changes in ATPase domain (Nollen et al., 2001). Therefore, the interaction between Leu-Ile and Hsc70 may result in conformational and functional regulation of Hsc70.

Hsc70/Hsp90 chaperones are specially considered to be an integrated co-chaperone machinery. They often work together as essential components of a process that alters the conformations of a certain number of signaling transducers to states that respond in signal transduction, such as glucocorticoid receptors, Akt and Src kinases (Pearl and Prodromou, 2001; Rajapandi et al., 2000). Moreover, activities of Hsc70/Hsp90 machinery are affected by a wide range of cofactor proteins that interact directly and specifically with either, and modulation of Hsc70 ATPase may affect Hsp90's functions towards its client proteins. We thus proposed that Leu-Ile, upon binding to Hsc70, influenced Hsc70/Hsp90 chaperoning function towards client signaling proteins, resulting in mobilization of downstream signaling. To explore this hypothesis, we first studied some tyrosine and serine/threonine kinases, including mitogen-activated protein kinases, Akt and Src, which are closely associated with Hsp90 and neuron survival (Richter and Buchner, 2001). Akt phosphorylation was elevated apparently by Leu-Ile, whereas other kinases showed no change, implying the functional modulation of Hsc70 by Leu-Ile and involvement of Hsc70/Hsp90 co-chaperone in the regulation of Akt phosphorylation. Thulasiraman et al. (2002) reported a similar finding that a small hydrophobic peptide, binding to ATPase domain of Hsc70, affects ATPase activity and Hsp90/Hsc70-dependent transformation of eukaryotic initiation factor 2α kinase into an active form.

Heat shock response has been implicated in mediating the neuroprotective effect of FK506 (Klettner and Herdegen, 2003; Gold et al., 2004) and in activating Akt by conformational regulation of this molecule (Matsuzaki et al., 2004; Konishi et al., 1999). However, Leu-Ile did not affect the expression of Hsc70, Hsp70 or Hsp90, indicating that it unlikely exerts neuroprotective action by a mechanism of heat shock response. Given the possibility that the binding of FK506 to Hsp90/steroid receptor complexes might dissociate Hsp90 from heat shock factor, thus inducing heat shock response (Gold et al., 2004; Klettner and Herdegen, 2003; Gold et al., 1999), Leu-Ile unlikely affect the association between Hsp90 and steroid receptor, which may underlie its incapability in inducing heat shock response.

GA is known to bind ATP-binding pocket of Hsp90 and to inhibit ATP binding and hydrolysis, thereby disrupting its function (Basso et al., 2002). GA significantly blocked the increased pAkt levels induced by Leu-Ile, whereas PI3-k inhibitor LY294002 failed. On the basis of these findings, Leu-Ile is considered to activate Akt through Hsp90. It is clear that Akt interacts with Hsp90 via its catalytic domain and that Hsp90 promotes Akt activity by reducing PP2A-mediated pAkt dephosphorylation at threonine 308 residue (Sato et al., 2000). Therefore, Leu-Ile, upon binding to Hsc70, may facilitate Hsp90-Akt interaction through conformational regulation, resulting in an increase in pAkt through protecting it from dephosphorylation. Immunoprecipitation assays demonstrated such hypothesis, as it revealed a significant increase in pAkt-Hsp90 interaction. We also observed an elevation in total Akt immunoprecipitated by Hsp90 in spite that the difference was not significant. Considering that GA

causes the ubiquitin-mediated degradation of client Akt (Prodromou et al., 1997), Leu-Ile may inhibit proteasomal degradation of Akt mediated by Hsc70/Hsp90, resulting in pAkt elevation accordingly. This notion is supported by previous studies, which shows that CAIR-1, upon binding to Hsc70 ATPase domain, increases Akt phosphorylation by inhibiting its shift from Hsp90 to Hsc70, where Akt is ubiquitinated and degraded (Doong et al., 2003). Our data cannot distinct from these two mechanisms. Anyway, Hsc70, upon binding to Leu-Ile, appears to be crucial for the transmitting of neurotrophic signals of this dipeptide, which brings about Hsp90/Akt signaling. Nakagomi et al (2003) reported that Hsp27 promotes survival in PC12 cells and ganglion neurons by promoting Akt activity, which is independent of upstream activator. A chaperone-like protein, β-synuclein, exerts neuroprotective effect by directly stabilizing Akt activity rather than by acting on PI3-k (Hashimoto et al., 2004). Therefore, these findings together with ours further support a notion that chaperones like Hsp90 may participate in neuroprotection by conformational or chaperoning modulation of Akt rather than by acting on upstream effectors of the pathways. Additionally, Akt activation is required for increased expression of astroglial GDNF induced by melatonin (Lee et al., 2006).

GA blocked CREB activation induced by Leu-Ile, indicating that CREB activation proceeds via Hsp90/Akt signaling. These findings are supported by earlier studies, which show that modulation of Akt based on Hsc70/Hsp90 co-chaperones results in the maintenance of downstream CREB activation (Doong et al., 2003). Although CRE exists in the promoter sequence of GDNF gene, there is no direct evidence showing the role of CREB in GDNF transcriptional regulation. Moreover, Akt promotes

phosphorylation of CREB, stimulates recruitment of CREB binding protein to promoters, and activates gene expression (Leinninger et al., 2004; Pugazhenthi et al., 2000). We thus intensively investigated the role of Leu-Ile-activated pCREB in GDNF expression. We found that Leu-Ile-induced GDNF mRNA production and protein expression were attenuated when CREB was inhibited. Furthermore, CREB activation was accompanied by an increased capacity to activate transcription of target genes, since pCREB-CRE-binding activity was promoted. These results demonstrated that CREB-dependent transcriptional regulation is responsible for the GDNF-inducing properties of Leu-Ile. Although GDNF expression likely involves combinatorial interactions with multiple transcription factors including CREB, NF-κB and AP-2 (Woodbury et al., 1998), Leu-Ile-induced CREB activation is sufficient for inducing GDNF expression, indicating that CREB functions as an important transcriptional factor for GDNF gene. Similarly, FK960 induces GDNF expression in CREB-dependent mechanisms (Koyama et al., 2004). The extent to which transcription factor is required for GDNF transcriptional regulation is likely to depend on the character, strength or duration of extracellular stimuli. For example, NF-κB seems to play a role in GNDF induction in response to cytokines (Tanaka et al., 2000), while CREB likely participate in GNDF induction by growth factors like bFGF (Lenhard et al., 2002). The defined Hsp90/Akt/CREB pathway may provide a novel significant signaling that regulates GDNF expression.

Several cascades have been implicated in underlying neurotrophic activity of FK506. For example, FK506 potentiates NGF-induced neurite outgrowth via Ras/Raf/MEK pathway and involves PI3-k signaling (Price et al., 2003; Price et al., 2005). Gold et al. (1999) reported that GA blocked

neurotrophic action of FK506, suggesting FK506 interaction with Hsp90 via binding to FKBP52 is important for its neuroregenerative properties. However, FKBP-12 is not necessary for its neurotrophic effects (Gold et al., 2005; Gold et al., 1999). As suggested from QCM findings, FK506 may not interact with Hsc70 directly. Although the role of Hsc70 in mediating neuroprotective action of FK506 is unclear at present, it is tempting to speculate that FK506 might regulate chaperoning function of Hsp90/Hsc70 through FKBP, and thus modulate certain signaling kinases. It remains to be investigated intensively.

GDNF is a promising therapeutic agent for the treatment of neurodegenerative diseases. However, the delivery of GDNF to central nervous system provides an interesting challenge, because GDNF is unable to cross the blood-brain-barrier (Kirik et al., 2004), and use of low-molecular-weight drugs is an interesting alternative. FK506 exerts neuroprotective action, which is thought to depend on its GDNF-promoting effect (Tanaka et al., 2003). However, it cannot be used in therapy for neurological disorders because of its immunosuppressive effects. Leu-Ile, a small hydrophobic molecule, can penetrate neuron and promote GDNF expression, whereas shows no immunosuppressive activity. Thus, it may represent a novel lead-compound for treatment of dopaminergic neuron or motoneuron diseases such as Parkinson disease.

In conclusion, Leu-Ile targets Hsc70/Hsp90 co-chaperone and thus then triggers Akt/CREB signaling, resulting in upregulation of GDNF expression. This defined cascade may provide a deep insight into the cellular mechanism of GDNF expression regulation.

References

Airaksinen MS, Saarma M (2002) The GDNF family: signalling, biological functions and therapeutic value. Nat Rev Neurosci 3:383–394.

Baecker PA, Lee WH, Verity AN, Eglen RM, Johnson RM (1999) Characterization of a promoter for the human glial cell line-derived neurotrophic factor gene. Mol Brain Res 69:209–222.

Basso AD, Solit DB, Chiosis G, Giri B, Tsichlis P, Rosen N (2002) Akt forms an intracellular complex with heat shock protein 90 (Hsp90) and Cdc37 and is destabilized by inhibitors of Hsp90 function. J Biol Chem 277:39858–39866.

Brami-Cherrier K, Valjent E, Garcia M, Pagès C, Hipskind RA, Caboche J (2002) Dopamine induces a PI3-kinase-independent activation of Akt in striatal neurons: a new route to cAMP response element-binding protein phosphorylation. J Neurosci 22:8911–8921.

Brunet A, Datta SR, Greenberg ME (2001) Transcription-dependent and -independent control of neuronal survival by the PI3K-Akt signaling pathway. Curr Opin Neurobiol 11:297–305.

Castro LM, Gallant M, Niles LP (2005) Novel targets for valproic acid: up-regulation of melatonin receptors and neurotrophic factors in C6 glioma cells. J Neurochem 95:1227–1236.

Chou CC, Forouhar F, Yeh YH, Shr HL, Wang C, Hsiao CD (2003) Crystal structure of the C-terminal 10-kDa subdomain of Hsc70. J Biol Chem 278:30311–30316.

Doong H, Rizzo K, Fang S, Kulpa V, Weissman AM, Kohn EC (2003) CAIR-1/BAG-3 abrogates heat shock protein-70 chaperone complex-mediated protein degradation. J Biol Chem 278:28490–28500.

Du K, Montminy M (1998) CREB is a regulatory target for the protein kinase Akt/PKB. J Biol Chem 273:2377–32379.

Flaherty KM, DeLuca-Flaherty C, McKay DB (1990) Three-dimensional structure of the ATPase fragment of a 70K heat-shock cognate protein. Nature 346:623–628.

Gold BG, Armistead DM, Wang MS (2005) Non-FK506-binding protein-12 neuroimmunophilin ligands increase neurite elongation and accelerate nerve regeneration. J Neurosci Res 80:56-65.

Gold BG, Densmore V, Shou W, Matzuk MM, Gordon HS (1999) Immunophilin FK506-binding protein 52 (not FK506-binding protein 12) mediates the neurotrophic action of FK506. J Pharmacol Exp Ther 289:1202–1210.

Gold BG, Voda J, Yu X, Gordon H (2004) The immunosuppressant FK506 elicits a neuronal heat shock response and protects against acrylamide neuropathy. Exp Neurol 187:160-170.

Hashimoto M, Bar-On P, Ho G, Takenouchi T, Rockenstein E, Crews L, Masliah E (2004) Beta-synuclein regulates Akt activity in neuronal cells. A possible mechanism for neuroprotection in Parkinson's disease. J Biol Chem 279:23622–23629.

Hernández MP, Sullivan WP, Toft DO (2002) The assembly and intermolecular properties of the hsp70-Hop-hsp90 molecular chaperone complex. J Biol Chem 277:38294–38304.

Johnson JR, Chu AK, Sato-Bigbee C. (2000) Possible role of CREB in the stimulation of oligodendrocyte precursor cell proliferation by neurotrophin-3. J Neurochem 74:1409–1417.

Kirik D, Georgievska B, Bjorklund A. (2004) Localized striatal delivery of GDNF as a treatment for Parkinson disease. Nat Neurosci 7:105–110.

Klettner A, Herdegen T (2003) The immunophilin-ligands FK506 and V-10,367 mediate neuroprotection by the heat shock response. Br J Pharmacol 138:1004–1012.

Konishi H, Fujiyoshi T, Fukui Y, Matsuzaki H, Yamamoto T, Ono Y, Andjelkovic M, Hemmings BA, Kikkawa U. (1999) Activation of protein kinase B induced by H_2O_2 and heat shock through distinct mechanisms dependent and independent of phosphatidylinositol 3-kinase. J Biochem 126: 1136–1143

Koyama Y, Egawa H, Osakada M, Baba A, Matsuda, T (2004) Increase by FK960, a novel cognitive enhancer, in glial cell line-derived neurotrophic factor production in cultured rat astrocytes. Biochem Pharmacol 68:275–282.

Lee SH, Chun W, Kong PJ, Han JA, Cho BP, Kwon OY, Lee HJ, Kim SS. (2006) Sustained activation of Akt by melatonin contributes to the protection against kainic acid-induced neuronal death in hippocampus. J Pineal Res

Lee 37 Lempomanes CR, Sikat PT, Greenfield AT, Allen PB, McEwen BS (2004) Estrogen induces phosphorylation of cyclic AMP response element binding (pCREB) in primary hippocampal cells in a time-dependent manner. Neuroscience 124:549–560.

Leinninger GM, Backus C, Uhler MD, Lentz SI, Feldman EL (2004) Phosphatidylinositol 3-kinase and Akt effectors mediate insulin-like growth factor-I neuroprotection in dorsal root ganglia neurons. FASEB J 18:1544–1556.

Lenhard T, Schober A, Suter-Crazzolara C, Unsicker K (2002) Fibroblast growth factor-2 requires glial-cell-line-derived neurotrophic factor for exerting its neuroprotective actions on glutamate-lesioned hippocampal neurons. Mol Cell Neurosci 20:181–197.

Matsuzaki H, Yamamoto T, Kikkawa U (2004) Distinct activation mechanisms of protein kinase B by growth-factor stimulation and heat-shock treatment. Biochemistry, 43:4284–4293.

Matsushita N, Fujita Y, Tanaka M, Nagatsu T, Kiuch T (1997) Cloning and structural organization of the gene encoding the mouse glial cell line-derived neurotrophic factor, GDNF. Gene 203:149–157.

McLaughlin SH, Smith HW, Jackson SE (2002) Stimulation of the weak ATPase activity of human Hsp90 by a client protein. J Mol Biol 315:787–798.

Morshauser RC, Hu W, Wang H, Pang Y, Flynn GC, Zuiderweg ER (1999) High-resolution solution structure of the 18 kDa substrate-binding domain of the mammalian chaperone protein Hsc70. J Mol Biol 289:1387–1403.

Motomiya Y, Ando Y, Haraoka K, Sun X, Iwamoto H, Uchimura T, Maruyama I (2003) Circulating level of α2-macroglobulin-α2-microglobulin complex in hemodialysis patients. Kidney Inter 64:2244–2252.

Muchowski, PJ (2002) Protein misfolding, amyloid formation, and neurodegeneration: a critical role for molecular chaperones? Neuron 35:9–12.

Nakagomi S, Suzuki Y, Namikawa K, Kiryu-Seo S, Kiyama H (2003) Expression of the activating transcription factor 3 prevents c-Jun N-terminal kinase-induced neuronal death by promoting heat shock protein 27 expression and Akt activation. J Neurosci 23:5187–5196.

Nitta A, Nishioka H, Fukumitsu H, Furukawa Y, Sugiura H, Shen L, Furukawa S (2004) Hydrophobic dipeptide Leu-Ile protects against neuronal death by inducing brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor synthesis. J Neurosci Res 78: 250–258.

Nollen EA, Kabakov AE, Brunsting JF, Kanon B, Hohfeld J, Kampinga HH (2001) Modulation of in vivo HSP70 chaperone activity by Hip and Bag-1. J Biol Chem 276:4677–4682.

Nollen EAA, Morimoto RI (2002) Chaperoning signaling pathways: molecular chaperones as stress-sensing 'heat shock' proteins. J Cell Sci 115:2809–2816.

Pearl LH, Prodromou C (2001) Structure, function and mechanism of the Hsp90 molecular chaperone. Adv Protein

Chem 59:157–186.

Pratt WB, Toft DO (2003) Regulation of signaling protein function and trafficking by the hsp90/hsp70-based chaperone machinery. Exp Biol Med 228:111–133.

Price RD, Yamaji T, Matsuoka N (2003) FK506 potentiates NGF-induced neurite outgrowth via the Ras/Raf/MAP kinase pathway. Br J Pharmacol 140:825-829.

Price RD, Yamaji T, Yamamoto H, Higashi Y, Hanaoka K, Yamazaki S, Ishiye M, Aramori I, Matsuoka N, Mutoh S, Yanagihara T, Gold BG. (2005) FK1706, a novel non-immunosuppressive immunophilin: neurotrophic activity and mechanism of action. Eur J Pharmacol 509:11–19.

Prodromou C, Roe SM, O'Brien R, Ladbury JE, Piper PW, Pearl LH (1997) Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell 90:65–75.

Pugazhenthi S, Nesterova A, Sable C, Heidenreich KA, Boxer LM, Heasley LE, Reusch JE (2000) Akt/protein kinase B up-regulates Bcl-2 expression through cAMP-response element-binding protein. J Biol Chem 275:10761–10766.

Rajapandi T, Greene LE, Eisenberg E (2000) The molecular chaperones Hsp90 and Hsc70 are both necessary and sufficient to activate hormone binding by glucocorticoid receptor. J Biol Chem 275:22597–22604.

Richter K, Buchner J (2001) Hsp90: chaperoning signal transduction. J Cell Physiol 188: 281–290.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19:4337–4348.

Rubio E, Valenciano AI, Segundo C, Sanchez N, de Pablo F, de la Rosa EJ (2002) Programmed cell death in the

neurulating embryo is prevented by the chaperone heat shock cognate 70. Eur J Neurosci 15:1646–1654.

Saini HS, Gorse KM, Boxer LM, Sato-Bigbee C (2004) Neurotrophin-3 and a CREB-mediated signaling pathway regulate Bcl-2 expression in oligodendrocyte progenitor cells. J Neurochem 89:951–961.

Sato S, Fujita N, Tsuruo T (2000) Modulation of Akt kinase activity by binding to Hsp90. Proc Natl Acad Sci USA 97:10832–10837.

Schinelli S, Zanassi P, Paolillo M, Wang H, Feliciello A, Gallo V (2001) Stimulation of endothelin B receptors in astrocytes induces cAMP response element-binding protein phosphorylation and c-fos expression via multiple mitogen-activated protein kinase signaling pathways. J Neurosci 21:8842–8853.

Tanaka K, Fujita N, Ogawa N (2003) Immunosuppressive (FK506) and non-immunosuppressive (GPI1046) immunophilin ligands activate neurotrophic factors in the mouse brain. Brain Res 970:250–253.

Tanaka M, Ito S, Kiuchi K (2000) Novel alternative promoters of mouse glial cell line-derived neurotrophic factor gene. Biochim Biophys Acta 1494:63–74.

Thulasiraman V, Yun BG, Uma S, Gu Y, Scroggins BT, Matts RL (2002) Differential inhibition of Hsc70 activities by two Hsc70-binding peptides. Biochemistry 41:3742–3753.

Verity AN, WyattTL, LeeW, Hajos B, Baecker PA, Eglen RM, Johnson RM (1999) Differential regulation of glial cell line-derived neurotrophic Factor (GDNF) expression in human neuroblastoma and glioblastoma cell lines. J Neurosci Res 55:187–197.

Woodbury D, Schaar DG, Ramakrishnan L, Black IB (1998) Novel structure of the human GDNF gene. Brain Res 803:95–104.

Xu W, Yuan X, Jung YJ, Yang, Y, Basso A, Rosen N, Chung EJ, Trepel J, Neckers L (2003) The heat shock protein 90 inhibitor geldanamycin and the ErbB inhibitor ZD1839 promote rapid PP1 phosphatase-dependent inactivation of AKT in ErbB2 overexpressing breast cancer cells. Cancer Res 63:7777–7784.

Yamashita T, Shimada S, Guo W, Sato K, Kohmura E, Hayakawa T, Takagi T, Tohyama M. (1997) Cloning and functional expression of a brain peptide/histidine transporter. J Biol Chem 272:10205–10211.

Yano S, Tokumitsu H, Soderling TR (1998) Calcium promotes cell survival through CaM-K kinase activation of the protein-kinase-B pathway. Nature 396:584–587.

Young D, Lawlor PA, Leone P, Dragunow M, During MJ (1999) Environmental enrichment inhibits spontaneous apoptosis, prevents seizures and is neuroprotective. Nat Med 5:448–453.

Yun BG, Matts RL. (2005) Hsp90 functions to balance the phosphorylation state of Akt during C2C12 myoblast differentiation. Cell Signal 17:1477-1485.

Zhang Y, Champagne N, Beitel LK, Goodyer CG, Trifiro M, LeBlanc A (2004) Estrogen and androgen protection of human neurons against intracellular amyloid beta1-42 toxicity through heat shock protein 70. J Neurosci 24:5315–5321.

FOOTNOTES

The abbreviations used are: GDNF, glial cell line-derived neurotrophic factor; CREB, cAMP response element binding protein; CRE, cAMP response element; PI3-k, phosphoinositide 3-kinase; Hsp90, heat-shock protein 90; Hsc70, 70-kDa heat-shock cognate protein; PKC, protein kinase C; MAP2, microtuble-associated protein-2; ERK1/2, extracellular signal-regulated kinase 1/2; CaMK, calmodulin kinases; PP-2A, protein proteases 2A; QCM, quartz crystal microbalance; ODN, oligodeoxynucleotide; GA, geldanamycin; pAkt, phosphorylated Akt; pCREB, phosphorylated CREB; GFAP, glial fibrillary acidic protein; PBS, phosphate-buffer saline; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis. CBB, Coomassie brilliant blue; FKBP, FK506 binding protein

Figure Legends

Figure 1. Transmembrane transport of Leu-Ile. **A**, Cultured neurons were exposed to FITC-Leu-Ile or FITC at various concentrations for 30 min, and uptake was analyzed according to intracellular fluorescent densities (n=3). **B**, Neurons were incubated with 10 μg/ml FITC-Leu-Ile or FITC at 37°C for the indicated time periods. Time-course uptake was analyzed (n=3). **C**, Neurons were exposed to FITC-Leu-Ile for 30 min in the presence of various concentrations of Leu-Ile, which were indicated by different symbols. Penetration of FITC-Leu-Ile was significantly inhibited by competitive Leu-Ile. **D**, High concentrations of Leu-Ile could not inhibit FITC transport.

Figure 2. Identification of the specific protein binding to Leu-Ile. **A**, The reaction complexes of brain homogenate and FITC-Leu-Ile were subjected to gel electrophoresis, followed by fluorescent scanning. FITC-Leu-Ile alone was used as a control. The protein binding with FITC-Leu-Ile is marked by arrow. **B**, Brain homogenate was incubated with Leu-Ile Affigel-10, followed by washing and elution. The eluates were separated by electrophoresis, followed by silver staining. The protein band (arrow) was analyzed by mass spectrometry. **C**, The figure incorporates the observed mass (Obs), expected nominal mass (Exp) and calculated mass (Cal), together with the Miss, Score, Rank from Mascot Search and Peptide sequence. **D**, The picture shows the amino acid sequence assigned to each peptide (underlined) and their position in Hsc70 sequence (NBCI, Gi:123647). **E**, Brain homogenate was reacted with Leu-Ile or not (control), and the reaction complex was subjected to SDS-PAGE,

followed by immunoblotting with anti-Hsc70 antibody. Leu-Ile-Hsc70 (closed arrow) and Hsc70 (open arrow) are shown. **F,** Recombinant Hsc70 was reacted with Leu-Ile or not (control), and the reaction complexes were subjected to SDS-PAGE, followed by CBB staining. Leu-Ile-Hsc70 (closed arrow) and Hsc70 (open arrow) are shown. **G,** Brain homogenate was incubated with Leu-Ile- or FK506-Affigel, followed by washing and elution. The eluates were separated by electrophoresis and probed with anti-Hsc70 antibody.

Figure 3. Affinity of Leu-Ile and Hsc70 was assayed by QCM. **A,** Time course of frequency change (-delta F) of dipeptide-immobilized QCM is shown, responding to the addition of Hsc70 to the aqueous solution. **B,** Binding behavior of Leu-Ile to Hsc70 is dependent on Leu-Ile concentration. **C,** Frequency change of FK506-immobilized QCM was not observed upon Hsc70.

Figure 4. Leu-Ile stimulates Akt phosphorylation. **A,** Neurons were exposed to Leu-Ile (10 μg/ml) for the indicated times. Cell lysates were subjected to SDS-PAGE and probed with various antibodies. The representative immunoblots are shown. **B,** Neurons were exposed to Leu-Ile (10 μg/ml) for the indicated times. Immunoblots were probed with antibodies against Hsp90, Hsp70 and Hsc70. **C,** Neurons were stimulated with Leu-Ile, Pro-Leu and Ile-Pro (10 μg/ml) for 30 min. Cell lysates were subjected to SDS-PAGE and probed with antibodies against pAkt and Akt.

Figure 5. Akt activation induced by Leu-Ile is mediated by Hsp90. **A-B**, Neurons were stimulated with Leu-Ile (10μg/ml) alone for 0, 10, 20 and 30 min, or pre-treated with GA (10 μM) for 3 h (A) or LY294002 (15 μM) for 2 h (B), followed by Leu-Ile (10 μg/ml) treatment for 30 min. Cell lysates were immunoblotted with antibodies against pAkt and Akt. Each column represents the mean ± SEM (n=4). Leu-Ile +GA, neurons were pre-treated with GA, followed by Leu-Ile; GA, neurons were pre-treated with GA alone; Leu-Ile+LY, neurons were pre-treated with LY294002, followed by Leu-Ile treatment; LY: neurons were pre-treated with LY294002. ** $p<0.01$ versus control (0 min); ## $p<0.01$ versus Leu-Ile (30 min); $$ $p<0.01$ versus LY294002. **C**, Cultures were exposed to 10 μg/ml of Leu-Ile for the periods indicated. Cell extracts were immunoprecipitated (IP) with anti-Hsp90 antibody or control rabbit IgG, followed by immunoblotting (WB). Densitometric data are presented as the mean ± SEM (n = 4). ** $p<0.01$ versus control (0 min).

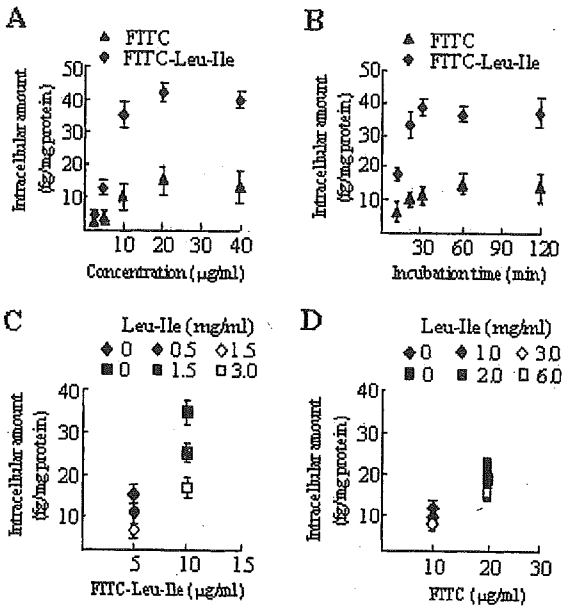
Figure 6. CREB is a downstream target of Hsp90/Akt signaling activated by Leu-Ile. **A,** Cultured neurons were exposed to 10 μg/ml of Leu-Ile for 0, 10, 20 and 30 min, and pCREB was measured by immunoblotting. **B**, Western blotting with anti-pCREB antibody reveals CREB activation induced by Leu-Ile but not Pro-Leu and Ile-Pro. **C**, Visualization of CREB phosphorylation (red) in MAP2-positive neurons (green) induced by Leu-Ile. **D**, Phosphorylated CREB (red) induced by Leu-Ile is not located in GFAP-positive cells (green). **E-F**, Neurons were treated with Leu-Ile (10 μg/ml) for 0 (control), 10, 20 and 30 min respectively, or pre-treated with GA (10 μM) for 3 h (E) or LY294002 (15

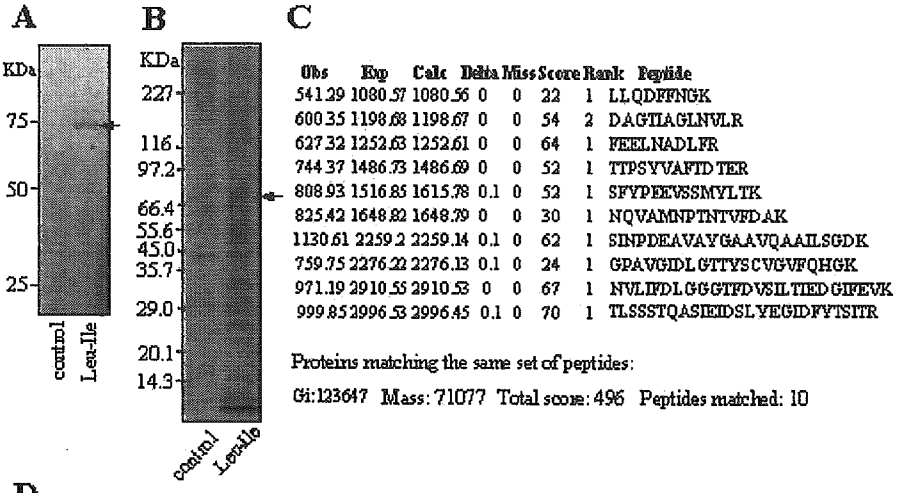
μM) for 2 h (F), followed by Leu-Ile (10 μg/ml) treatment for 30min. Each column represents the mean ± SEM (n = 4). Leu-Ile+GA, neurons were pre-treated with GA, followed by Leu-Ile; GA, neurons were pre-treated with GA; Leu-Ile+LY, neurons were pre-treated with LY294002, followed by Leu-Ile; LY: neurons were pre-treated with LY294002. ** $p<0.01$ versus control (0 min); ## $p<0.01$ versus Leu-Ile (30 min); $$ $p<0.01$ versus LY294002. **G**, PKC, pERK1/2 and pCaMKIIα/β were measured after Leu-Ile (10 μg/ml) treatment by immunoblotting.

Figure 7. Leu-Ile increases GDNF expression in CREB-dependent manner. **A,** Leu-Ile significantly increased GDNF expression, whereas Pro-Leu and Ile-Pro showed no GDNF-inducing activities. ** $p<0.01$ versus control (n = 4); **B,** GDNF mRNA levels induced by Leu-Ile for various periods were studied by real-time RT-PCR. * $p<0.05$ and ** $p<0.01$ versus control (0 h); **C,** CREB expression was blocked by CREB antisense ODN, as revealed by Western blotting. **D,** Neurons were incubated with Leu-Ile (10 μg/ml) for 24 h in the presence of CREB antisense ODN or sense ODN. Data are expressed as a percentage of the control (mean ± SEM, n = 4). ** $p<0.01$ versus control and * $p<0.05$ versus Leu-Ile or Leu-Ile plus CREB sense ODN. **E,** Neurons were incubated with Leu-Ile (10 μg/ml) for 24 h in the presence of CREB antisense ODN or sense ODN. Data are expressed as a percentage of control (mean ± SEM, n = 4). *** $p<0.001$ versus control; ## $p<0.01$ versus Leu-Ile or Leu-Ile plus CREB sense ODN. **F,** Neurons were labeled with anti-GDNF (green) and anti-pCREB

antibodies (red). **G**, CRE-pCREB binding activities were quantified after Leu-Ile treatment for 30 min.

** $p<0.01$ versus control; ### $p<0.001$ verse mutant ODN ($n = 4$).


A
FITC
FITC-Leu-Ile
Intracellular amount (fg/mg protein)
Concentration (μg/ml)
B
FITC
FITC-Leu-Ile
Intracellular amount (fg/mg protein)
Incubation time (min)
C
Leu-Ile (mg/ml)
0 0.5 1.5
0 1.5 3.0
Intracellular amount (fg/mg protein)
FITC-Leu-Ile (μg/ml)
D
Leu-Ile (mg/ml)
0 1.0 3.0
0 2.0 6.0
Intracellular amount (fg/mg protein)
FITC (μg/ml)



D **dnak-type molecular chaperone Hsc70 – mouse**

MSKGPAVGIDLGTTYSCVGVFQHGKVEIIANDQGNRTTPSYVAFTDTERLIGDAAKNQVAMNPTNTVFDAKRLI
GRRFDDAVVQSDMKHWPFMVVNDAGRPKVQVEYKGETKSFYPEEVSSMVLTKMKEIAEAYLGKTVTNAVVT
VPAYFNDSQRQATKDAGTIAGLNVLRIINEPTAAAIAYGLDKKVGAERNVLIFDLGGGTFDVSILTIEDGIFEVKS
TAGDTHLGGEDFDNRMVNHFIAEFKRKHKKDISENKRAVRRLRTACERAKRTLSSSTQASIEIDSLYEGIDFYTSI
TRARFEELNADLFRGTLDPVEKALRDAKLDKSQIHDIVLVGGSTRIPKIQKLLQDFFNGKELNKSINPDEAVAYG
AAVQAAILSGDKSENVQDLLLLDVIPLSLGIETAGGVMTVLIKRNTTIPTKQTQTFTTYSDNQPGVLIQVYEGER
AMTKDNNLLGKFELTGIPPAPRGVPQIEVTFDIDANGILNVSAVDKSTGKENKITITNDKGRLSKEDIERMVQEAE
KYKAEDEKQRDKVSSKNSLESYAFNMKATVEDEKLQGKINDEDKQKILDKCNEIISWLDKNQTAEKEEFEHQQ
KELEKVCNPIITKLYQSAGGMPGGMPGGFPGGGAPPSGGASSGPTIEEVD