

Section: Cellular/molecular neuroscience *Senior Editor: Dr. Marie T. Filbin*

An analogue of dipeptide-like structure of FK506 increases GDNF expression through CREB activated by Hsp90/Akt signaling pathway.

Abbreviated title: A dipeptide increases GDNF expression by Akt/CREB

Xiaobo Cen,^{1,2} Atsumi Nitta,^{1,8} Shin Ohya,¹ Yinglan Zhao,¹ Naoya Ozawa,¹ Akihiro Mouri,¹ Daisuke Ibi,^{1,7} Li Wang,² Makiko Suzuki,³ Kuniaki Saito,³ Yasutomo Ito,⁴ Tetsuya Kawagoe,⁵ Yukihiko Noda,^{1,6} Yoshihisa Ito,^{1,7} Shoei Furukawa,⁸ Toshitaka Nabeshima^{1*}

¹ Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan, ²National Chengdu Center for Safety Evaluation of Traditional Chinese Medicine, West China Hospital, Sichuan University, Chengdu 610041, China, ³Department of Laboratory Medicine, Gifu University School of Medicine, Gifu 500-8705, Japan, ⁴ Equipment Center for Research and Education, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan, ⁵ Department of Research and Development, Initium Incorporation, Tokyo, 107-0062, Japan, ⁶ Division of Clinical Science in Clinical Pharmacy Practice, Management and Research, Faculty of Pharmacy, Meijo University, Nagoya 468-8503, Japan, ⁷ Department of Pharmacology, College of Pharmacy, Nihon University, 7-7-1 Narashinodai, Funabashi-shi, Chiba 274-8555, Japan, ⁸Laboratory of Molecular Biology, Gifu Pharmaceutical University, Gifu 502-8585, Japan

* Address correspondence to Toshitaka Nabeshima:

Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Tsuruma-Cho, Showa-ku, Nagoya 466-8560, Japan.

E-mail: tnabeshi@med.nagoya-u.ac.jp

Tel: 81-52-744-2674 Fax: 81-52-744-2979

§ A.N. and X.C. contributed equally to this work.

Number of figures : 7

Key words: GDNF, Dipeptide, FK506, Hsp90, Hsc70, CREB

Acknowledgments: This study was supported in part by a Grant-in-Aid for Science Research and Special Coordination Funds for Promoting Science and Technology, Target-Oriented Brain Science Research Program KAKENHI and 21st Century Center of Excellence Program "Integrated Molecular Medicine

for Neuronal and Neoplastic Disorders” from the Ministry of Education, Culture, Sports, Science and Technology of Japan; by a Grant-in-Aid for Health Science Research on Regulatory Science of Pharmaceuticals and Medical Devices, and Dementia and Fracture from the Ministry of Health, Labor and Welfare of Japan; by a Smoking Research Foundation Grant for Biomedical Research.

Abstract

Glial cell line-derived neurotrophic factor (GDNF) is an important neurotrophic factor that has therapeutic implications for neurodegenerative disorders. We previously showed that Leu-Ile, an analogue of dipeptide-like structure of FK506 (tacrolimus), induces GDNF expression both *in vivo* and *in vitro*. In this investigation we sought to clarify the cellular mechanisms underlying GDNF-inducing effect of this dipeptide. Leu-Ile transport was investigated using FITC-Leu-Ile in cultured neurons, and the results showed the transmembrane mobility of this dipeptide. By liquid chromatography-mass spectrometry and quartz crystal microbalance assay, we identified heat-shock cognate protein 70 (Hsc70) as a protein binding specifically to Leu-Ile, and molecular modeling showed that ATPase domain is the predicted binding site. Leu-Ile stimulated Akt phosphorylation, which was attenuated significantly by Hsp90 inhibitor geldanamycin (GA). Moreover, enhanced interaction between phosphorylated Akt and Hsp90 was detected by immunoprecipitation. Leu-Ile elicited an increase in cAMP response element binding protein (CREB) phosphorylation, which was inhibited by GA, indicating that CREB is a downstream target of Hsp90/Akt signaling. Leu-Ile elevated the levels of GDNF mRNA and protein expression, whereas inhibition of CREB blocked such effects. Leu-Ile promoted the binding activity of phosphorylated CREB with cAMP response element. These findings show that CREB plays a key role in transcriptional regulation of GDNF expression induced by Leu-Ile. In conclusion, Leu-Ile activates Hsp90/Akt/CREB signaling, which contributes to the upregulation of GDNF expression. It may represent a novel lead-compound for treatment of dopaminergic neuron or

motoneuron diseases.

Introduction

GDNF is an important neurotrophic factor that regulates the development, migration and survival of neurons, and has therapeutic implications for neurodegenerative disorders (Airaksinen and Saarna, 2002). We have demonstrated that Leu-Ile, an analogue of dipeptide-like structure of FK506, shows non-immunosuppressive activity and promotes neuronal survival through induction of GDNF both in *vivo* and in *vitro* studies (Nitta et al., 2004), but the mechanism is unclear.

Studies have indicated the complex regulatory mechanisms of GDNF expression. It can be induced by diverse extracellular stimuli (Verity et al., 1999; Castro et al., 2005), and multiple transcription factors binding sites have been identified in the promoter sequence of GDNF gene (Woodbury et al., 1998), such as CREB binding element (Matsushita et al., 1997; Baecker et al., 1999). Earlier studies showed that CREB activation is associated with GDNF expression (Lenhard et al., 2002; Young et al., 1999), implying CREB may participate in regulating GDNF expression as a transcriptional factor. Phosphorylation of CREB at Ser¹³³ within the kinase-inducible domain is critical for its function as a stimulus-dependent transcriptional activator, and multiple kinases have been implicated as its activator in neurons, including protein kinase C (PKC) (Roberson et al., 1999), calmodulin kinase (CaMK) II (Lee et al., 2004), extracellular signal-regulated kinase 1/2 (ERK1/2) (Schinelli et al., 2001) and serine/threonine kinase Akt (Brunet et al., 2001). Different extracellular stimuli may activate distinct signalings, which contribute to CREB phosphorylation and cellular responses. In particular, CREB is

considered to be a regulatory target for Akt, and Akt can promote cell survival by stimulating the expression of cellular genes via CREB-dependent pathway (Pugazhenthii et al., 2000; Du and Montminy, 1998).

Although phosphoinositide 3-kinase (PI3-k) is an important activator for Akt, increasing evidence has indicated that Akt can be regulated in PI3-k independent manners in neurons, such as ERK1/2 and CaMK cascades (Brami-Cherrier et al., 2002; Yano et al., 1998). Moreover, Akt is a well-characterized Hsp90-dependent kinase (Basso et al., 2002; Xu et al., 2003), and chaperones Hsp90 and Hsc70 have been demonstrated to play a role in Akt regulation through distinct mechanisms. For instance, Hsp90-Akt interaction increases Akt activity by protecting it from dephosphorylation by protein phosphatase 2A (PP-2A) (Sato et al., 2000; Yun and Matts, 2005); the binding of client molecule to Hsc70, maintains Akt phosphorylation and downstream cascade activation by inhibiting its proteasomal degradation based on Hsc70/Hsp90 machinery (Doong et al., 2003). Therefore, modulation of Hsp90 resulted from a variety of physiological or pharmacological factors may alter Akt signaling, contributing to the regulation of cellular function and response (Pratt and Toft, 2003). It is known that Hsp90 ATPase activity is highly regulated by the binding of co-chaperone or client proteins, such as FK506 binding protein (FKBP) and Hsc70 (McLaughlin et al., 2002).

We defined a signaling cascade that Leu-Ile promotes CREB phosphorylation via Hsp90/Akt signaling, which plays an important role in the transcriptional regulation of GDNF gene. Moreover, Hsc70 is likely to cooperate with Hsp90 as a co-chaperone to modulate Akt activity.

Materials and Methods

Materials

Leucine-Isoleucine (Leu-Ile), Proline-Leucine (Pro-Leu) and Isoleucine-Proline (Ile-Pro) were synthesized by Kokusan Chemical Co. Ltd. (Tokyo, Japan). Leu-Ile labeled with fluorescein isothiocyanate (FITC) was prepared by Thermo Electron Corporation (Ulm, Germany). LY294002 and geldanamycin were purchased from Sigma. FK506 was gifted from Fujisawa Pharmaceutical Co., Ltd. (Osaka, Japan). Anti-Hsp70, anti-Hsp90, Anti-pAkt (Thr308), anti-Akt, anti-pCREB (Ser133), anti-CREB, anti-ERK1/2, anti-pERK1/2 (Thr202/Tyr204), anti-pCaMKII α,β , anti-pP38MAPK (Thr180/Tyr182), anti-pSARK/JNK (Thr183/Tyr185) and anti-microtubule-associated protein-2 (MAP-2) antibodies were purchased from Cell Signaling Technology Inc. (Beverly, MA). Anti-PKC, anti-c-Src and anti-actin antibodies were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA). Anti-Hsc70 antibody and recombinant Hsc70 were from Stressgen Biotechnologies Corp. (Victoria, Canada). Anti-GDNF antibody was from R&D Systems, Inc. (Minneapolis, MN). Anti-glial fibrillary acidic protein (GFAP) antibody was from Chemicon International Inc. (Temecula, CA).

Primary hippocampal neuron cultures

Primary hippocampal neuronal cultures were prepared from day 17 embryos of rats. Briefly, hippocampi were dissected and digested with 0.25% trypsin at 37°C for 30 min. Hippocampal cells were plated in poly-ornithine-coated plates in DMEM/F12 medium containing 10% fetal bovine serum.

The medium was replaced with DMEM/F12 medium containing 1% N2 supplement (Invitrogen) 24 h later. MAP-2 positive cells accounted for over 95% of the total in cultures.

Transmembrane transport of Leu-Ile

Cultured neurons were incubated with FITC-Leu-Ile or FITC at various concentrations for the indicated periods at 37°C. Cells were washed three times with phosphate-buffer saline (PBS) and collected in 300 μ l PBS using a rubber scratcher. After samples were sonicated and centrifuged at 10,000 g for 30 min at 4°C, the supernants (200 μ l) were collected for fluorescent density measurement at an excitation wavelength of 485 nm and an emission wavelength of 518 nm by Fluoroskan Ascent (Thermo Labsystems). Intracellular amount of FITC-Leu-Ile or FITC was calculated according to the standard curve.

Identification of binding protein for Leu-Ile in mouse brain

Brains were removed from 7-week aged male ICR mice (NIPPON SLC, Shizuoka, Japan), and homogenized in RIPA buffer (20mM Tris-HCl, pH7.4, 0.25M NaCl, 5mM EDTA, 1% Triton X-100, 1mM PMSF and 1 μ g/ml each of leupeptin, aprotinin and pepstatin A). After centrifugation at 10,000 g for 60 min at 4°C, the supernants were collected and reacted with FITC-Leu-Ile for 60 min at 37°C. Samples from the reaction complex were subjected to gel electrophoresis, and the gels were analyzed directly by FluorImager595 (Molecular Dynamics, CA).

Preparation of Sepharose Affigel-10 (Amersham Biosciences) coupled with Leu-Ile was performed according to manufacturer's instructions. The column with Affigel-10 coupled with Leu-Ile was loaded

with brain homogenates and equilibrated with 10 mM Tris-HCl buffer (pH 7.4) at 4°C overnight. The column was washed with 0.1M PBS (pH 7.4) extensively, followed by elution with 0.17 M glycine-HCl buffer (pH 3.0). The eluants were separated by gel electrophoresis. To identify the binding protein by liquid chromatography-mass spectrometry (LC/MS), the specific protein band was excised from the gel after silver staining and digested in-gel with trypsin. The digested peptide fragments were directly sprayed into a Q-TOF hybrid mass spectrometer equipped with an electrospray source (Q-ToF 2; Micromass, Manchester, UK). The analysis was conducted using Mascot Search (Matrix Science, MA) with reference to the protein sequence database at the National Center for Biotechnology Information (NCBI nr: <http://www.ncbi.nlm.nih.gov/Entrez>). To validate the binding protein, samples from the reaction complex of brain homogenates with Leu-Ile were subjected to gel electrophoresis, and then immunoblotted with anti-Hsc70 antibody. In addition, recombinant Hsc70 protein was reacted with Leu-Ile at 37°C for 60 min, and the reaction complex was separated by gel electrophoresis, followed by Coomassie brilliant blue (CBB) staining.

Leu-Ile- or FK506-conjugated Affigel-10 was incubated with brain homogenates at 4°C overnight. The columns were washed extensively, followed by elution with 0.17 M glycine-HCl buffer (pH 3.0). The eluants were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotted with anti-Hsc70 antibody.

Interaction between Leu-Ile and Hsc70

The binding of Leu-Ile or FK506 for Hsc70 was examined by quartz crystal microbalance (QCM), which is useful for studying mass-measuring and molecular interaction in aqueous solution (Motomiya et al., 2003). Briefly, 100 μ l of each dipeptide (10 μ g/ml in PBS) or FK506 [10 μ g/ml in chloroform or chloroform/ethanol (1:1)] was immobilized into a QCM plate for 1 h at room temperature, and then removed. After washing three times with PBS, the plates were soaked in PBS at 25°C. Hsc70 or heat-denatured Hsc70 was applied to the equilibrated solution, and the change in resonance frequency was recorded by an AFFINIX Q User Analysis (AQUA) (Initium, Tokyo, Japan). The binding affinity was indicated by frequency changes of QCM, and disassociation constant (Kd) was calculated with the AQUA software.

Modeling of functional domains of Hsc70 and the predicted binding site for Leu-Ile

To further understand the interaction between Hsc70 and Leu-Ile, molecular models of the ATPase domain, substrate-binding domain and C'-terminal domain of Hsc70 were generated using the three-dimensional structural data from Protein Data Bank (<http://pdbeta.rcsb.org/pdb/Welcome.do>) in Research Collaboratory for Structural Bioinformatics (Chou et al., 2003; Flaherty et al., 1990; Morshauser et al., 1999). Interaction of each domain with Leu-Ile was analyzed using Molecular Operating Environment (MOE) software (Chemical Computing Group, Montreal, Canada). All calculations used an MMFF94x force field and a cutoff distance of 9.5Å for non-binding interactions. Alpha Site Finder of the MOE program was used for docking stimulation.

Western blotting

Cultured neurons were lysed in RIPA buffer (20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM sodium orthovanadate, 2 mM EDTA, 50 mM NaF, 1% Nonidet P-40, 1 mM PMSF and 2 µg/ml each of aprotinin, leupeptin and pepstatin). Lysates were sonicated and centrifuged at 9,000 g for 15 min. Protein concentrations were determined by Protein Assay Reagents (Bio-Rad). The samples were subjected to SDS-PAGE, and then electrotransferred to polyvinylidene difluoride membranes. The membranes were immunoblotted and developed using chemiluminescence detection reagents. To calculate the amount of phosphorylated form versus total protein, the same membranes were stripped, incubated with the primary antibody for total protein and examined as described above. The relative amount of immunoreactive protein in each band was assayed by scanning densitometric analysis using the ATTO Densito Graph 4.1 System (ATTO, Tokyo, Japan).

Immunoprecipitation

After centrifugation the supernatants of cell lysates were normalized for protein concentration. A fraction (500 µg) of the total protein was incubated by gently rocking at 4°C overnight in the presence of anti-Hsp90 antibody. The immunocomplexes were captured by protein A sepharose (Amersham Biosciences), washed out by lysis buffer, and then subjected to SDS-PAGE and immunoblotting.

Immunostaining

Cultured neurons attached to glass coverslips were fixed with 4% paraformaldehyde in PBS for 20 min, and then blocked in 3% normal serum and 0.1% Triton X-100 for 1 h. The coverslips were incubated with the primary antibodies at 4°C overnight, washed with PBS, and then incubated with appropriate

secondary antibodies (Molecular Probes, OR) for 2 h. After being washed and mounted, stained neurons were observed under a fluorescent microscope (Axioscop 2 plus; Zeiss, NY).

Real-time RT-PCR

Level of GDNF mRNA was determined by real-time RT-PCR using an iCycler System (Bio-Rad). Briefly, isolation of total RNA was performed using RNeasy[®] Mini Kit (QIAGEN). For reverse transcription, 1 µg RNA was converted into a cDNA by a standard 20 µl reverse transcriptase reaction using oligo (dT) primers (Invitrogen) and Superscript II RT (Life Technologies). Total cDNA (1 µl) was amplified in a 25 µl reaction mixture using 0.1 µM each of forward and reverse primers and Platinum[®] Quantitative PCR SuperMix-UDG (Invitrogen). Ribosomal mRNA was used and determined as control for RNA integrity with TaqMan Ribosomal RNA Control Reagents (Applied Biosystems). The Primer and dye probes were designed by Nippon Gene Co. Ltd. (Tokyo, Japan) using Primer Express software. GDNF forward was 5'-AGCTGCCAGCCCAGAGAATT-3' (bp 288-307), with reverse being 5'-GCACCCCGATTTTGC-3' (bp 354-370) and dye probe being 5'-CAGAGGGAAAGGTCGCAGAGGCC-3' (bp 309-331).

Antisense oligonucleotide of CREB

CREB expression was inhibited by an oligodeoxynucleotide (ODN) targeting the initiation codon of CREB mRNA as previously reported (Saini et al., 2004; Johnson et al., 2000). The phosphorothioate ODNs were synthesized by Nisshinbo Industries, Inc. (Tokyo, Japan). The sequence of antisense ODN was 5'-GCTCCAGAGTCCATGGTCAT-3', with a sense ODN with the sequence

5'-ATGACCATGGACTCTGGAGC-3' as a control. Transfections were carried out using Lipofectamine reagent (Invitrogen), and oligonucleotide was added to culture medium at a final concentration of 4 μ M. The inhibition of CREB expression after transfection was assessed by Western blotting. To investigate the role of CREB in transcriptional regulation of GDNF expression, cultures were incubated with CREB ODN before Leu-Ile treatment.

pCREB-CRE binding activity

Cultured neurons were collected and nuclear extracts were prepared by using BDTM TransFactor Extraction Kits (BD Biosciences, USA) according to the manufacturer's protocol. The cAMP response element (CRE)-pCREB binding activity was determined using TransAMTM pCREB/CREB Transcription Factor Assay Kits (Active Motif North America, CA). Briefly, nuclear extract was applied to each well immobilized with oligonucleotide containing CRE 5'-TGACGTCA-3', and incubated for 3 h. After washing, the wells were incubated with anti-pCREB antibody, followed by HRP-conjugated secondary antibody. After development with tetramethylbenzidine, the absorbance was measured with a microplate reader at 450 nm with reference at 655 nm. The specificity of the binding of pCREB to CRE was confirmed by conducting competitive experiments with 20 pmol of wild-type oligonucleotide probe or mutant probe containing the consensus CRE.

Statistical analysis

All data were expressed as means \pm SEM. Statistical significance was determined by a one-way ANOVA, followed by the Student-Newman-Keuls test for multigroup comparisons. Differences were considered significant when $P < 0.05$.

Results

Transmembrane transport

As shown in Fig.1A, uptake of FITC-Leu-Ile by neurons was increased with the elevation of extracellular concentration. Time-course studies showed that uptake of FITC-Leu-Ile by neurons was a quick process and appeared to be saturated after incubation for 60 min (Fig. 1B). Although transmembrane transport of FITC was observed in dose- and time-course studies, its penetration amount was much lower than that of FITC-Leu-Ile (Fig.1A, 1B). Because specific inhibitor for neuronal peptide transporters is not available, competitive transport was investigated using high concentrations of Leu-Ile. Simultaneous incubation with various concentrations of Leu-Ile for 30 min significantly inhibited FITC-Leu-Ile transport in a concentration-dependent manner (Fig. 1C), but failed to inhibit FITC transport (Fig. 1D), suggesting that FITC-Leu-Ile and Leu-Ile are transported by the same pathway. Together, these results strongly imply that FITC-Leu-Ile transport is mainly due to the transmembrane activity of Leu-Ile rather than FITC and that the kinetics of FITC-Leu-Ile transport, at least in some degree, reflects that of Leu-Ile.

Identification of target protein for Leu-Ile in mouse brain

FITC-Leu-Ile was incubated with mouse brain homogenate, and the reaction complexes were subjected to electrophoresis. By fluorescent scanning, one specific fluorescent protein band with a molecular weight of ~70 kDa was detected, suggesting that this protein has the specific affinity for

Leu-Ile (Fig. 2A, arrow). To further identify the protein binding to Leu-Ile, brain homogenate was applied to Leu-Ile-conjugated Affigel-10, and the eluants were subjected to electrophoresis and detected by silver staining. We found a specific protein band of ~70 kDa with stronger density in the gel (Fig. 2B, arrow), which was similar to that detected by fluorescent scanning. It has been known that the family of heat shock protein 70 represents an important cellular mechanism in neuroprotection (Rubio et al., 2002; Zhang et al., 2004); moreover, Leu-Ile derives from FK506, which exerts neuroprotective action through Hsp70 (Gold et al., 2004). Therefore, the band of ~70 kDa was selected and processed to generate tryptic peptides, which was analyzed by direct nano-flow LC-MS. All of the digested peptide fragments could be assigned to Hsc70 with 100% homology by Mascot Search, and the matching score was 496 (Fig. 2C,2D). To confirm the mass spectrometric-based identification of the Leu-Ile-binding protein, reaction complexes were subjected to SDS-PAGE and immunoblotted with Hsc70 antibody. We detected Hsc70 (open arrow) as well as an Hsc70-Leu-Ile complex (closed arrow), which showed a slight retardation of electrophoretic mobility because of the increased molecular weight compared with that of Hsc70 (Fig. 2E). Moreover, Hsc70-Leu-Ile (closed arrow) and Hsc70 (open arrow) were also identified by CBB staining in the reaction complex of Leu-Ile with recombinant Hsc70 (Fig.2F). These results confirm that Hsc70 is a specific binding protein for Leu-Ile.

To study whether FK506 binds to Hsc70, Leu-Ile- or FK506 Affigel-10 were incubated with brain homogenate at 4°C overnight. The eluants were subjected to SDS-PAGE and probed with anti-Hsc70

antibody. Interestingly, Hsc70 was detected in the eluants from both Leu-Ile- and FK506 Affigel-10, suggesting that Hsc70 may bind to FK506 directly or indirectly (Fig.2G).

Interaction between Leu-Ile and Hsc70

QCM was applied to investigate more directly the interaction between Leu-Ile and Hsc70. The resonance frequency change ($-\Delta F$) of QCM responding to Leu-Ile decreased over time, indicating that Hsc70 had a significant affinity for Leu-Ile in a time-dependent manner (Fig. 3A). However, Pro-Leu and Ile-Pro had no effect on the resonance frequency change. The resonance frequency was decreased dose-dependently by Hsc70, showing the affinity of Hsc70 for Leu-Ile with K_d equal to 1.83×10^{-8} M (Fig. 3B). Leu-Ile had no influence on resonance frequency change when it was incubated with heat-denatured Hsc70 (data not shown). These results indicate the binding specificity of these two molecules and the requirement of the three-dimensional conformations of Hsc70 and Leu-Ile. However, resonance frequency change was not observed when Hsc70 was added to the QCM plate immobilized with FK506, suggesting that FK506 may not bind directly to Hsc70 (Fig. 3C).

ATPase domain of Hsc70 is the predicted binding site of Leu-Ile

To get further insights for Leu-Ile-Hsc70 interaction, three-dimensional structural models of ATPase domain, substrate-binding domain or C'-terminal domain of Hsc70 were produced, and the potential interaction of each domain with Leu-Ile was analyzed by MOE software. The ATPase domain showed the strongest interaction potential with Leu-Ile among these three domains. The predicted binding site of Leu-Ile in this domain appears to be a pocket structure, which is near to ADP docking site

(supplementary Fig.1A). It has been demonstrated that substrates, binding at this domain, affect ATP cycle and cause conformational regulation of Hsc70 and its co-chaperones (Hernández et al., 2002). Substrate-binding domain showed no stable docking site for Leu-Ile (supplementary Fig.1B).

Leu-Ile stimulates Akt phosphorylation

Since Hsc70/Hsp90 co-chaperons modulate the activities of a restricted number of tyrosine and serine/threonine kinases (Nollen and Morimoto, 2002), we first investigated whether c-Src, p38MAPK, SARK/JNK and Akt were affected by Leu-Ile. The phosphorylated Akt (pAkt) level was elevated by Leu-Ile (10 µg/ml) treatment for 20 or 30 min (Fig. 4A), whereas no changes in levels of other kinases were observed. In addition, expressions of Hsp90, Hsp70 and Hsc70 were not affected by Leu-Ile treatment for the indicated time points, showing that Leu-Ile is not a heat shock response inducer (Fig. 4B). Another two peptides, Pro-Leu and Ile-Pro, could not promote Akt phosphorylation (Fig. 4C).

Leu-Ile-induced Akt phosphorylation is mediated by Hsp90

As shown in Fig.5A, Akt phosphorylation was stimulated after Leu-Ile treatment for 20 or 30 min [$F_{(5,18)}=11.30$, $p<0.01$ and $p<0.01$ respectively, compared with the control (0 min)]. Since Hsp90 is a modulator for Akt, we thus investigated whether Akt activation by this dipeptide was mediated through Hsp90. The cultures were exposed to an Hsp90 inhibitor GA (10 µM) for 3 h, followed by Leu-Ile stimulation for 30 min. We found that the increase in pAkt level induced by Leu-Ile was obviously abolished by GA pre-treatment ($p<0.01$, compared with Leu-Ile treatment for 30 min). GA did not cause toxicity to neurons in our cultures (data not shown). To evaluate the involvement of PI3-k, an

upstream activator for Akt, the cultures were stimulated by Leu-Ile after pre-treatment with a PI3-k inhibitor LY294002 (15 μ M) for 2 h. Although pAkt level induced by Leu-Ile was inhibited by LY294002 in some degree, it was still much higher than that of LY294002 alone ($F_{(5,18)}=11.05$, $p<0.01$) (Fig.5B), suggesting that Leu-Ile-activated Akt may not be mediated by PI3-k. Furthermore, immunoblotting of immunoprecipitates with anti-Hsp90 antibody was performed using anti-Akt or anti-pAkt antibody. The level of immunoprecipitated pAkt was elevated significantly by Leu-Ile stimulation for 20 or 30 min [$F_{(3,12)}=7.75$, $p<0.01$ and $p<0.01$ respectively, versus control (0 min)] (Fig. 5C, top panel), indicating the enhanced interaction between Hsp90 and pAkt. Although immunoprecipitated Akt did not differ significantly, the increase tendency was obvious (Fig.5C, middle panel). Direct interaction between Hsc70 and Akt was not observed in our immunoprecipitation assays (data not shown). Taken together, Leu-Ile is considered to activate Akt in Hsp90-dependent manner.

CREB is a downstream target of Hsp90/Akt signaling activated by Leu-Ile

CREB was chosen to study intensively, since it is a regulatory target of Akt and closely associated with GDNF expression. The amount of phosphorylated CREB (pCREB) at Ser¹³³ was increased after Leu-Ile stimulation for 20 or 30 min (Fig.6A), whereas both Pro-Leu and Ile-Pro could not promote CREB phosphorylation (Fig.6B). Moreover, increased pCREB immunoreactivity and nucleus translocation induced by Leu-Ile were observed in MAP2-positive cells (Fig. 6C, middle panel), which is thought to be an early step for gene transcriptional regulation of CREB. To study the possibility of Leu-Ile acting on glial cells, both GFAP and pCREB were stained in spite that the culture contained

few glia cells. We found that enhanced pCREB immunoreactivity and nucleus translocation induced by Leu-Ile were not located in GFAP-positive cells, which showed lower pCREB immunoreactivity (Fig. 6D). To assess the involvement of Hsp90/Akt signaling in CREB phosphorylation induced by Leu-Ile, we investigated the change of pCREB after inhibition of this pathway. The cultures were stimulated with Leu-Ile alone for 10, 20, or 30 min, or pre-treated with GA (10 μ M) for 3 h, followed by Leu-Ile treatment for 30 min. We found that the pCREB level was elevated after Leu-Ile exposure for 20 or 30 min [$F_{(5,18)}=11.32$, $p<0.01$ and $p<0.01$ respectively, compared with control (0 min)], whereas the increase was inhibited by GA pre-treatment ($p<0.01$ versus Leu-Ile for 30 min) (Fig.6E). Double-staining supported these results, showing the loss of pCREB immunoreactivity and nucleus translocation in GA-treated neurons (Fig.6C, bottom panel). The increase in pCREB induced by Leu-Ile could not be inhibited by LY294002, and showed a higher level compared with LY294002 group ($F_{(5,18)}=10.36$, $p<0.01$) (Fig. 6F). Since a wide range of neuromodulators can converge on CREB via various cascades in neurons, we examined pERK1/2, PKC and pCaMKII α/β . However, no changes of these kinases were observed responding to Leu-Ile (Fig. 6G). Collectively, these results show that CREB is a downstream target of Hsp90/Akt signaling activated by Leu-Ile.

Leu-Ile increases GDNF expression in CREB-dependent manner

After the cultures were exposed to 10 μ g/ml Leu-Ile, Pro-Leu or Ile-Pro for 24 h respectively, the levels of GDNF expression were measured. We found that Leu-Ile, but not Pro-Leu and Ile-Pro, significantly promoted GDNF production (Fig.7A). Moreover, the levels of GDNF mRNA were