 100 μg/ml of cycloheximide for 1 h and then treated with 50 ng/ml of NGF in the absence or presence of 0.5 μM MG132 for the indicated times. Cell lysates were separated by 12% SDS-PAGE and immunoblotted with anti-TrkA and anti-actin antibodies. (B) PC12 cells were treated with 50 ng/ml for 5, 15, 30, and 60 min. After incubation, surface TrkA receptors were stained with anti-TrkA extracellular domain antibodies and exposed to FITC-labeled secondary antibodies. Surface expression of TrkA receptors was detected using a FACS flow cytometer (Beckton & Dickinson), and the fluorescence data were analyzed using Cellquest Software. Mean fluorescence intensity ± S.D determined from three independent experiments is shown (P value < 0.05).

ated signaling is known to induce rapid internalization of TrkA receptors via clathrin-coated pits and trafficking of these receptors to the signaling endosome (6, 20-22). Thus, sustained expression of TrkA receptors might be caused by inhibition of the internalization of these receptors.

Our results show that TrkA receptors were internalized upon treatment with NGF and that this internalization was enhanced in the presence of MG132 (Fig. 3B). NGF treatment for 5 min led to a decrease in the surface expression of TrkA receptors to about 61%, whereas co-treatment with NGF and MG132 decreased the expression to 44%. Internalization of surface TrkA receptors upon co-treatment with NGF and MG132 occurred more rapidly compared to treatment with NGF alone. These results suggest that TrkA receptor signaling by NGF was regulated by the proteasome, which altered the stability of TrkA receptors.

K63 ubiquitin is necessary for the stability of TrkA

Proteasome inhibition resulted in the rapid internalization of surface TrkA receptor as well as stabilization of intracellular TrkA receptors. NGF is known to stimulate polyubiquitination of TrkA receptors, which is essential for TrkA internalization

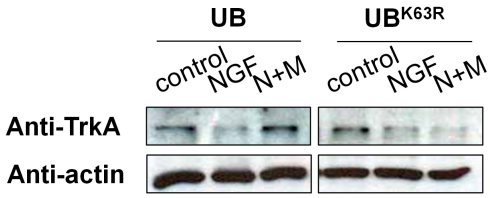

Fig. 4. The ubiquitin mutant UB^{K63R} is necessary for the stability of TrkA. PC12 cells were transiently transfected with a His$_6$-UB and His$_6$-UB^{K63R} expression construct using Lipotectamine^{TM} 2000 according to the manufacturer's instructions. After 24 h, PC12 cells were treated with 100 μg/ml of cycloheximide for 1 h and then treated with 50 ng/ml of NGF in the absence or presence of 0.5 μM MG132 for 1 h. Cell lysates were separated by 12% SDS-PAGE and immunoblotted with anti-TrkA and anti-actin antibodies.

and signaling. Therefore, we examined whether or not TrkA receptor stabilization in the presence of the proteasome inhibitor MG132 is dependent on ubiquitination. In cells overexpressing UB, NGF treatment led to the degradation of TrkA receptors, and the extent of degradation was decreased in the presence of MG132, consistent with that shown in Fig. 3A (Fig. 4). However, UB^{K63R} overexpression resulted in the degradation of TrkA receptors even in the presence of both NGF and MG132. These results indicate that stabilization of TrkA receptors by MG132 was dependent on the UB^{K63R} chain.

It is known that tyrosine kinase receptors are either recycled back to the cell surface or degraded by lysosomes. However, some papers found that lysosomal degradation of tyrosine kinase receptors is preceded by proteasome-dependent deubiquitination (4, 23). Wooten and coworkers suggested that proteasomal deubiquitinating enzymes trim K63-ubiquitin chains from the TrkA receptor prior to its delivery to lysosomes for degradation. Our results also suggest that the stability of the TrkA receptor is regulated by the proteasome. Taken together, the ubiquitin-proteasome system plays an important role in regulating the stability of TrkA receptors prior to lysosomal degradation.

In conclusion, the proteasome inhibitor MG132 facilitated NGF-induced neurite outgrowth and potentiated the activation of the ERK and AKT pathways as well as the activation of TrkA receptors. NGF-treated TrkA receptors were stabilized in the presence of a proteasome inhibitor, and the UB^{K63R} chain was proven essential for this stability. These results indicate that the ubiquitin-proteasome system regulates neurite formation by influencing the stability of TrkA receptors.

MATERIALS AND METHODS

Reagents

MG132 was obtained from Calbiochem (La Jolla, CA, USA). Anti-TrkA antibodies were purchased from Upstate Biotechnology (MA, USA). Anti-TrkA serum, which recognizes the extracellular domain of TrkA, was kindly provided by Louis

Reichardt, University of California, San Francisco (24). The ubiquitin and ubiquitin mutant construct (UB, UB^{K48R}, and UB^{K63R}) were obtained from Michael Rape, University of California, Berkeley (25). Antibodies against phospho-$TrkA^{Y490}$, TrkA, phospho-AKT^{Ser473}, AKT, phospho-$ERK1/2^{Thr202/Tyr204}$, and ERK1/2 were obtained from Cell Signaling (Berkeley, MA, USA).

Neurite outgrowth

To measure neurite outgrowth, PC12 cells were grown in high-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% horse serum and 5% fetal bovine serum and maintained at 37°C in a incubator with a humidified atmosphere of 5% CO_2. Cells were seeded onto 12-well poly-L-lysine-coated plates at a density of 1×10^5 cells per plate 1 day before treatment. The next day, cells were treated with 50 ng/ml of NGF for 24 h in the presence or absence of 0.5-20 μM MG132. Images of the cells were obtained (for ε1,000 cells), and the extent of neurite outgrowth was assessed by counting the number of cells with neurites. Cells with neurites were defined as cells possessing at least one neurite more than 1 cell body diameter in length.

Western blot analysis

To detect phosphorylation of ERK, AKT, and TrkA receptor proteins, the cells were washed three times with cold phosphate-buffered saline (PBS), lysed with lysis buffer A (50 mM Tris-Cl, pH 7.4; 150 mM NaCl; 2 mM EDTA; 0.5% NP-40, protease inhibitor cocktail [Roche], 1 mM sodium orthovanadate), and centrifuged at 12,000 rpm for 15 min. Supernatants were analyzed for protein content using a Micro BCA protein assay kit (Pierce, Rockford, IL, USA). Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes. The membranes were probed with antibodies against phospho-$TrkA^{Y490}$, TrkA, phospho-AKT^{Ser473}, AKT, phospho-$ERK1/2^{Thr202/Tyr204}$, and ERK1/2.

Flow cytometric analysis

Flow cytometric analysis was performed to analyze the extent of internalization of TrkA receptors. Cells were trypsinized, collected by centrifugation, and incubated with 50 ng/ml of NGF in the presence or absence of 0.5 μM MG132 at 37°C for 5, 15, 30, and 60 min. All subsequent labeling steps were performed on ice. Cells were washed twice in fluorescence-activated cell sorting (FACS) buffer (0.5% bovine serum albumin [BSA], 0.05% sodium azide, 1 mM $MgCl_2$, and 1 mM $CaCl_2$ in PBS) and incubated with antibodies capable of recognizing the extracellular domain of TrkA in FACS buffer for 45 min (24). The cells were washed with FACS buffer, probed with secondary antibody labeled with anti-rabbit-fluorescein isothiocyanate (FITC) for 30 min, and then washed twice in FACS buffer. The fluorescence intensity of the labeled cells was analyzed using a FACS flow cytometer (Beckton & Dickinson). The degree of internalization was calculated using CellQuest Software.

Acknowledgements

We gratefully acknowledge the support of a National Research Foundation of Korea (NRF) grant funded by the Ministry of Education, Science and Technology (MEST) (Systems Biology Research Grant: 20090065565) and the Korea Institute of Science and Technology (A studies on Metabolomics: 2E21520).

REFERENCES

1. Alfa, R. W., Tuszynski, M. H. and Blesch, A. (2009) A novel inducible tyrosine kinase receptor to regulate signal transduction and neurite outgrowth. *J. Neurosci. Res.* **87**, 2624-2631.
2. Huang, E. and Reichardt, L. (2001) Neurotrophins: roles in neuronal development and function. *Annu. Rev. Neurosci.* **24**, 677-736.
3. Huang, E. and Reichardt, L. (2003) Trk receptors: roles in neuronal signal transduction. *Annu. Rev. Biochem.* **72**, 609-642.
4. Geetha, T. and Wooten, M. W. (2008) TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent. *Traffic* **9**, 1146-1156.
5. Moises, T., Wüller, S., Saxena, S., Senderek, J., Weis, J. and Krüttgen, A. (2009) Proteasomal inhibition alters the trafficking of the neurotrophin receptor TrkA. *Biochem. Biophys. Res. Commun.* **387**, 360-364.
6. Chao, M. V. and Hempstead, B. L. (1995) p75 and Trk: a two-receptor system. *Trends Neurosci.* **18**, 321-326.
7. Vaudry, D., Stork, P. J., Lazarovici, P. and Eiden, L. E. (2002) Signaling pathways for PC12 cell differentiation: Making the right connections. *Science* **296**, 1648-1649.
8. Hershko, A. and Ciechanover, A. (1998) The ubiquitin system. *Annu. Rev. Biochem.* **67**, 425-430.
9. Verma, R., Oania, R., Graumann, J. and Deshaies, R. J. (2004) Multiubiquitin chain receptors define a layer of substrate selectivity in the ubiquitin-proteasome system. *Cell* **118**, 99-110.
10. Pickart, C. M. (2001) Ubiquitin enters the new millennium. *Mol. Cell* **8**, 499-504.
11. Rubinsztein, D. C. (2006) The roles of intracellular protein-degradation pathways in neurodegeneration. *Nature* **443**, 780-786.
12. Geetha, T., Jiang, J. and Wooten, M. W. (2005) Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling. *Mol. Cell* **20**, 301-312.
13. Geetha, T., Seibenhener, M. L., Chen, L., Madura, K. and Wooten, M. W. (2008) p62 serves as a shuttling factor for TrkA interaction with the proteasome. *Biochem. Biophys. Res. Commun.* **374**, 33-37.
14. Jadhav, T., Geetha, T., Jiang, J. and Wooten, M. W. (2008) Identification of a consensus site for TRAF6/p62 polyubiquitination. *Biochem. Biophys. Res. Commun.* **371**, 521-524.
15. Obin, M., Mesco, E., Gong, X., Haas, A. L., Joseph, J. and Taylor, A. (1999) Neurite outgrowth in PC12 cells. *J. Biol. Chem.* **274**, 11789-11795.

16. Inoue, M., Zhai, H., Sahazaki, H., Furuyama, H., Fukuyama, Y. and Hirima, M. (2004). TMC-95A, a reversible proteasome inhibitor, induces neurite outgrowth in PC12 cells. *Bioorg. Med. Chem. Lett.* **14**, 663-665.
17. Momose, I., Sekizawa, R., Iinuma, H. and Takeuchi, T. (2002). Inhibition of proteasome activity by tyropeptin A in PC12 cells. *Biosci. Biotechnol. Biochem.* **66**, 2256-2258.
18. Song, E. J., Hong, H. M. and Yoo, Y. S. (2009) Proteasome inhibition induces neurite outgrowth through posttranslational modification of TrkA receptor. *Int. J. Biochem. Cell. Biol.* **41**, 539-545.
19. Pasquini, L. A., Paez, P. M., Besio Moreno, M. A., Pasquini, J. M. and Soto, E. F. (2003) Inhibition of the protein by lactacystin enhances oligodendroglial cell differentiation. *J. Neurosci.* **23**, 4635-4644.
20. Bronfman, F. C., Tcherpakov, M., Jovin, T. M. and Fainzilber, M. (2003) Ligand-induced internalization of the p75 neurotrophin receptor: a slow route to the signaling endosome. *J. Neurosci.* **23**, 3209-3220.
21. Kuruvilla, R., Zweifel, L. S., Glebova, N. O., Lonze, B. E., Valdex, G., Ye, H. and Ginty, D. D. (2004) A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling. *Cell* **118**, 243-255.
22. Makkerh, J. P., Ceni, C., Auld, D. S., Vaillancourt, F., Dorval, G. and Baker, P. A. (2005) p75 neurotrophin receptor reduces ligand-induced Trk receptor ubiquitination and delays Trk receptor internalization and degradation. *EMBO Rep.* **6**, 936-941.
23. Alwan, H. A., van Zoelen, E. J. and van Leeuwen, J. E. (2003) Ligand-induced lysosomal epidermal growth factor receptor (EGFR) degradation is preceded by proteasome-dependent EGFR de-ubiquitination. *J. Biol. Chem.* **278**, 35781-35790.
24. Chen, Z. Y., Ieraci, A., Tanowitz, M. and Lee, F. S. (2005) A novel endocytic recycling signal distinguishes biological responses of Trk neurotrophin receptors. *Mol. Biol. Cell* **6**, 5761-5772.
25. Jin, L., Williamson, A., Banerjee, S., Philipp, I. and Rape, M. (2008) Mechanism of ubiquitin-chain formation by the human anaphase-promoting complex. *Cell* **133**, 653-665.