

Single Transmembrane Domain Insulin-Like Growth Factor-II/Mannose-6-Phosphate Receptor Regulates Central Cholinergic Function by Activating a G-Protein-Sensitive, Protein Kinase C-Dependent Pathway

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The insulin-like growth factor-II/mannose-6-phosphate (IGF-II/M6P) receptor is a single-pass transmembrane glycoprotein that plays an important role in the intracellular trafficking of lysosomal enzymes and endocytosis-mediated degradation of IGF-II. However, its role in signal transduction after IGF-II binding remains unclear. In the present study, we report that IGF-II/M6P receptor in the rat brain is coupled to a G-protein and that its activation by Leu²⁷IGF-II, an analog that binds rather selectively to the IGF-II/M6P receptor, potentiates endogenous acetylcholine release from the rat hippocampal formation. This effect is mediated by a pertussis toxin (PTX)-sensitive GTP-binding protein and is dependent on protein kinase C α (PKC α)-induced phosphorylation of downstream substrates, myristoylated alanine-rich C kinase substrate, and growth associated protein-43. Additionally, treatment with Leu²⁷IGF-II causes a reduction in whole-cell currents and depolarization of cholinergic basal forebrain neurons. This effect, which is blocked by an antibody against the IGF-II/M6P receptor, is also sensitive to PTX and is mediated via activation of a PKC-dependent pathway. These results together revealed for the first time that the single transmembrane domain IGF-II/M6P receptor expressed in the brain is G-protein coupled and is involved in the regulation of central cholinergic function via the activation of specific intracellular signaling cascades.

Key words: cholinergic neurons; G-protein-coupled receptor; intracellular signaling; insulin-like growth factors; neurotransmitter release; protein kinase C

Introduction

The insulin-like growth factor-II (IGF-II) receptor, which is identical to the cation-independent mannose-6-phosphate (M6P) receptor, is a single-pass transmembrane glycoprotein containing a large extracellular domain and a small cytoplasmic tail (Morgan et al., 1987; MacDonald et al., 1988; Jones and Clemmons, 1995; Ghosh et al., 2003). The receptor binds IGF-II with higher affinity than IGF-I and does not bind insulin. The receptor also interacts, via distinct sites, with M6P-bearing lysosomal enzymes and a variety of other ligands, such as transforming growth factor- β (TGF β), leukemia inhibitory factor (LIF), proliferin, and retinoic acid (Dore et al., 1997; Kang et al., 1997; Blanchard et

al., 1999). A majority of IGF-II/M6P receptors are expressed within *trans*-Golgi network/endosomal compartments, in which they segregate newly synthesized lysosomal enzymes for subsequent sorting to endosomes and lysosomes (Kornfeld, 1992; Braulke, 1999). A subset of receptors located on the plasma membrane regulates endocytosis of secreted lysosomal enzymes, mediates internalization and subsequent degradation of IGF-II, LIF, and proliferin, and potentiates activation of latent TGF β (Hille-Rehfeld, 1995; Braulke, 1999).

Unlike its participation in intracellular trafficking, the role of the IGF-II/M6P receptor in the transmembrane signaling of IGF-II remains unclear. The biological effects of IGF-II are mostly mediated via the IGF-I receptor or insulin receptor isoform A receptor, whereas the IGF-II/M6P receptor acts as a “clearance receptor” to stabilize local IGF-II concentration (Frasca et al., 1999; Hawkes and Kar, 2004). In some non-neuronal tissues, however, the IGF-II/M6P receptor mediates specific biological responses after IGF-II binding, including amino acid uptake (Shimizu et al., 1986), motility of human rhabdomyosarcoma cells (Minniti et al., 1992), and stimulation of Na⁺/H⁺ exchange and inositol triphosphate (IP₃) production (Rogers et al., 1990). The underlying mechanisms whereby the IGF-II/M6P receptor mediates these effects remain unclear. Nev-

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ertheless, some studies, including the demonstration of a putative G-protein binding site within the cytoplasmic domain of the receptor, suggest a possible role for G-proteins in the ligand-induced responses of the receptor (Murayama et al., 1990; Ikezu et al., 1995). However, failure of the receptor to couple with a G-protein under certain conditions (Sakano et al., 1991; Körner et al., 1995) has raised doubt about the role of the receptor in mediating biological responses after IGF-II binding (Kiess et al., 1994; Dahms and Hancock, 2002).

In the adult rat brain, IGF-II/M6P receptor is known to be distributed in various regions, including cortex, striatum, and hippocampus (Couce et al., 1992; Kar et al., 1993a; Nagano et al., 1995; Hawkes and Kar, 2003). Evidence for the distinct distribution of the IGF-II/M6P receptor from that of IGF-I or insulin receptors and specific alterations in IGF-II/M6P receptor levels in response to various pharmacological/surgical manipulations (Kar et al., 1993b, 1997a; Breese et al., 1996; Walter et al., 1999) raises the possibility that this receptor may have a role in the regulation of certain brain functions. The present study shows that the single transmembrane domain IGF-II/M6P receptor is coupled to a G-protein in the brain and is involved in the potentiation of acetylcholine (ACh) release by activating a G-protein-sensitive, protein kinase C α (PKC α)-dependent pathway.

Materials and Methods

Materials. Adult (3 months) and postnatal (21–25 d) male Sprague Dawley rats (Charles River Canada, Montreal, Quebec, Canada) were kept in accordance with Institutional and Canadian Council on Animal Care Guidelines. Recombinant IGF-I was purchased from ICN Biomedicals (Montreal, Quebec, Canada), whereas Leu²⁷IGF-II was obtained from GroPep (Adelaide, Australia) and also as a gift from Dr. K. Sakano (Daiichi Pharmaceutical, Tokyo, Japan). Nonhydrolyzable GTP analogs, guanosine-5'-[γ -thio]triphosphate (GTP γ S) and guanosine 5'-[β , γ -imido]triphosphate [Gpp(NH)p], were purchased from Roche Diagnostics (Laval, Quebec, Canada), whereas IGF-II, insulin, pertussis toxin (PTX), cholera toxin (CTX), and polymyxin B (PMB) were from Calbiochem (San Diego, CA). Labeled [¹²⁵I]IGF-I, [¹²⁵I]IGF-II, [¹²⁵I]insulin (2000 Ci/mmol), and [³H]cAMP kit were purchased from Amersham Biosciences (Toronto, Ontario, Canada). [γ -³²P]ATP, [³H]choline (75 Ci/mmol), and [¹⁴C]acetyl-CoA were obtained from PerkinElmer (Mississauga, Ontario, Canada). Tetrodotoxin (TTX), cGMP, adenosine 5'-[β , γ -imido]triphosphate [App(NH)p], isoproterenol, vesicular ACh transporter (VACHT) antibody, and iberiotoxin (IBTX) were obtained from Sigma (St. Louis, MO), IGF-I receptor antagonist JB-1 was from Bachem (Torrance, CA), and anti-IGF-I receptor antibody was purchased from Oncogene (San Diego, CA). Polyclonal rat IGF-II/M6P receptor antibodies and the purified receptor were from our laboratory, as well as from Dr. S. P. Nissley (National Institutes of Health, Bethesda, MD). Anti-phosphotyrosine, anti-PKC α , anti-phospho-myristoylated alanine-rich C kinase substrate (MARCKS), anti-G β , anti-G α , anti-G β , anti-horseradish peroxidase-conjugated antibodies, purified G β , and protein A/G-PLUS agarose were purchased from Santa Cruz Biotechnology (Santa Cruz, CA), and oligo-dT, Superscript II RT, and *Taq* polymerase were from Invitrogen (Burlington, Ontario, Canada). Anti-phospho-PKC α and anti-phospho-PKC ϵ antibodies were purchased from Upstate Biotechnology (Lake Placid, NY), anti-growth associated protein-43 (GAP-43) antibody was from Chemicon (Temecula, CA), and fluorescence-conjugated secondary antibodies were from Jackson ImmunoResearch (West Grove, PA). All other chemicals of analytical grade were purchased from Sigma, Invitrogen, or Fisher Scientific (Pittsburgh, PA).

Receptor binding assays. Animals were killed by decapitation, and their brains were processed for either membrane binding assay or receptor autoradiography as described previously (Jafferali et al., 2000). For [¹²⁵I]IGF-I or [¹²⁵I]IGF-II membrane binding, hippocampal regions were dissected out, homogenized in 50 mM Tris-HCl, pH 7.4, centri-

fuged, and then incubated either with 25 pM [¹²⁵I]IGF-I at 22°C for 2 h in 50 mM Tris-HCl buffer containing 10 mM MgCl₂, 0.1% bovine serum albumin (BSA), and 0.1% Bacitracin or with 25 pM [¹²⁵I]IGF-II at 4°C for 18 h in 50 mM Tris-HCl buffer containing 0.025% BSA. For the insulin binding, hippocampal P₂ synaptosomal fractions were incubated with 50 pM [¹²⁵I]insulin at 4°C for 6 h in 10 mM HEPES containing 0.5% BSA, 0.025% Bacitracin, 0.0125% *N*-ethylmaleimide (NEM), and 100 kIU/ml aprotinin. For each radioligand, competition binding was performed in the presence of 10⁻⁶ to 10⁻¹² M IGF-I, IGF-II, Leu²⁷IGF-II, and insulin. Incubations were terminated either by filtration or centrifugation and then radioactivity was measured. All experiments were performed three to five times, each in triplicate, and data were analyzed using the GraphPad Prism software (GraphPad Software, San Diego, CA).

For *in vitro* receptor autoradiography, frozen brain sections (20 μ m) through the hippocampus were processed as described previously (Kar et al., 1993a, 1997a). In brief, for [¹²⁵I]IGF-I binding, sections were incubated with 25 pM [¹²⁵I]IGF-I for 2 h at 22°C in Tris-HCl (50 mM), pH 7.4, buffer containing 10 mM MgCl₂, 0.1% BSA, and 0.1% Bacitracin with or without 10⁻¹² to 10⁻⁶ M IGF-I, IGF-II, Leu²⁷IGF-II, or insulin. The slides were then washed and exposed against Hyperfilm for 4 d. For [¹²⁵I]IGF-II binding, sections were incubated with 25 pM [¹²⁵I]IGF-II in 50 mM Tris-HCl buffer containing 0.025% BSA with or without 10⁻¹² to 10⁻⁶ M IGF-I, IGF-II, Leu²⁷IGF-II, or insulin. After 18 h at 4°C, sections were washed and exposed for 3 d against Hyperfilm. The insulin binding was performed by incubating sections with 50 pM [¹²⁵I]insulin for 18 h at 4°C in 10 mM HEPES, pH 7.4, containing 0.5% BSA, 0.025% Bacitracin, 0.0125% NEM, and 100 kIU/ml aprotinin with or without 10⁻¹² to 10⁻⁶ M IGF-I, IGF-II, Leu²⁷IGF-II, or insulin. Sections were washed and then exposed to Hyperfilm for 5 d. All autoradiograms were analyzed using a microcomputer imaging device (MCID) image analysis system (Kar et al., 1997a).

Affinity cross-linking. For cross-linking experiments, hippocampal membranes were incubated with either 25 pM [¹²⁵I]IGF-II in 50 mM Tris-HCl for 18 h at 4°C or 25 pM [¹²⁵I]IGF-I in 50 mM Tris-HCl for 2 h at 22°C in the presence or absence of 10⁻⁷ M IGF-I, IGF-II, or Leu²⁷IGF-II as mentioned in receptor binding assays. The bound ligands were then cross-linked to their respective receptors by incubating for an additional 50 min at 4°C with 2.5–7.5 \times 10⁻⁴ M disuccinimidyl suberate (MacDonald, 1991). The reactions were quenched with 500 μ l of 0.1 M Tris-HCl, pH 7.4, centrifuged, and then separated by 4–20% SDS-PAGE. Gels were dried and exposed to Hyperfilm for 7–14 d as described previously (MacDonald, 1991).

IGF-II/M6P receptor and G-protein. To establish whether the IGF-II/M6P receptor is linked to a G-protein, hippocampal membranes were prepared as described previously and then incubated with 25 pM [¹²⁵I]IGF-II in 50 mM Tris-HCl, pH 7.4, buffer containing 0.025% BSA with or without 10⁻⁷ to 10⁻³ M GTP γ S, Gpp(NH)p, cGMP, App(NH)p, 5–20 μ g/ml preactivated (1 h incubation in 50 mM DTT) PTX or 10⁻⁸ to 10⁻⁶ M CTX. Nonspecific binding was determined in the presence of 10⁻⁷ M unlabeled IGF-II or Leu²⁷IGF-II. In parallel, [¹²⁵I]IGF-I binding was also performed in the presence of 10⁻⁷ to 10⁻³ M GTP γ S and Gpp(NH)p. The binding reaction in both cases was terminated by rapid filtration, and radioactivity was measured with a gamma counter. In certain instances, cross-linking experiments were performed after [¹²⁵I]IGF-II binding assay with or without GTP γ S, Gpp(NH)p, IGF-II, or Leu²⁷IGF-II as described above.

IGF-II/M6P receptor Western blotting and immunocytochemistry. Adult male rats were decapitated, and their brain areas of interest [i.e., medial septum/diagonal band of Broca (DBB), lateral septum, nucleus basalis of Meynert (NBM), and hippocampus] were homogenized in Tris-lysis buffer [50 mM Tris-HCl, pH 7.4, 10 mM MgCl₂, 0.1% BSA, 5 mM phenylmethylsulfonyl fluoride (PMSF), 10 μ g/ml leupeptin, and 10 μ g/ml aprotinin] as described previously (Hawkes and Kar, 2003). Proteins were separated by gel electrophoresis and then incubated with anti-IGF-II/M6P receptor antibody (1:8000). Membranes were exposed to anti-rabbit IgG and visualized using an ECL kit. For immunolabeling, Bouin's-fixed brain sections were incubated overnight with a cholinergic marker, VACHT antibody (1:250), and then exposed to FITC-conjugated anti-goat IgG for 2 h. The sections were reincubated with anti-IGF-II/

M6P receptor antibody (1:250) and then exposed to Texas Red-conjugated anti-rabbit IgG for 2 h (Hawkes and Kar, 2003). Mounted sections were examined under a Nikon (Tokyo, Japan) PCM2000 confocal microscope.

Determination of ACh release. Brain slices from adult rats were superfused as described previously (Kar et al., 1997b; Seto et al., 2002). Briefly, rats were decapitated, and hippocampus and striatum were dissected out, sliced at 400 μm , and then superfused with oxygenated Krebs' buffer (in mM: 120 NaCl, 4.6 KCl, 2.4 CaCl_2 , 1.2 KH_2PO_4 , 1.2 MgSO_4 , 9.9 D-glucose, and 25 NaHCO_3 , adjusted to pH 7.4) using Brandel (Gaithersburg, MD) superfusion apparatus. The buffer also contained physostigmine (30 μM) to prevent degradation of ACh and choline chloride (10 μM) to support a constant supply of precursor for the synthesis of ACh. Effluents for the first 45 min were discarded to establish a stable basal efflux of ACh, and thereafter samples were collected every 20 min. After 1 h basal efflux, tissues were stimulated with high- K^+ Krebs' buffer (25 mM KCl with equimolar reduction in NaCl to conserve isotonicity) for an additional 1 h in either the presence or absence of 10^{-12} to 10^{-8} M Leu²⁷IGF-II or 10^{-9} M IGF-II. In some experiments, hippocampal slices were superfused with 25 mM K^+ Krebs' buffer alone or containing 10^{-8} M Leu²⁷IGF-II with or without 10 μg TTX, 25 $\mu\text{g}/\text{ml}$ PTX, 25 μM PMB, or 20 $\mu\text{g}/\text{ml}$ JB-1. The JB-1 concentration (i.e., 20 $\mu\text{g}/\text{ml}$) used in this study was based on previous results (Fernandez et al., 1999; Quesada and Micevych, 2004). The use of IGF-II/M6P receptor blocking antibody was not feasible because of the permeability problem across 400 μm brain slices as reported previously (Ronn et al., 1995). The superfusates collected every 20 min were processed to detect ACh levels using radioenzymatic assay as described previously (Kar et al., 1997b). Evoked release represents the net release above the basal efflux and is expressed as picomoles of ACh per minute per milligram of protein. The data, which are presented as mean \pm SEM, were analyzed using two-tailed Student's *t* test, with significance set at $p < 0.05$.

Immunoprecipitation. Rat hippocampal slices were prepared as described for *in vitro* ACh release and exposed to 10^{-8} M Leu²⁷IGF-II, 10^{-7} M IGF-I, 1 μM isoproterenol, 10^{-7} M IGF-I plus 20 $\mu\text{g}/\text{ml}$ JB-1, or 10^{-8} M Leu²⁷IGF-II plus 25 $\mu\text{g}/\text{ml}$ PTX. Tissue slices were then lysed in cold radioimmunoprecipitation assay (RIPA) buffer [50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% Igepal CA-630, 0.1% SDS, 50 mM NaF, 1 mM NaVO_3 , 5 mM PMSF, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 10 $\mu\text{g}/\text{ml}$ aprotinin] and processed for IGF-II/M6P receptor/ $\text{G}_i\alpha_2/\text{G}_s\alpha/\text{G}_q\alpha$ immunoprecipitation by incubating supernatant as well as pellets at 4°C overnight with either IGF-II/M6P receptor, mouse $\text{G}_i\alpha_2$, $\text{G}_s\alpha$, or $\text{G}_q\alpha$ antibodies. The immune complexes were precipitated by protein A/G PLUS-agarose, separated by gel electrophoresis, and then blotted with anti- $\text{G}_i\alpha_2/\text{G}_s\alpha/\text{G}_q\alpha$ or IGF-II/M6P receptor antisera, respectively (Zheng et al., 2000). As for $\text{G}_s\alpha$ control, isoproterenol-treated slices were immunoprecipitated with an β -adrenergic receptor antibody (1:5000) and then blotted with anti- $\text{G}_s\alpha$ antibody. For IGF-I receptor immunoprecipitation, the supernatant was incubated with an anti-IGF-I receptor, and then IGF-I receptor phosphorylation was determined with an anti-phosphotyrosine antibody as described previously (Seto et al., 2002).

Intracellular signaling. Hippocampal slices were superfused for 5, 10, 20, 40, or 60 min with or without 10^{-8} M Leu²⁷IGF-II and then processed for radioimmunoassay to measure cAMP levels. Additionally, hippocampal slices exposed to either 10^{-8} M Leu²⁷IGF-II or 10^{-8} M Leu²⁷IGF-II plus 25 μM PMB for 5, 10, 20, 40, or 60 min were simultaneously processed for Western blotting to evaluate PKC/MARCKS/GAP-43 levels. For [³H]cAMP assay, hippocampal slices were homogenized in 0.5 M Tris-EDTA buffer, pH 7.4, and then assayed as per the instructions of the manufacturer. For Western blotting, tissues were homogenized in RIPA lysis buffer, and subcellular membrane and cytosolic fractions were prepared (Zheng et al., 2000). Proteins were subsequently separated by 4–20% SDS-PAGE and incubated overnight with anti-PKC α (1:5000), anti-phospho-PKC α (1:5000), anti-PKC γ (1:5000), anti-phospho-PKC ϵ (1:5000), anti-phospho-MARCKS (1:1000), or anti-phospho-GAP-43 (1:5000) antibodies. Membranes were then exposed to the secondary antibody, visualized using an ECL detection kit, and quantified using an MCID system (Hawkes and Kar, 2003).

Dissociation of neurons for electrophysiology. Brains were removed from

decapitated male 21–25 d postnatal rats, and the area containing the DBB was dissected out as described previously (Jassar et al., 1999). Acutely dissociated neurons were then prepared by treating the 350 μm slices with trypsin (0.65 mg/ml) at 30°C, followed by mechanical trituration. Cells were then plated on poly-L-lysine-coated coverslips and viewed under a Zeiss (Oberkochen, Germany) inverted microscope.

Voltage- and current-clamp recordings. All whole-cell patch-clamp recordings were performed under visual guidance using either Axopatch-1D or Axopatch 200B amplifiers (Molecular Devices, Union City, CA). Patch electrodes were flame polished to yield resistances of 3–6 M Ω . Whole-cell patch-clamp recordings were obtained using pipette solution that contained the following (in mM): 140 K-methylsulfate, 10 EGTA, 5 MgCl_2 , 1 CaCl_2 , 10 HEPES, 2.2 Na_2 -ATP, and 0.3 Na-GTP, pH 7.2. After whole-cell configuration was established with voltage-clamp mode (holding potential of -80 mV), we waited at least 5 min for the cell to stabilize then either started voltage-clamp studies or switched to current-clamp recording mode. All treatments (i.e., Leu²⁷IGF-II, PTX, PMB, JB-1, IBTX, preimmune Ig, and anti-IGF-II/M6P receptor antibody) were applied either via bath perfusion or through a focal applicator from a micromanifold tip. The current and membrane voltages were recorded using a low-pass filter at 5 kHz and digitized at 10 kHz. Cells were held in voltage clamp at -80 mV, which was close to the resting membrane potential of DBB neurons (Jhamandas et al., 2003). Based on the previous observations (Jassar et al., 1999), to record whole-cell currents, we used a voltage-ramp protocol in which the cells were held at -80 mV and received a 1 s hyperpolarizing pulse (to remove K^+ channel inactivation) before being subjected to a slow voltage ramp from -110 to $+30$ mV at the rate of 20 mV/s. All data, which were analyzed using pClamp6 and 9 software, are presented as mean \pm SEM. Student's two-tailed *t* test was used for determining significance of effect.

Single-cell reverse transcription-PCR. Neurons were harvested after electrophysiological recordings for reverse transcription (RT)-PCR as described previously (Jhamandas et al., 2003). Primer sequences for choline acetyltransferase (ChAT) and glutamate decarboxylase (GAD) have been described previously (Jhamandas et al., 2003), and that for β -actin was obtained from GenBank (the lower primer, 5'-GAT AGA GCC ACC AAT CCA C; the upper primer, 5'-CCA TGT ACG TAG CCA TCC A). All primers were synthesized at the Department of Biochemistry (University of Alberta). The contents were mixed together and placed in the thermal cycler (Jhamandas et al., 2003). A portion of the product was run on a 2% Tris-acetate-EDTA agarose gel, and then the gel was placed in 2 $\mu\text{g}/\text{ml}$ ethidium bromide. After 10 min, DNA bands were visualized with ultraviolet transilluminator and photographed.

Results

Characterization of Leu²⁷IGF-II in the rat brain

To determine the receptor specificity of Leu²⁷IGF-II, we first performed competition binding studies in the rat hippocampus, which is enriched with cholinergic terminals and receptors for IGF-I, IGF-II/M6P, and insulin (Hawkes and Kar, 2004). Specific [¹²⁵I]IGF-II binding sites were competed potently by IGF-II > Leu²⁷IGF-II > IGF-I >> insulin (Fig. 1A). The most potent competitors were IGF-II (IC_{50} of 0.2 nM) and Leu²⁷IGF-II (IC_{50} of 1 nM), whereas IGF-I was much less potent (IC_{50} of 15 nM), followed by insulin (IC_{50} of 3 μM). In contrast, specific [¹²⁵I]IGF-I binding was inhibited in a dose-dependent manner by IGF-I > IGF-II > insulin > Leu²⁷IGF-II (Fig. 1B). The IC_{50} values for IGF-I, IGF-II, insulin, and Leu²⁷IGF-II were 0.3 nM, 4.8 nM, 0.2 μM , and 0.8 μM , respectively. Competition studies with [¹²⁵I]insulin binding sites revealed that insulin (IC_{50} of 1.4 nM) competed potently than IGF-II (IC_{50} of 15 nM) > IGF-I (IC_{50} of 60 nM) >> Leu²⁷IGF-II (IC_{50} of 8 μM) (Fig. 1C).

At the regional level, [¹²⁵I]IGF-II, [¹²⁵I]IGF-I, and [¹²⁵I]insulin receptor binding sites exhibited distinct distribution profiles in the hippocampus (Fig. 2A–C). High levels of [¹²⁵I]IGF-II binding sites, as reported previously (Lesniak et al., 1988; Kar et

al., 1993a), were evident in the pyramidal cell layer (12.5 ± 1.7 fmol/mg tissue) of the Ammon's horn and the granular cell layer (17.5 ± 2.3 fmol/mg tissue) of the dentate gyrus (DG), whereas all other layers showed low levels (2.5 ± 0.3 to 3.8 ± 0.5 fmol/mg tissue) of specific labeling (Fig. 2A). The [125 I]IGF-II binding was competed potently by IGF-II > Leu 27 IGF-II > IGF-I \gg insulin (Fig. 2D, G, J, M). High amounts of [125 I]IGF-I binding sites, which were localized in the CA2–CA3 subfields and in the molecular layer of the DG, were competed potently by IGF-I > IGF-II > insulin > Leu 27 IGF-II (Fig. 2B, E, H, K, N). As for [125 I]insulin binding, a high density of binding was evident in the molecular layer of the DG (Fig. 2C), whereas moderate labeling was noted in other layers of the Ammon's horn. Specific [125 I]insulin binding was competed potently by insulin > IGF-II > IGF-I \gg Leu 27 IGF-II (Fig. 2F, I, L, O).

Affinity cross-linking experiments with rat hippocampal membranes revealed that [125 I]IGF-II bound to a 250 kDa band corresponding to the IGF-II/M6P receptor (MacDonald, 1991), which was completely displaced by 10^{-7} M IGF-II or Leu 27 IGF-II (Fig. 2P). Affinity labeling with [125 I]IGF-I showed two bands of ~ 135 and 240 kDa, corresponding to the size of the IGF-I receptor α -subunit and α -subunit dimers, respectively (Zeeh et al., 1997). Radiolabel was displaced from both bands by 10^{-7} M IGF-I but not with 10^{-7} M Leu 27 IGF-II (Fig. 2Q).

IGF-II/M6P receptor and PTX-sensitive G-protein

To establish the possible link of the IGF-II/M6P receptor to a G-protein, [125 I]IGF-II binding assays were performed in hippocampal membranes with or without 10^{-7} to 10^{-3} M cGMP, App(NH)p, GTP γ S, and Gpp(NH)p. Both Gpp(NH)p and GTP γ S, which promote the shift of receptors from the coupled to the uncoupled form (Stiles et al., 1984), but not cGMP or App(NH)p, inhibited [125 I]IGF-II receptor binding with IC_{50} values of 230 and 270 μ M (Fig. 3A), respectively, as reported for seven-transmembrane G-protein-coupled receptors (Inui et al., 1989). In contrast, binding of ligands by the tyrosine kinase IGF-I receptor was unaffected by either analog (Fig. 3B). The interaction of the IGF-II/M6P receptor with Gpp(NH)p and GTP γ S was further supported by our cross-linking data, which showed that binding of [125 I]IGF-II to its receptor is dose dependently inhibited by GTP analogs (Fig. 3C). To determine the subset of G-proteins associated with the IGF-II/M6P receptor, competition binding experiments were performed using ADP-ribosyltransferase toxins, PTX and CTX, which inhibit G_i/G_o - and G_s/G_{12} -proteins, respectively (Yamane and Fung, 1993). Incubation of hippocampal membranes with CTX did not alter [125 I]IGF-II receptor binding, whereas PTX decreased binding in a dose-dependent manner (Fig. 3D). To substantiate these data, rat hippocampal proteins were immunoprecipitated with anti-IGF-II/M6P receptor antibody after exposure to 10^{-8} M Leu 27 IGF-II and then blotted with an anti- G_{i2} , anti- G_s , or anti- G_q antibody. Our results demonstrate that a G_{i2} protein ($\sim 15\%$), but not G_s or G_q protein, coimmunoprecipitated with the IGF-II/M6P receptor (Fig. 3E, G, I) (G_q data not shown). We were also able to detect IGF-II/M6P receptor ($\sim 18\%$) in hippocampal proteins after coimmunoprecipitation

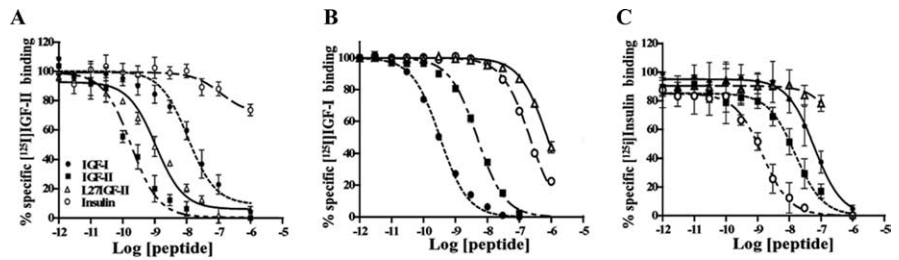


Figure 1. Comparative competition binding profiles of IGF-I, IGF-II, insulin, and Leu 27 IGF-II (L27IGF-II) against [125 I]IGF-II (A), [125 I]IGF-I (B), and [125 I]insulin (C) in adult rat hippocampal membrane preparations. The binding profiles indicate that each ligand bound its respective receptor with higher affinity than related peptides. Leu 27 IGF-II competed with high affinity for [125 I]IGF-II but rather poorly for [125 I]IGF-I and [125 I]insulin receptor binding sites. Each point represents the mean \pm SEM of data obtained from three to five experiments, each performed in triplicate and expressed as percentage of specific binding.

with an anti- G_{i2} antibody (Fig. 3F, H). As for control, a G_s protein, as expected, was coimmunoprecipitated with a β -adrenergic receptor antibody after isoproterenol stimulation (Fig. 3I) (Gurdal et al., 1997). The interaction between IGF-II/M6P receptor and the G_{i2} protein is validated by the observation that PTX treatment reduced the receptor level coimmunoprecipitated by G_{i2} antibody (Fig. 3J).

IGF-II/M6P receptor in cholinergic neurons

As a prelude to examining the effects of Leu 27 IGF-II on cholinergic function, we determined the presence of IGF-II/M6P receptor in the basal forebrain nuclei, which provide cholinergic projection to the hippocampus (Fig. 4A). Our immunoblotting results reveal that the IGF-II/M6P receptor is expressed in the NBM, lateral septum, medial septum/DBB, and hippocampus (Fig. 4B). At the cellular level, IGF-II/M6P receptor immunoreactivity is evident in neurons and their processes in the septum, striatum, NBM, DBB, and hippocampus (Fig. 4C, E). Additionally, all VAcHT-positive cholinergic neurons located in the basal forebrain are found to express the receptor (Fig. 4C–E).

Effect of Leu 27 IGF-II on ACh release

Based on the abundance of IGF-II/M6P receptors in cholinergic neurons, we sought to examine the effect of Leu 27 IGF-II and IGF-II on ACh release by stimulating hippocampal slices with 25 mM K^+ buffer. Leu 27 IGF-II significantly ($p < 0.01$) increased ACh release in a dose-dependent manner (Fig. 5A). The potentiation of ACh release was apparent during the final phase of stimulation with 10^{-8} and 10^{-9} M Leu 27 IGF-II (Fig. 5B), whereas no alterations in evoked release were evident at lower concentrations (i.e., 10^{-10} to 10^{-12} M). IGF-II (10^{-9} M), as observed for Leu 27 IGF-II, was also found to significantly potentiate evoked ACh release from rat hippocampal slices (Fig. 5C). Apart from hippocampus, Leu 27 IGF-II at 10^{-8} M augmented evoked ACh release in rat striatal slices (Fig. 5D). The stimulatory response of Leu 27 IGF-II was insensitive to TTX but sensitive to PTX (Fig. 5E). To determine whether Leu 27 IGF-II potentiates release by regulating ACh synthesis, the effects of Leu 27 IGF-II on ChAT activity and high-affinity choline uptake (HACU) were examined (Collier, 1988). However, neither ChAT activity nor HACU was altered by Leu 27 IGF-II (supplemental Fig. 1A, B, available at www.jneurosci.org as supplemental material). Finally, to confirm that the potentiation of ACh release by Leu 27 IGF-II is not mediated by the IGF-I receptor, hippocampal slices were exposed to 10^{-8} M Leu 27 IGF-II or 10^{-7} M IGF-I and then processed for IGF-I receptor immunoprecipitation, followed by blotting with

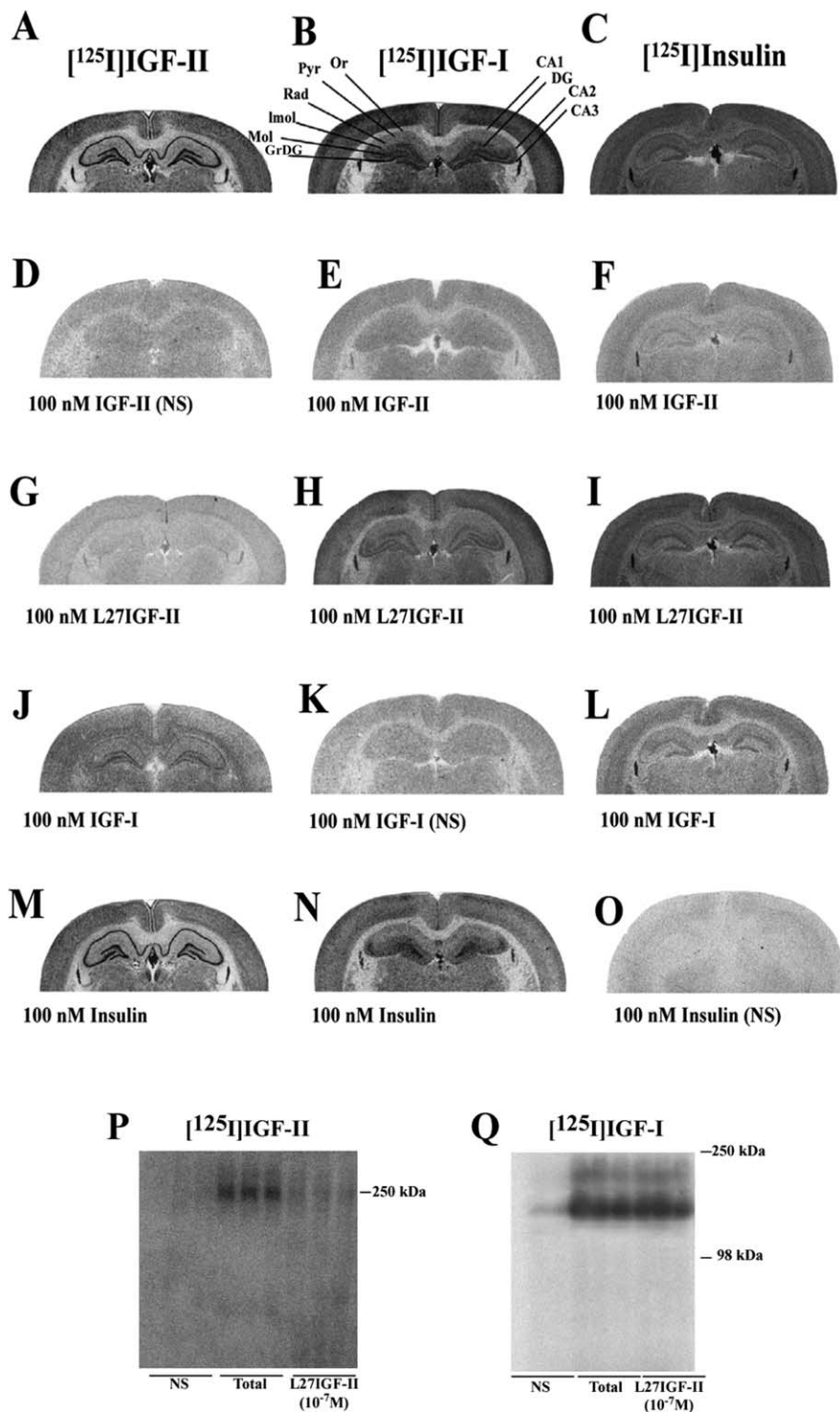


Figure 2. *A–Q*, Photomicrographs showing the autoradiographic distribution of [¹²⁵I]IGF-II (*A, D, G, J, M*), [¹²⁵I]IGF-I (*B, E, H, K, N*), and [¹²⁵I]insulin (*C, F, I, L, O*) binding sites in the absence or presence of 100 nM IGF-II, Leu²⁷IGF-II (L27IGF-II), IGF-I, and insulin in the adult rat hippocampus. [¹²⁵I]IGF-II binding in the hippocampus was competed potently by IGF-II > Leu²⁷IGF-II > IGF-I (*D, G, J*) but not much by insulin (*M*). [¹²⁵I]IGF-I binding was competed potently by IGF-I > IGF-II > insulin > Leu²⁷IGF-II (*K, E, N, H*). [¹²⁵I]Insulin binding was competed by insulin > IGF-II > IGF-I >> Leu²⁷IGF-II (*O, F, L, I*). *P, Q*, Affinity cross-linking of [¹²⁵I]IGF-II (*P*) and [¹²⁵I]IGF-I (*Q*) to rat hippocampal membranes showing that Leu²⁷IGF-II displaces radiolabel from the 250 kDa band corresponding to the IGF-II/M6P receptor but not from the 240 or 135 kDa bands bound by [¹²⁵I]IGF-I. NS, Nonspecific binding; Or, stratum oriens; Pyr, pyramidal cell layer; Rad, stratum radiatum; Imol, stratum lacunosum moleculare; Mol, molecular layer of the DG; GrDG, granular cell layer of the DG.

phosphotyrosine antibodies. Our data reveal that, although IGF-I increased tyrosine phosphorylation of the IGF-I receptor, Leu²⁷IGF-II did not (Fig. 5*F*). Furthermore, the well characterized IGF-I receptor antagonist JB-1 did not alter the potentiating effect of 10⁻⁸ M Leu²⁷IGF-II on hippocampal ACh release (Fig. 5*C*).

Intracellular pathway involved in Leu²⁷IGF-II-mediated ACh release

IGF-II binding to the IGF-II receptor has been shown to alter the activity of adenylate cyclase and PKC, two intracellular substrates involved in the regulation of ACh release (Zhang et al., 1997; McKinnon et al., 2001). In our study, Leu²⁷IGF-II did not alter [³H]cAMP levels during the course of the experiment (supplemental Fig. 1*C*, available at www.jneurosci.org as supplemental material). Interestingly, Leu²⁷IGF-II also did not alter either membrane or cytosolic PKC α levels at any time over the 1 h course of the experiment (data not shown), but membrane levels of phospho-PKC α (Fig. 6*A*) and its downstream substrates MARCKS and GAP-43 (Newton, 2001; Rhee, 2001) (Fig. 6*B, C*) were significantly increased during the initial 10 min of stimulation and then subsequently declined with their translocation into the cytosol. However, unlike phospho-PKC α or MARCKS, translocation of phospho-GAP-43 into the cytosol did not occur until 20 min exposure to Leu²⁷IGF-II. In contrast to phospho-PKC α , no alterations in phospho-PKC ϵ or PKC γ were evident after Leu²⁷IGF-II exposure (Fig. 6*D*). To confirm the involvement of PKC, hippocampal slices were superfused with 10⁻⁸ M Leu²⁷IGF-II in the presence or absence of 25 μ M PMB, a PKC inhibitor that has been shown to inhibit ACh release (Iannazzo et al., 2000). Although PMB itself did not alter evoked release, it did block the potentiation of ACh release induced by Leu²⁷IGF-II (Fig. 6*E*). Additionally, altered levels of phospho-PKC α (Fig. 6*F*), MARCKS, and GAP-43 (data not shown), observed after treatment with Leu²⁷IGF-II, were also found to be inhibited by PMB.

Effects of Leu²⁷IGF-II on DBB neurons

The effects of Leu²⁷IGF-II on ACh release were further examined using whole-cell patch-clamp recordings in dissociated DBB neurons (Figs. 7, 8). In 17 DBB neurons, application of 50 nM Leu²⁷IGF-II resulted in a reversible, significant decrease ($p < 0.01$) in the outward whole-cell currents in the voltage range from -30 to +30 mV (Fig. 7*A*), and the response did

not desensitize. In five DBB neurons, Leu²⁷IGF-II did not evoke a significant change in peak current at +30 mV (control, 5.4 ± 0.6 nA; Leu²⁷IGF-II, 5.5 ± 0.5 nA; $p = 0.58$). Under current-clamp conditions, Leu²⁷IGF-II evoked a reversible depolarization of the DBB neurons (11.2 ± 3.4 mV; $n = 6$) (Fig. 7F). Single-cell RT-PCR analysis revealed that Leu²⁷IGF-II-responsive cells expressed ChAT mRNA-derived product, whereas non-responsive cells expressed GAD mRNA (Fig. 7B).

Effects of blocking calcium-activated potassium currents (I_{Ca}) on the Leu²⁷IGF-II response

The contribution of $I_{K,Ca}$ or I_C (BK channels) to the voltage-activated currents can be determined by using the selective blockers of this family of voltage-sensitive calcium-activated potassium channels. The more sensitive and specific one is IBTX (IC_{50} of 0.25 nM), a snail toxin from *Buthus tamulus* (Galvez et al., 1990). Figure 7C shows an average of voltage-ramp relationship of currents from four cells evoked under control conditions, with 25 nM IBTX alone and with Leu²⁷IGF-II plus IBTX. IBTX applied alone caused a reduction in outward currents in the same voltage range as Leu²⁷IGF-II. When Leu²⁷IGF-II was applied in the presence of IBTX, there was no additional significant reduction in whole-cell currents than evoked by Leu²⁷IGF-II alone (IBTX, 4.5 ± 0.6 nA; Leu²⁷IGF-II plus IBTX, 4.3 ± 0.5 nA; $p > 0.05$). The inset in Figure 7C illustrates Leu²⁷IGF-II-evoked reduction of whole-cell currents under control conditions and in the presence of IBTX. Thus, 81% of the Leu²⁷IGF-II-evoked decrease in whole-cell currents is composed of an IBTX-sensitive calcium-activated potassium conductance.

Effects of Leu²⁷IGF-II on transient outward (I_A) and the delayed rectifier (I_K) potassium currents

I_A and I_K are voltage-sensitive currents, and their activation and inactivation are strongly voltage-dependent. I_A requires the holding potential to be relatively hyperpolarized (approximately -110 mV) for removal of its inactivation, whereas it is inactivated at -40 mV. Conversely, I_K is not inactivated at -40 mV. These biophysical properties of I_A and I_K can, thus, be used to isolate these currents. Therefore, a conditioning pulse to -40 mV will activate I_K without any significant contamination by I_A (Connor and Stevens, 1971; Jhamandas et al., 2001). A conditioning pulse to -120 mV will activate both I_A and I_K . The difference currents obtained by subtracting the currents evoked by depolarizing pulses after a conditioning pulse to -40 mV from those evoked

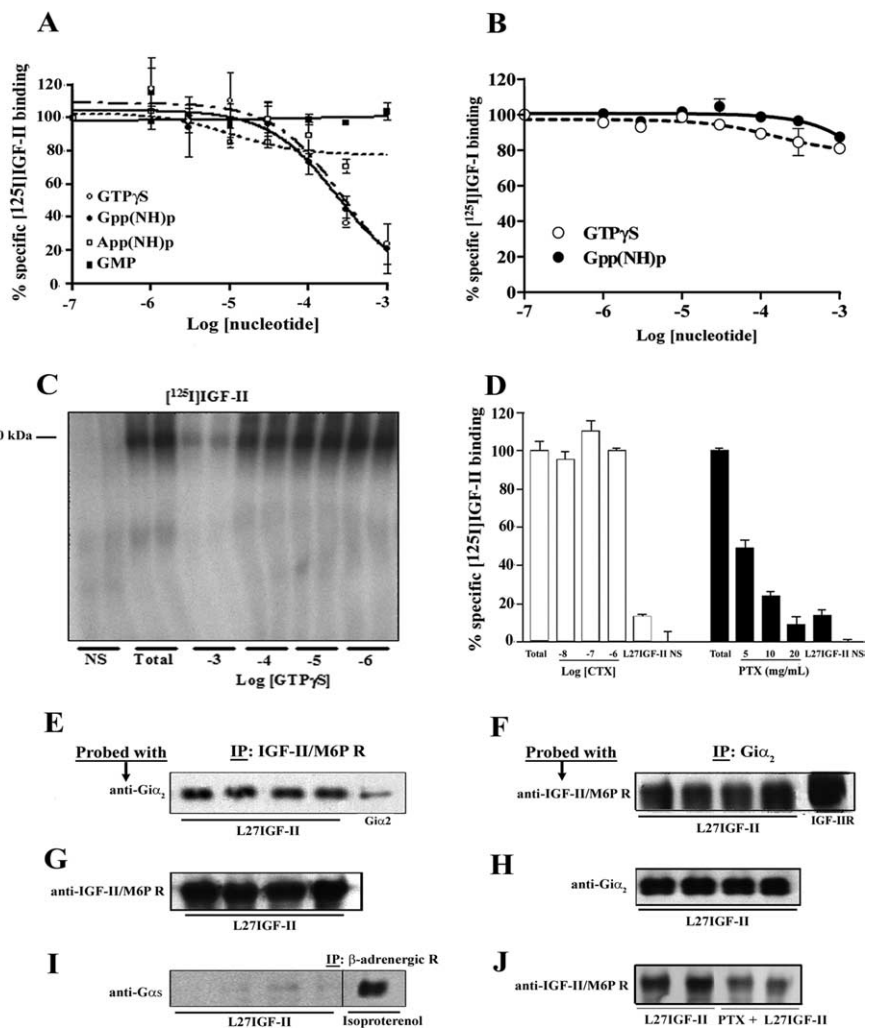


Figure 3. *A, B*, Competition binding experiments showing that GTP γ S and Gpp(NH)p, but not cGMP or App(NH)p, produced a dose-dependent decrease in [¹²⁵I]IGF-II binding in adult rat hippocampal membranes (*A*). [¹²⁵I]IGF-I binding was unaffected by either GTP γ S or Gpp(NH)p (*B*). *C*, Affinity cross-linking of [¹²⁵I]IGF-II to rat hippocampal membranes depicting that GTP γ S and IGF-II displace radiolabel from the 250 kDa band corresponding to the IGF-II/M6P receptor. *D*, Binding experiments showing that PTX, but not CTX, decreased [¹²⁵I]IGF-II binding in a dose-dependent manner. *E–H*, Western blots depicting reciprocal coimmunoprecipitation of the IGF-II/M6P receptor and $G_{i\alpha_2}$ -protein from hippocampal samples of four different adult rats. These panels show the results of immunoprecipitation (IP) by anti-IGF-II/M6P receptor, followed by Western blotting with anti- $G_{i\alpha_2}$ antibody (*E*) and the reciprocal immunoprecipitation Western blotting experiment (*F*). Purified $G_{i\alpha_2}$ (*E*, far right lane) or IGF-II/M6P receptor (*F*, far right lane) was loaded as controls. Antibody specificity was confirmed by immunoprecipitation of either IGF-II/M6P receptor followed by blotting with anti-IGF-II receptor antibody (*G*) or $G_{i\alpha_2}$ followed by blotting with anti- $G_{i\alpha_2}$ (*H*). *I*, Western blot showing that $G_{i\alpha_2}$ -protein can be coimmunoprecipitated from hippocampal samples by a specific β -adrenergic receptor antibody after isoproterenol stimulation (far right lane) but not by an IGF-II/M6P receptor antibody after Leu²⁷IGF-II treatment (left 4 lanes). *J*, Western blot showing that PTX treatment reduced the IGF-II/M6P receptor level coimmunoprecipitated by the $G_{i\alpha_2}$ antibody. All competition binding data, which are presented as the mean \pm SEM of percentage of specific binding, were obtained from three to five experiments, each performed in triplicate. Nonspecific (NS) and Leu²⁷IGF-II (L27IGF-II) binding detected in the presence of 10^{-7} M IGF-II and 10^{-7} M Leu²⁷IGF-II, respectively.

after a conditioning pulse to -120 mV provide an accurate estimate of I_A . Figure 7D, inset, shows the currents recorded from a DBB neuron with a conditioning pulse to -40 mV for 150 ms, representing mainly I_K , under control conditions and in the presence of Leu²⁷IGF-II. Figure 7E shows the difference currents recorded from the same neuron representing mainly I_A , under control conditions and in the presence of Leu²⁷IGF-II. Figure 7, D and E, shows the current–voltage (I – V) relationships of averaged peak I_K ($n = 4$) and I_A ($n = 5$), respectively, using the voltage step

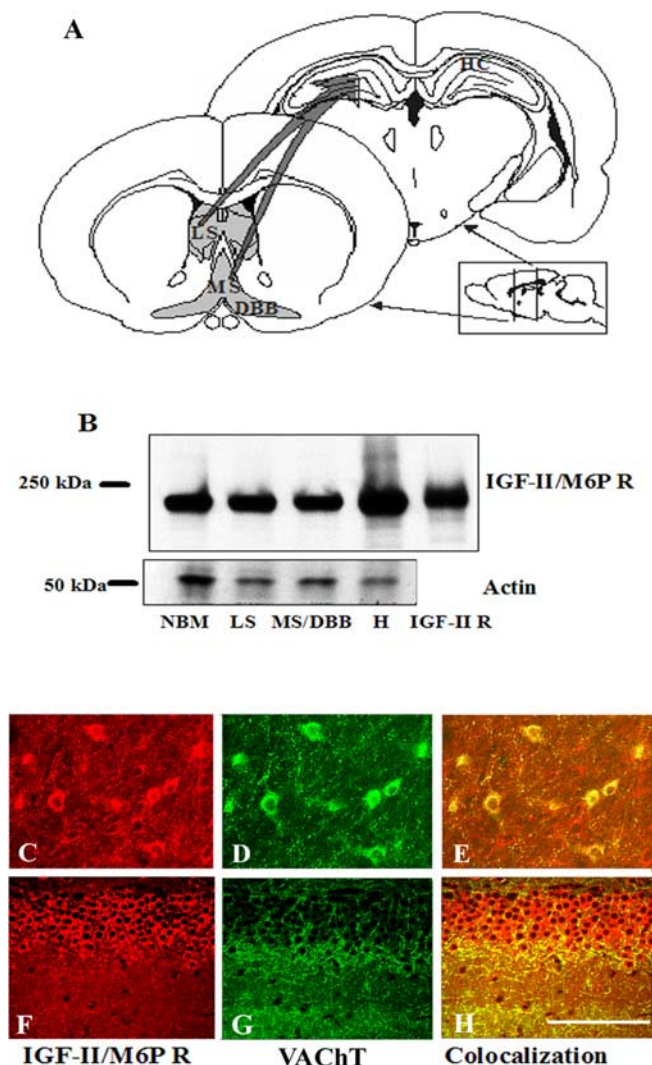


Figure 4. *A*, Schematic diagram of septohippocampal cholinergic projections in the adult rat brain. *B*, Western blot showing the presence of IGF-II/M6P receptor in the NBM, lateral septum (LS), medial septum/diagonal band complex (MS/DBB), and hippocampus (H) of the adult rat brain, with purified IGF-II/M6P receptor loaded as control. *C–H*, Immunofluorescence photomicrographs showing the IGF-II/M6P receptor (*C, F*) and VAcHT immunoreactivities (*D, G*) and their colocalization (*E, H*) in the basal forebrain (*C–E*) and hippocampal granule cell layers (*F–H*) of the adult rat brain. Scale bar, 10 μ m.

protocols shown in the insets of this figure. Leu²⁷IGF-II reduced I_K by 11% but did not affect I_A (peak currents measured at +30 mV).

Specificity of the Leu²⁷IGF-II response in DBB neurons

To establish that the effects of Leu²⁷IGF-II on DBB neurons were mediated via the IGF-II/M6P receptor, dissociated neurons were pretreated with or without a specific IGF-II/M6P receptor blocking antibody (1:100) (for antibody characterization, see supplemental Fig. 1*D*, available at www.jneurosci.org as supplemental material), and then their response to Leu²⁷IGF-II was assessed. Neurons treated with the IGF-II/M6P receptor antibody, but not preimmune Ig (1:100), exhibited a drastically reduced response to Leu²⁷IGF-II compared with the untreated neurons (untreated neurons, 20.0 ± 1.5 vs antibody-treated neurons, 6.8 ± 0.8 ; $p < 0.01$) (Fig. 8*A*) (supplemental Fig. 1*E*, available at www.jneurosci.org as supplemental material). In addition, the re-

sponse of 50 nM Leu²⁷IGF-II was found to be completely maintained in the presence of the IGF-I receptor antagonist JB-1 (20 μ g/ml) (supplemental Fig. 1*F*, available at www.jneurosci.org as supplemental material).

Effects of Leu²⁷IGF-II on DBB neurons in the presence of PTX and PKC inhibitor

To determine whether the Leu²⁷IGF-II-evoked effects on DBB neurons were mediated by a G-protein-coupled mechanism, neurons were pretreated with and without PTX (1 μ g/ml) for 5–8 h and assessed for their response to Leu²⁷IGF-II. Leu²⁷IGF-II did not evoke a significant reduction in whole-cell currents in PTX-treated neurons (control, 5.1 ± 0.4 nA; Leu²⁷IGF-II plus PTX, 5.0 ± 0.4 nA; $n = 12$; $p = 0.42$), whereas the analog caused a significant reduction in whole-cell currents in neurons not exposed to PTX (Fig. 8*B, C*). Additionally, Leu²⁷IGF-II had no effect on whole-cell currents in the presence of the PKC inhibitor PMB (10 μ M) when compared with control (control, 4.4 ± 0.7 nA; Leu²⁷IGF-II alone, 2.4 ± 0.2 nA; PMB alone, 4.0 ± 0.3 nA; Leu²⁷IGF-II plus PMB, 3.9 ± 0.2 nA; $p = 0.51$ for control vs Leu²⁷IGF-II plus PMB) (Fig. 8*D, E*).

Discussion

Using a combination of experimental approaches, the present study provides the very first evidence that the single-pass transmembrane IGF-II/M6P receptor expressed in the brain is G-protein coupled and is involved in the regulation of central cholinergic function by activating PKC-dependent signaling cascades. Traditional G-protein-coupled receptors exhibit a seven-transmembrane spanning conformation that mediate receptor–G-protein interactions (Neves et al., 2002; Pierce et al., 2002). Previous studies done in a cell-free non-neuronal system have suggested a possible interaction between the IGF-II/M6P receptor and G-proteins in response to IGF-II binding (Okamoto et al., 1990; Takahashi et al., 1993; Ikezu et al., 1995). However, failure of the IGF-II/M6P receptor to interact with a G-protein in mouse L-cell membrane and phospholipid vesicles (Sakano et al., 1991; Körner et al., 1995) has challenged the relevance of these results (Dahms and Hancock, 2002). The present study clearly demonstrates an interaction between rat hippocampal IGF-II/M6P receptors and a G-protein. This is supported by four different lines of evidence. First, only GTP γ S and Gpp(NH)p, which promote affinity reduction of ligand/receptor binding (Stiles et al., 1984), inhibited [¹²⁵I]IGF-II interaction with its receptor. Second, PTX, which causes ADP-ribosylation of a cysteine residue in the G_{i/o}-proteins (Yamane and Fung, 1993), inhibited [¹²⁵I]IGF-II receptor binding. Third, G_i α proteins can be coimmunoprecipitated with the IGF-II/M6P receptors and are sensitive to PTX treatment. Fourth, pretreatment with PTX abolished Leu²⁷IGF-II-mediated ACh release and the response of dissociated cholinergic neurons to this peptide. These results, however, did not provide any evidence of direct interaction between the IGF-II/M6P receptor and the G-protein. Thus, it is possible that the IGF-II/M6P receptor and PTX-sensitive G-proteins may constitute part of a larger protein complex, and the interaction observed in the present study is mediated indirectly via other protein(s). Although other receptors that lack seven transmembrane domains have also been shown to interact with G-proteins (Cunha et al., 1999; Dalle et al., 2001), our results provide compelling evidence that the single transmembrane domain IGF-II/M6P receptor in the adult rat brain is linked to and can mediate cell signaling by activating a G-protein.

The presence of IGF-I, IGF-II, and insulin receptors on most

cell types and their ability to bind insulin, IGF-I, and IGF-II has made it difficult to delineate a specific role for the IGF-II/M6P receptor in the mediation of a given biological response. The development of the Leu²⁷IGF-II analog, which interacts selectively with the IGF-II/M6P receptor in a variety of non-neuronal studies, has provided an opportunity to reexamine the role of IGF-II/M6P receptor in cell signaling (Burgisser et al., 1991; Minniti et al., 1992; McKinnon et al., 2001). Our receptor binding assays show that specific [¹²⁵I]IGF-I, [¹²⁵I]IGF-II, and [¹²⁵I]insulin binding sites are concentrated in distinct hippocampal regions. The IGF-I and insulin receptors bind preferentially their own ligands and interact with related ligands at lower affinity. Conversely, the IGF-II/M6P receptor recognizes IGF-II with higher affinity than IGF-I and does not interact with insulin. Leu²⁷IGF-II binds the IGF-II/M6P receptor with high affinity and does not interact substantially with either the IGF-I or insulin receptors. This is supported by our cross-linking studies, which revealed that Leu²⁷IGF-II competes for the IGF-II/M6P receptor but not the IGF-I receptor. These results show that Leu²⁷IGF-II in the adult rat brain, as reported for other tissues (Beukers et al., 1991; Roth et al., 1991; Rosenthal et al., 1994), acts as a rather selective analog for the IGF-II/M6P receptor. To substantiate further that the effects of Leu²⁷IGF-II are mediated by the IGF-II/M6P receptor, we have used a well characterized IGF-II/M6P receptor blocking antibody (MacDonald et al., 1989) that has been shown to distinguish a biological role for the receptor in non-neuronal tissues (Minniti et al., 1992; McKinnon et al., 2001).

Our electrophysiological data reveal that Leu²⁷IGF-II evokes a reduction of whole-cell currents in DBB cholinergic neurons within a voltage range in which a suite of potassium conductances is activated. Among the potassium conductances affected, Leu²⁷IGF-II mostly decreases the calcium-activated potassium conductance, *I_C*, with lesser effects on the delayed rectifier potassium conductance, *I_K*, and no discernible effects on the transient outward potassium conductance, *I_A*. Because *I_C* provides a drive for the repolarization phase of the action potential and plays an important role in the process of spike frequency adaptation (Vergara et al., 1998), the Leu²⁷IGF-II-induced blockade of *I_C* could explain the increase in excitability and depolarization observed under current-clamp conditions. In fact, we have shown previously that, in DBB neurons, inhibition of *I_C* by IBTX, a specific blocker of *I_C* or BK channels, results in membrane depolarization (Jhamandas et al., 2002).

The increase in the cellular activity of cholinergic neurons is

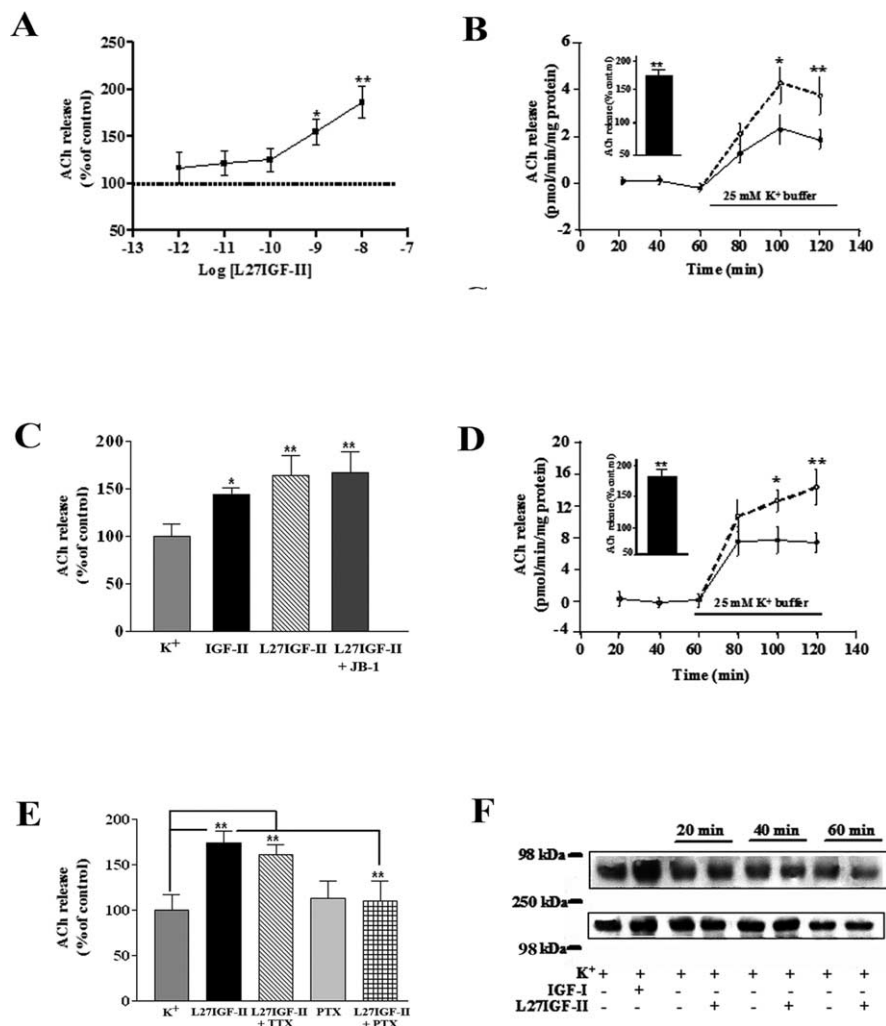


Figure 5. *A*, Dose-dependent potentiating effects of Leu²⁷IGF-II (L27IGF-II) on 25 mM K⁺-evoked ACh release from adult rat hippocampal slices. *B*, Time course effects of Leu²⁷IGF-II on the evoked ACh release from the hippocampus. Tissue slices were stimulated with 25 mM K⁺ buffer in the presence (dotted lines) or absence (solid lines) of 10⁻⁸ M Leu²⁷IGF-II, and evoked release was potentiated during late phase of stimulation. The inset in *B* represents total release as percentage of control over the 60 min stimulation period. *C*, Histograms showing that Leu²⁷IGF-II and IGF-II can potentiate ACh release from adult rat hippocampal slices and the effect of Leu²⁷IGF-II was unaltered in presence of IGF-I receptor antagonist JB-1 (20 μg/ml). *D*, Time course effects of Leu²⁷IGF-II on the evoked ACh release from the striatum. Tissue slices were stimulated with 25 mM K⁺ buffer in the presence (dotted lines) or absence (solid lines) of 10⁻⁸ M Leu²⁷IGF-II and evoked release was potentiated during late phase of stimulation. The inset in *D* represents total release as percentage of control over the 60 min stimulation period. *E*, Histograms showing that 10⁻⁸ M Leu²⁷IGF-II-mediated potentiation of evoked ACh release was unaffected by 10 μM TTX but attenuated in the presence of 25 μg/ml PTX in rat hippocampal slices. *F*, Effect of 10⁻⁷ M IGF-I and 10⁻⁸ M Leu²⁷IGF-II on tyrosine phosphorylation of the hippocampal IGF-I receptor as determined by immunoprecipitation followed by Western blotting. IGF-I, but not Leu²⁷IGF-II, increased the tyrosine phosphorylation of the IGF-I receptor. The blot shown in the bottom panel represents IGF-I receptor level after reprobing. The blots are representative of experiments that were replicated three times. All ACh release results are expressed as the mean ± SEM (*n* = 15–18). **p* < 0.05; ***p* < 0.01.

consistent with the observed potentiation of ACh release evoked by Leu²⁷IGF-II in brain slices. The latter effect was TTX insensitive, indicating that, within the hippocampus, Leu²⁷IGF-II may possibly act either in close proximity or directly on the cholinergic terminals. The localization of the IGF-II/M6P receptors on cholinergic terminals may provide an anatomical substrate for a direct action of Leu²⁷IGF-II, but it is possible that potentiation of ACh release can be mediated indirectly via other neurotransmitters, because Leu²⁷IGF-II requires prolonged depolarization to potentiate ACh release and IGF-II/M6P receptors in the hip-

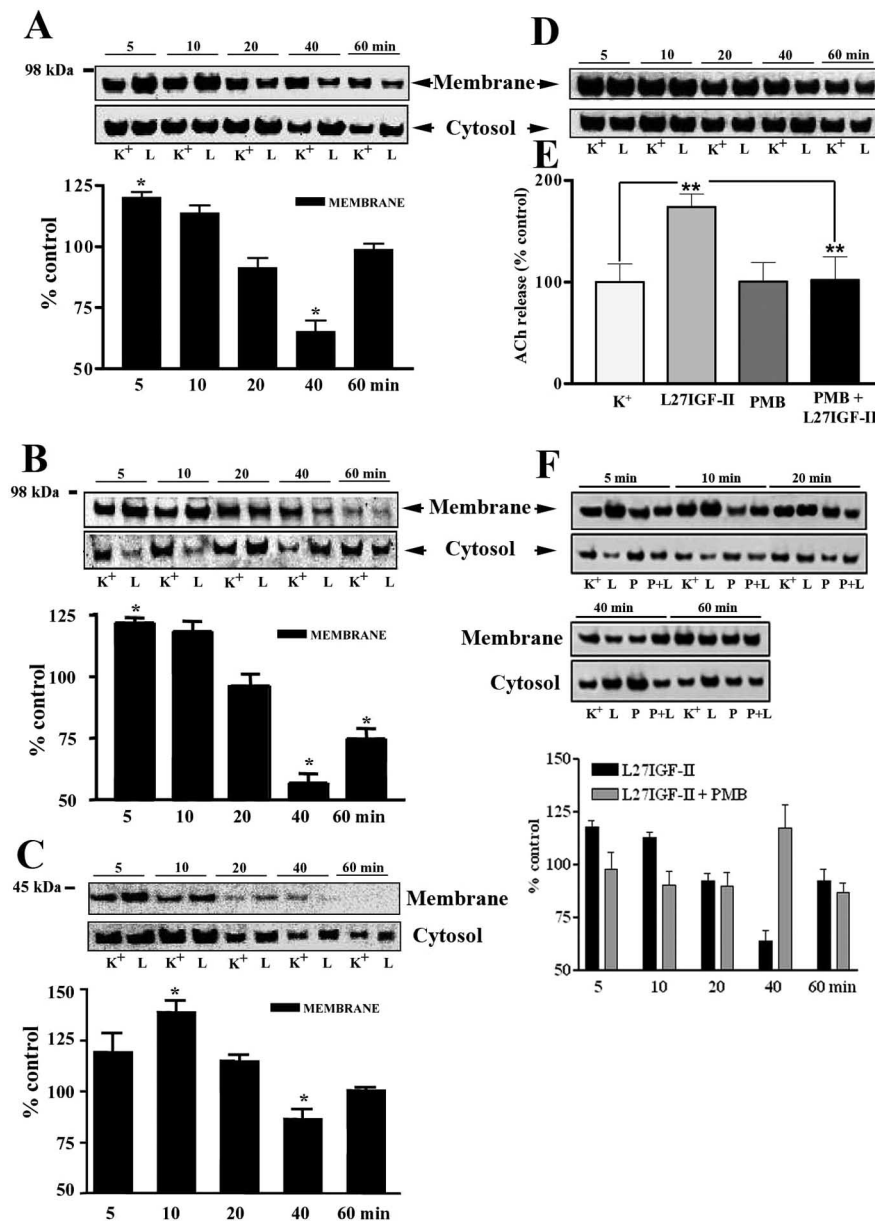


Figure 6. A–D, Leu²⁷IGF-II (L) significantly increased membrane levels of phospho-PKCα (A), phospho-MARCKS (B), and phospho-GAP-43 (C) within the first 10 min of stimulation compared with K⁺-treated (K) controls (top blots). This activation induced their translocation into the cytosolic fraction (bottom blots). Leu²⁷IGF-II had no effect on phospho-PKCε levels (D). Quantification of blots was performed as described previously, and histograms were compiled by dividing the optical density value of Leu²⁷IGF-II-treated samples by that of the corresponding K⁺-treated control samples, for every time point. E, Histogram showing that the PKC inhibitor PMB attenuated Leu²⁷IGF-II-mediated potentiation of hippocampal ACh release. Results are expressed as the mean ± SEM (n = 12–15). **p < 0.01. F, Western blots of phospho-PKCα levels in membrane (top blots) and cytosolic (bottom blots) fractions of hippocampal tissues treated with either high-K⁺ buffer alone (K) or in combination with Leu²⁷IGF-II (L), PMB (P), or PMB plus Leu²⁷IGF-II (P + L). Leu²⁷IGF-II-induced increase in membrane levels of phospho-PKCα was attenuated in the presence of PMB.

pocampus are located primarily on the cell bodies rather than in the cholinergic terminals. Because neither ChAT activity nor HACU is affected, it appears that IGF-II/M6P receptor activation modulates ACh release without altering ACh synthesis. Furthermore, given the evidence that Leu²⁷IGF-II binds rather selectively to the IGF-II/M6P receptor and the analog-induced depolarization of DBB neurons can be blocked by an IGF-II/M6P receptor antibody but not by preimmune serum or an IGF-I receptor antagonist, it is apparent that the potentiation of ACh release by Leu²⁷IGF-II is mediated via this receptor rather than

IGF-I receptor. This is further supported by three distinct lines of evidence: (1) Leu²⁷IGF-II, unlike IGF-I, does not induce tyrosine phosphorylation of the IGF-I receptor, (2) the IGF-I receptor antagonist JB-1 did not affect Leu²⁷IGF-II-mediated potentiation of ACh release, and (3) IGF-I has been shown to inhibit hippocampal ACh release indirectly via GABAergic neurons (Seto et al., 2002). The PTX-sensitive effects of Leu²⁷IGF-II on the reduction of whole-cell currents and the potentiation of ACh release not only reinforce our receptor binding data on G-protein-sensitive toxin but also implicate a role for the G_{i/o}-protein in the regulation of IGF-II/M6P receptor function.

Synaptic release of ACh is modulated by a variety of intracellular messengers, including cAMP and PKC, two molecules known to be regulated by IGF-II (Zhang et al., 1997; McKinnon et al., 2001). Our results indicate that cAMP level is not altered, but phospho-PKCα level and its downstream signaling molecules MARCKS and GAP-43 are time dependently increased in membrane and then in cytosolic fractions after exposure to Leu²⁷IGF-II. These results are in agreement with the established role of PKC in the regulation of ACh release (Vaughan et al., 1999; Iannazzo et al., 2000). Cellular subfractionation studies have demonstrated the recruitment of inactive cytosolic PKCα to the plasma membrane after the generation of second messengers [i.e., IP₃, diacyl glycerol (DAG), and Ca²⁺] for subsequent activation (Newton, 2001). This translocation occurs within 10 min of stimulation and is then gradually down-regulated (Stable and Parker, 1991). IGF-II/M6P receptor activation has been reported to increase the levels of IP₃ via phospholipase C (PLC), the enzyme that catalyzes IP₃ and DAG production (Rogers et al., 1990; Poiraudau et al., 1997). Given the evidence that PLC can be directly activated by the βγ subunits of G-proteins (Singer et al., 1997; Rhee, 2001), it is likely that, in our paradigm, Leu²⁷IGF-II stimulates PKCα phosphorylation after IGF-II/M6P receptor-mediated coupling to a G-protein. Activated PKCα subsequently phosphorylates membrane-associated MARCKS, which then translocates into the cytoplasm, allowing for a partial breakdown of the actin cytoskeleton barrier (Vaughan et al., 1999). At the same time, phospho-PKCα also activates membrane-associated GAP-43 proteins, facilitating calcium/calmodulin-dependent kinase activation and increased release of vesicular ACh. The observation that the PKC inhibitor PMB blocked Leu²⁷IGF-II-induced potentiation of ACh release as well as activation of phospho-PKCα, MARCKS, and GAP-43 reinforces a role for the PKC-dependent

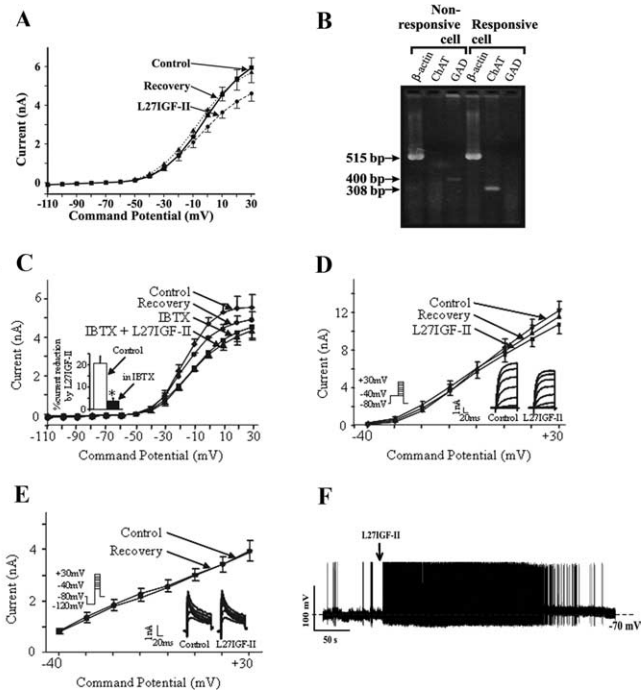


Figure 7. *A*, *I*–*V* relationship from 17 DBB neurons showing whole-cell currents evoked under control conditions, in the presence of 50 nM Leu²⁷IGF-II (L27IGF-II), and after recovery. *B*, Examples of single-cell RT-PCR analysis from two DBB neurons tested with Leu²⁷IGF-II. The Leu²⁷IGF-II-responsive neuron is cholinergic (ChAT positive), whereas the nonresponsive is GABAergic (GAD positive). For each cell, the presence of β -actin acts as a positive control. Lengths of fragments arising from amplification of mRNAs of β -actin of \sim 515 bp, ChAT of \sim 308 bp, and GAD of \sim 400 bp are indicated. *C*, *I*–*V* relationship of mean whole-cell currents evoked by applying voltage ramps under control conditions, in the presence of calcium-activated potassium channel blocker IBTX (25 nM) alone, and Leu²⁷IGF-II (50 nM) with IBTX. IBTX application resulted in a reduction in the outward currents in the voltage range from -30 to $+30$ mV. In the presence of IBTX, the Leu²⁷IGF-II response is significantly attenuated. In *D*, voltage protocol for recording I_K is depicted on the left with holding potential of -80 mV and a 150 ms conditioning pulse to -40 mV. In this protocol, outward currents are mediated mostly through I_K (delayed rectifier), and the effects of Leu²⁷IGF-II (50 nM) on a DBB neuron are shown in the inset (right). Voltage-dependent changes in mean I_K from four cells are shown in an *I*–*V* plot. *E*, In the same neuron as *D*, Leu²⁷IGF-II (50 nM) did not affect the transient outward K^+ current (I_A) (inset, right). I_A was obtained as difference currents by subtracting the currents obtained by the voltage protocol shown in *D* from that obtained by applying the voltage protocol shown in *E* (inset, left), in which cells were held at -80 mV and a 150 ms conditioning pulse to -120 mV was applied. Voltage-dependent changes in mean I_K from five cells are shown in an *I*–*V* plot. *F*, Under whole-cell current-clamp recording conditions, focal application of Leu²⁷IGF-II depolarized a DBB neuron and evoked action potential generation. The dashed line represents the resting membrane potential of this cell, -70 mV.

pathway in mediating response of the IGF-II/M6P receptor in cholinergic neurons. This is further substantiated by our electrophysiological data, which showed that PMB can inhibit Leu²⁷IGF-II-mediated response of whole-cell currents in dissociated cholinergic neurons.

The present study suggests that the IGF-II/M6P receptor can regulate central cholinergic function by activating a specific intracellular signaling pathway. These results are underscored by *in vitro* data that have shown that IGF-II upregulates the expression of ChAT enzyme in septal cultured neurons by activating the IGF-II/M6P receptor (Konishi et al., 1994) and promotes proliferation/survival of ChAT/calbindin-positive neurons in rat septal cultures (Silva et al., 2000). We also recently reported that IGF-II/M6P receptor level is decreased, as a function of apolipoprotein E $\epsilon 4$ allele, in the hippocampus of Alzheimer's disease brain (Kar et al., 2006), in which loss of cholinergic markers have been

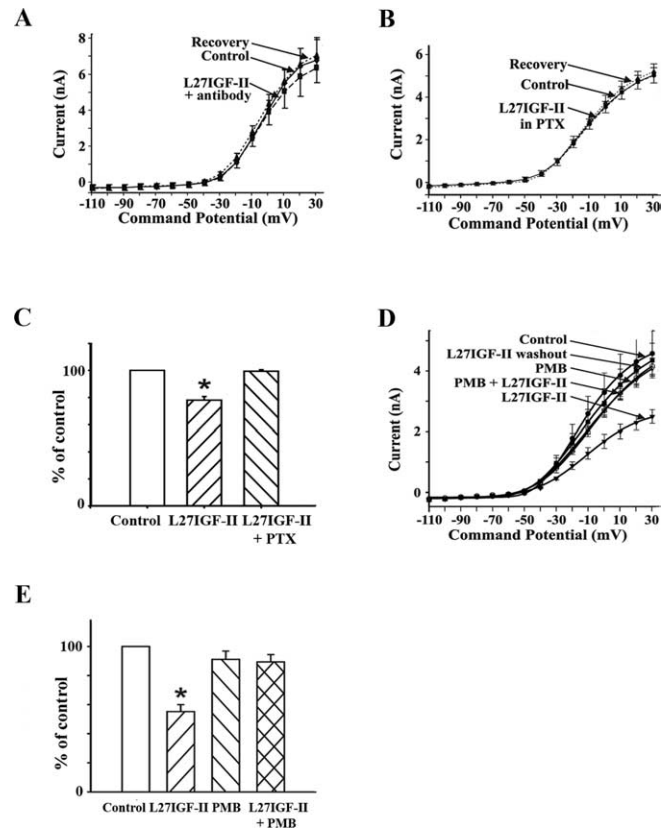


Figure 8. *A*, *I*–*V* relationship from DBB neurons ($n = 9$) pretreated with IGF-II/M6P receptor antibody (1:100), which showed a drastically reduced response to Leu²⁷IGF-II (L27IGF-II; 50 nM) in whole-cell currents. *B*, *I*–*V* relationship from DBB neurons ($n = 12$) pretreated with PTX (1 μ g/ml), in which Leu²⁷IGF-II (50 nM) did not evoke a significant reduction in whole-cell currents. *C*, Histograms show the effects of Leu²⁷IGF-II alone ($n = 17$) and in the cells pretreated with PTX ($n = 12$) as a percentage of control whole-cell currents at $+30$ mV; $*p < 0.01$. *D*, *I*–*V* relationship from DBB neurons ($n = 7$) in which Leu²⁷IGF-II evoked a significant and reversible reduction in whole-cell currents. In the same cells, inclusion of PMB (10 μ M) in the perfusion medium blocks the Leu²⁷IGF-II-induced reduction of whole-cell currents. *E*, Histograms depict the effects of Leu²⁷IGF-II, PMB, and Leu²⁷IGF-II in the presence of PMB ($n = 7$) as a percentage of control whole-cell currents at $+30$ mV; $*p < 0.01$.

associated with impairment of cognitive functions (Francis et al., 1999). These results together suggest that the IGF-II/M6P receptor may have a role not only in the normal brain but also in degenerative disorders characterized by preferential loss of cholinergic neurons.

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