Nerve Growth Factor-induced Glutamate Release Is via p75 Receptor, Ceramide, and Ca\(^{2+}\) from Ryanodine Receptor in Developing Cerebellar Neurons*

Received for publication, April 28, 2003, and in revised form, August 4, 2003
Published, JBC Papers in Press, August 5, 2003, DOI 10.1074/jbc.M304409200

Tadahiro Numakawa§§, Hitoshi Nakayama§§, Shingo Suzuki**, Takekazu Kubo‡‡, Futoshi Nara‡‡, Yuniko Numakawa§§, Daisaku Yokomaku†, Toshiyuki Araki**, Tetsuya Ishimoto‡, Akihiko Ogura**, and Takahisa Taguchi‡

From the §Neurones R. G. Special Division for Human Life Technology National Institute of Advanced Industrial Science and Technology (AIST) Midorigaoka, Ikeda, Osaka 563-8577, Japan, §Department of Mental Disorder Research, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, 4-1-1, Ogawa-higashicho, Kodaira-city, Tokyo 187-8502, Japan, ¶Nara Medical University Kashihara, Nara 634-8521, Japan, **Institute for Protein Research, Osaka University, Suita, Osaka 565-0871, Japan, §§Stankyo Co., Ltd., 2-58 Hiromachi 1-chome, Shinagawa-ku, Tokyo 140-8710, Japan, §§§Human Stress, AIST, Ikeda 563-8577, Japan, and ¶¶Graduate School of Frontier Biosciences, Faculty of Science, Osaka University, 1-1 Machikaneyama-cho, Toyonaka, Osaka 560-0043, Japan

Very little is known about the contribution of a low affinity neurotrophin receptor, p75, to neurotransmitter release. Here we show that nerve growth factor (NGF) induced a rapid release of glutamate and an increase of Ca\(^{2+}\) in cerebellar neurons through a p75-dependent pathway. The NGF-induced release occurred even in the presence of the Trk inhibitor K252a. The release caused by NGF but not brain-derived neurotrophic factor was inhibited, suggesting that a ceramide produced by sphingomyelinase was required for the NGF-stimulated Ca\(^{2+}\) release, suggesting that a ceramide produced by sphingomyelinase was required for the NGF-induced Ca\(^{2+}\) release was inhibited, suggesting that the NGF-regulated the endogenous p75 expression, the NGF-increased Ca\(^{2+}\) was essential for the NGF-induced glutamate release. Furthermore, scyphostatin, a sphingomyelinase inhibitor, blocked the NGF-dependent Ca\(^{2+}\) increase and glutamate release, suggesting that a ceramide produced by sphingomyelinase was required for the NGF-stimulated Ca\(^{2+}\) increase and glutamate release. This action of NGF only occurred in developing neurons whereas the brain-derived neurotrophic factor-mediated Ca\(^{2+}\) increase and glutamate release was observed at the mature neuronal stage. Thus, we demonstrate that NGF-mediated neurotransmitter release via the p75-dependent pathway has an important role in developing neurons.

Neurotrophins have been recognized as key molecules in the long term maintenance of the CNS through processes such as development, morphological differentiation, and survival (1–3). Recently, studies have shown that neurotrophins play a fundamental role in the acute effects on neuronal plasticity. In particular, BDNF has important roles in neuronal transmissions and activity-dependent neuronal plasticity (4, 5). As an example, BDNF was reported to induce excitatory transmissions (6), an influx of Ca\(^{2+}\) and Na\(^{+}\) (7), the generation of action potentials (8), and increased intracellular Ca\(^{2+}\) (9). We also reported that BDNF induced the release of excitatory neurotransmitter glutamate through a TrkB/PLC-γ1/P\(_{i}\)-dependent pathway in cultured cortical and cerebellar neurons (10, 11). On the whole, these actions of BDNF were dependent on the activation of a specific receptor, TrkB, which is broadly expressed in the brain. On the other hand, the expression of TrkA, a high affinity receptor for NGF, is very restricted, and only limited neuronal populations (for instance, cholinergic neurons in the basal forebrain) exhibited biological responses to NGF (12, 13).

p75 is the first neurotrophin receptor to be discovered in the receptor family that includes Fas and tumor necrosis factor receptors (14) and is expressed in a wide range of both neuronal and non-neuronal cells (15, 16). NGF also binds to the p75 receptor, which binds other neurotrophins with similar affinity, including BDNF, neurotrophin-4/5 (NT-4/5), and NT-3. The p75 receptor was found to be necessary for amplifying NGF/TrkA-mediated biological effects in early reports (17–19). In addition to increasing the response of cells co-expressing p75 and Trks, p75 is also able to transduce signals in the absence of Trks; for example, NGF activated the transcription factor nuclear factor κB, c-Jun amino-terminal kinase, and ceramide generation via p75 (20–22). In particular, studies on neurotrophin-dependent neuronal cell death have focused on the role of c-Jun amino-terminal kinase, mixed lineage kinase, and p53 (23–26).

Endogenous ceramide can be generated by the hydrolysis of sphingomyelin stimulated by neurotrophins via activation of p75 (20) and is known to be a second messenger that works in diverse intracellular pathways, with effects on neuronal outgrowth/survival and death (27, 28). Several reports (29–31)
NGF-induced p75-dependent Glutamate Release

indicated that the NGF-selective binding of p75 resulted in the promotion of survival and protection from apoptosis. Interestingly, Yamashita et al. (32) reported that GTPase RhoA activity, modulated by p75, participated in axonal outgrowth. Furthermore, Bocchigl and Sirrenberg (33) reported that NGF affected dopamine release via p75 in cultured mesencephalic dopaminergic neurons. These studies suggest that not only Trks but also p75 receptor-mediated pathways contribute to the neuronal system. However, very little is known about p75 receptor-dependent regulation in the glutamatergic system, and the intracellular mechanism including the Ca2+-dependent secretion of transmitter release following p75 activation is still unclear.

In this study, we found that NGF induced a rapid release of glutamate from cultured cerebellar neurons. It was revealed that the release did not require the activation of Trks but did require the p75 receptor, which induced the activation of sphingomyelinase, the generation of ceramide, and an increase in Ca2+ through the ryanoend Ca2+ receptor. Furthermore, we found that the NGF-induced release of glutamate was only observed in immature cerebellar neurons. We propose that an acute action of NGF through the p75 receptor is involved in the release of neurotransmitters in developing cerebellar neurons.

EXPERIMENTAL PROCEDURES

Cell Culture—Primary dissociated cultures were prepared from 4- to 5-day-old rat (SLC, WistarST, Japan) cerebellum as reported previously (12). Briefly, cells were gently dissociated with a plastic pipette after digestion with papain (90 units/ml; Worthington) at 37 °C. For the measurement of amino acids, the dissociated cells were plated on polyethyleneimine-coated 12- and 24-well plates (4- or 2-cm² surface area/well, respectively; Corning Glass). The cultures were maintained for 1–14 days. The cultures were maintained for 1–14 days.

Preparation and Titration of Adenovirus Vectors—cDNA of NLS-LacZ and rat p75 were subcloned into the Smal site of the cosmid vector pXScAvw under the control of the CAG promoter (34, 35). These cosmid vectors and the EcoT221-digested DNA-terminal protein complex (DNA-TPC) of Ad5-dix, which is a human type 5 adenovirus lacking the E3 region, were co-transfected into HEK 293 cells by the calcium phosphate method. The recombinant adenoviruses AdXCanAALS-LacZ and AdXCaAP75 generated by homologous recombination in HEK 293 cells were isolated. As the recombinant adenoviruses did not include the E1A region, PCR amplification of this region was performed to ensure no contamination by wild type adenovirus (Ad5-dix). The recombinant adenoviruses, which showed no PCR amplification of E1a, were then used, propagated in HEK 293 cells, and purified by cesium gradient centrifugation (36). The viral titer was determined by a plaque assay on HEK 293 cells. We performed adenovirus-mediated gene transfer 24 h prior to the neurotrophin stimulation.

Immunocytochemistry—Cells were stained with two or p75-antibodies or an anti-MAP2 antibody (rabbit IgG; a gift from Dr. H. Murofushi, University of Tokyo). Briefly, for staining with Mc-192 (conditioned medium from hybridoma cells, a gift from Dr. M. V. Chao), and p75-antibody, cells were fixed in 4% paraformaldehyde at room temperature for 20 min and then incubated overnight at 4 °C with Mc192 diluted 1:200 in 0.05% Triton X solution. The other p75-antibody (Promega) was incubated at a 1:500 dilution. The anti-MAP2 antibody was used at 1:1000. Cells were visualized using a Vectorsain ABC kit (Vector Laboratories) together with 0.02% (w/v) 3,3'-diaminobenzidine tetrahydrochloride and 0.01% (v/v) H2O2 in 0.05 M Tris-HCl buffer, pH 7.6, containing 0.1% (v/v) H2O2. The amount of amino acids released from the cultures was measured as described previously (11, 38). Briefly, the amino acids released into the modified HEPES-buffered Krebs-Ringer assay buffer (130 m NaCl, 5 mM KCl, 1.2 mM NaHPO4, 1.8 mM CaCl2, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4) were measured by high pressure liquid chromatography (Shimadzu, Kyoto, Japan) with a fluorescence detector (excitation wavelength, 340 nm; emission wavelength, 445 nm; Shimadzu, Kyoto, Japan). NGF and BDNF dissolved in phosphate-buffered saline containing bovine serum albumin (1 mg/ml) were added to the cultures. The amount of amino acids increased every minute. After exposing the cultures to these neurotrophins (1 min), basal fractions (1 min) were collected. The high KCl (100 mM) solution consisted of 85 mM NaCl, 50 mM KCl, 1.2 mM NaHPO4, 1.8 mM CaCl2, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4. The Ca2+-free solution was prepared by omitting the CaCl2.

Thapsigargin (1.0 μM) (Research Biochemicals, Natick, MA) was added 30 min prior to NGF stimulation. BAPTA-AM (Research Biochemicals) was used at 100 μM. Dantrolene (Tocris Cookson Ltd.), a ryanoend calcium channel receptor antagonist, was applied at 0.1, 1.0, or 10 μM for 20 min before the assay. Cyphostatin (a gift from Sankyo Inc., Tokyo), a sphingomyelinase inhibitor, was applied for 20 min at 0.1 and 1.0 μM. Mouse osteospongic C (Calbiochem), an IP3 calcium channel receptor antagonist, was applied at 10 μM for 1 h before the assay. When the effects of the various drugs on NGF-, BDNF- or ceramide-induced glutamate release were examined, the neurons were washed three times, and sample fractions in the presence of these drugs were collected. C2-ceramide, C6-ceramide, and C6-dihydroceramide were purchased from Biomol Research Labs., Inc. The glutamate release experiments were performed at least four times with independent cultures to confirm reproducibility. Representative data from a sister culture (n indicates the number of wells of a plate) are shown in the figures.

Ca2+-Imaging—Cells were cultured on polyethyleneimine-coated plates (Matsunami, Osaka, Japan) attached to fibercept (IN VITRO). After being washed, the cells were incubated for 1 h at 37 °C with 10 μM Fluo-3 AM (Molecular Probes, Eugene, OR) dilute in HEPES-buffered Krebs Ringer assay buffer. The dye intensity was monitored using a confocal laser microscope (RCM 8000; Nikon, Tokyo, Japan). Cells were irradiated with an excitation blue light beam (488 nm) produced by an argon ion laser. The emitted fluorescence was guided through a ×40 water-immersion objective to a pinhole diaphragm at 520 nm using a diachronic mirror. The intensity of emission was scanned at a dwell time of 1/30 s with a monitor video enhancer. Image data were obtained every 2 or 4 s. The data were stored in an RCM work station and analyzed. The fluorescent dye intensity stimulated by neurotrophins or ceramide analogs was normalized to the baseline before stimulation. It was confirmed that the dye fluorescence intensity was stable with minimum bleach for more than 3 min in vehicle solution. All imaging experiments were performed at least six times with separate cultures. Representative data from neurons in a sister culture are shown in the figures.

Immunoblotting—The cerebrums and cerebellums were segregated and homogenized in lysis buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.15 M NaCl, 10 mM NaF, 1 mM Na2VO4, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 10 mM Na3P2O7, and 100 μM phenylsine oxide). Cultured neurons were washed once with ice-cold phosphate-buffered saline and then lysed in the same buffer. Lysates were centrifuged at 15,000 rpm for 10 min at 4 °C, and the supernatant was collected and stored at -80 °C without any buffer. Immunoblotting was carried out as described previously (11). The anti-TrkB monoclonal antibody (clone 47) was purchased from BD Biosciences (1:1000). The anti-p75 polyclonal antibody was from Promega (1:1000). The anti-phospho-Trk antibody and the anti-pan Trk polyclonal antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) (1:1000), and the anti-β-arrestin-2 antibody was from Jackson ImmunoResearch Laboratories, Inc. (1:1000). The anti-ClassIII β-tubulin (TUJ1) was purchased from Berkeley Antibody Company (Richmond, CA) (1:1000).

Mg2+-Dependent Neutral Sphingomyelinase Activity—The Mg2+-dependent neutral sphingomyelinase activity was determined as described previously (39) except for the preparation of cell extracts. The cerebellar neurons cultured for 7 days on 100-mm dishes were stimulated with NGF or BDNF (both at 100 ng/ml) for the periods indicated in the figure. The cells were lysed in 5 mM Tris-HCl, pH 7.4, containing 1% Triton X-100.
FIG. 1. NGF induced the rapid release of glutamate in cultured cerebellar neurons. A, dose-dependent effect of NGF on glutamate release. Cerebellar neurons were cultured from postnatal 4–5-day-old rats and maintained for 6 days in vitro before the assay. NGF at 100 ng/ml exerted the maximum effect. NGF had no effect at 3 or 10 ng/ml. The vehicle alone (bovine serum albumin (BSA) at 2 ng/ml final) also had no effect. B, K252a, a Trk inhibitor (200 nm), completely inhibited the BDNF-induced release. In contrast, NGF still induced the glutamate release with K252a. Before exposing the culture to NGF or BDNF (1 min), basal fractions (1 min) were collected (DIV6). C, effect of neurotrophins on the phosphorylation of Trks. Cultured cerebellar neurons were incubated with NGF, BDNF, and NT-4/5. All neurotrophins were applied at 100 ng/ml for 30 s. BDNF and NT-4/5 phosphorylated the Trks, but NGF did not. Immunoblotting was performed with anti-phospho-Trk (p-Trks; upper) or anti-Trk (lower) antibodies. Immunoblotting with anti-class III β-tubulin (TUJ1) antibody was also performed. D, time course analysis of the glutamate release induced by NGF. The NGF-induced release was maintained for 2–3 min and then returned to the basal level. NGF was applied at 100 ng/ml (DIV6). Data represent the mean ± S.D. (n = 6). Statistical analysis was performed with Student’s t test. **, p < 0.01 versus basal.

RESULTS
NGF Induced a Rapid Release of Glutamate in Cultured Cerebellar Neurons—To clarify the p75-dependent neurotransmitter release, we investigated the NGF-induced glutamate release in cultured cerebellar neurons that are known to lack TrkA, a specific receptor for NGF. First, we examined the dose-dependent effect of NGF on the glutamate release. As shown in Fig. 1A, NGF significantly induced the release of glutamate at high concentrations (50 or 100 ng/ml) although no release was triggered at 3 or 10 ng/ml. The vehicle alone (bovine serum albumin; 2 ng/ml) had no effect. The BDNF-induced release was marked at 3 or 10 ng/ml (43), implying the involvement of the low affinity receptor p75 in the NGF-induced release. Previously (10, 11), we found that the BDNF-induced glutamate release occurred through the PLC-γ pathway following TrkB activation. Because the dose-dependence of NGF on the release was greater than that of BDNF, we further examined whether Trk receptors were involved in the NGF-induced release. In the presence of K252a, a Trk receptor inhibitor, BDNF did not induce any glutamate release, but NGF did, indicating that the NGF-induced release was not through Trks (Fig. 1B). Furthermore, we confirmed the tyrosine phosphorylation of Trks stimulated by neurotrophins. Treatment with BDNF or NT-4/5 (100 ng/ml, respectively) for 30 s caused significant Trks phosphorylation whereas NGF (100 ng/ml for 30 s) had no effect (Fig. 1C). These results indicated that the glutamate release induced by NGF occurs through a Trk-inde-
ependent pathway. A time course analysis of the NGF-induced release was also performed (Fig. 1D). Glutamate release was observed immediately after the application of NGF at 100 ng/ml and was maintained for 2–3 min.

**Overexpression of p75 Enhances NGF-induced Glutamate Release**—To clarify the possibility of p75 involvement in the NGF-mediated glutamate release, we measured glutamate release in cerebellar neurons overexpressing p75. At 24 h after transfection with the adenovirus-mediated gene transfer system, the NGF-induced release in cultured cells that expressed either p75 at two different intensities (m.o.i. of 3 or 10) or lacZ alone (control, m.o.i. of 10) was measured. In this system, the NGF-induced glutamate release was enhanced in a p75 expression-dependent manner; that is, the release was greater in p75-overexpressing neurons than lacZ-transfected cells (Fig. 2, A and C). In contrast, the BDNF-induced release was not increased in the p75-overexpressing cultures (Fig. 2C). The intensity of p75 expression was confirmed by immunocytochemical analysis using two anti-p75 antibodies (Fig. 2B). The intensity of p75 expression depended on the m.o.i. of p75 adenovirus (a and b, m.o.i. of 10 (p75); c and d, m.o.i. of 3 (p75); e and f, m.o.i. of 10 (lacZ)). In addition, the level of p75 overexpression in the adenovirus-infected cultures was determined by Western analysis (Fig. 2B, g). The expression of TrkB or TUJ1 (a neuronal marker) was not changed by p75 overexpression. lacZ-infected cultures showed no change in endogenous p75 expression. In our culture, the p75 overexpression did not induce cell death within 24 h after the transfection (data not shown). Further, to reveal whether the NGF-dependent release was through p75, we tested the counteracting effect of the p75 antibody in untransfected cells. MC192 (p75 antibody) was known to inhibit the NGF-dependent biological effects on neuronal cultures (31, 33) although MC192 did not block NGF binding to p75 (44). It was revealed that MC192 blocked the NGF-induced glutamate release (Fig. 2D). In contrast, the BDNF-induced release was not blocked by MC192. Heat-inactivated NGF did not trigger the release of glutamate (Fig. 2D). Furthermore, we examined the effect of p75-siRNA on the NGF-induced glutamate release. It was shown that endogenous p75 expression in Schwann cells was inhibited by p75-siRNA (37). The previous study (45) described that siRNA could be readily introduced into cultured neurons. After transfection of p75-siRNA in this study, the NGF-induced release was reduced significantly (Fig. 2E, a). Scramble (control) siRNA-transfected cells still responded to NGF (Fig. 2E, a). We confirmed that the expression of endogenous p75 was significantly down-regulated by p75-siRNA transfection (Fig. 2E, b, None; 1.00 ± 0.08, p75-siRNA; 0.54 ± 0.10, ratio (per control), n = 3, quantification by densitometry). In contrast, the expression of endogenous TrkB was not changed (Fig. 2E, b). Although the degree of reduced p75 expression in the presence of p75-siRNA was about 50%, a complete loss of the NGF-induced glutamate release was observed, implying that there was a threshold in the p75 expression to activate the pathway downstream of p75. The effect of p75-siRNA on the BDNF-induced glutamate release was examined. An inhibitory influence on the BDNF-induced release was not observed (None; 2.57 ± 0.20, p75-siRNA; 2.72 ± 0.23, ratio (BDNF-induced/basal), n = 6, respectively). All these results strongly suggested that the NGF-induced release was p75-dependent.

**Intracellular Ca\(^{2+}\) Increase Derived from Ryanodine Receptors Was Essential for the NGF-induced Glutamate Release**—In a previous study (10, 11), we reported that the PLC-γ/IP₃/Ca\(^{2+}\) pathway downstream of TrkB was essential for the BDNF-induced release of glutamate. However, we needed to identify the type of Ca\(^{2+}\) dependence in the NGF-induced release. First, we analyzed the change in the intracellular concentration of Ca\(^{2+}\) induced by exposure to NGF. The application of NGF induced an acute increase in the intracellular Ca\(^{2+}\) in cerebellar neurons (Fig. 3, A and B, a). This increase was observed in both neurites and soma, and the time dependence of the increase in the cell bodies was similar to that in neuritis. In this study, quantitative data on the intracellular concentration of Ca\(^{2+}\) were obtained by analyzing each cell body selected (activated cells), because we could detect a much higher intensity of dye emission in cell bodies than in neurites. The Ca\(^{2+}\) increase still occurred in the extracellular Ca\(^{2+}\)-free condition (Fig. 3C). However, BAPTA-AM (an intracellular Ca\(^{2+}\) chelator) blocked the NGF-induced increase in Ca\(^{2+}\) (Fig. 3C). Thapsigargin, which causes a depletion of intracellular Ca\(^{2+}\) stores, also blocked any increase (Fig. 3, B and C, b), indicating that Ca\(^{2+}\) derived from intracellular Ca\(^{2+}\) storage sites was important. Thus, we tested the effect of xestospongin C, a cell-permeable IP₃ receptor antagonist, on the increase in Ca\(^{2+}\) caused by NGF and found that the increase occurred even in the presence of xestospongin C (Fig. 3D), suggesting that another intracellular Ca\(^{2+}\) receptor, in addition to the IP₃-sensitive channel receptor, was critical for a rise in Ca\(^{2+}\) levels. Therefore, we examined the involvement of the ryanodine Ca\(^{2+}\) channel receptor (RyR). Dantrolene, a RyR blocker, eliminated the increase in intracellular Ca\(^{2+}\) induced by NGF (Fig. 3D). We next investigated the Ca\(^{2+}\) dependence of the NGF-induced glutamate release. In the extracellular Ca\(^{2+}\)-free condition, NGF was still able to induce the release (Fig. 4A), indicating that no Ca\(^{2+}\) influx from the extracellular Ca\(^{2+}\) was required. On the other hand, BAPTA-AM and thapsigargin completely eliminated the NGF-induced release (Fig. 4A), suggesting that the Ca\(^{2+}\) from intracellular Ca\(^{2+}\) storage sites was important. As expected, dantrolene blocked the release of glutamate induced by NGF in a dose-dependent manner (Fig. 4B). In contrast, xestospongin C failed to block the NGF-induced release (Fig. 4B). These results strongly suggested that the Ca\(^{2+}\) increase through ryanodine-sensitive Ca\(^{2+}\) pools is essential for the NGF-induced glutamate release.

**Ceramide Works as a Second Messenger in the NGF-induced Glutamate Release**—Many reports have shown that NGF-regulated sphingomyelin hydrolysis is critical for NGF action via the p75 signal cascade in neuronal biological responses. For example, ceramide, which is produced by sphingomyelinase, is involved in NGF-mediated neuronal cell death (24, 28). Therefore, we attempted to clarify whether ceramide production was involved in the NGF-induced glutamate release. First, we tested the effect of C₂-ceramide, a ceramide analog, on the release. We found that C₂-ceramide evoked the release of glutamate in a dose-dependent manner (Fig. 5A). C₂-ceramide at a low concentration (10 nM) was effective, although a much higher level of release was achieved at 10 μM (9- to 10-fold the basal level). The vehicle (Me₂SO) had no effect. In the following experiment, we used C₂-ceramide at a final concentration of 1.0 μM, because some reports have indicated that a high concentration causes apoptosis (46) and thus is not suitable for the regulation of neurotransmitter release. Furthermore, C₃-ceramide, or dihydroceramide (an inactive analog), was examined. In addition to C₂-ceramide, C₃-ceramide also induced glutamate release, but dihydroceramide did not (Fig. 5B). It was found that dantrolene significantly decreased the C₂-ceramide-induced glutamate release (Fig. 5B). The effect of sphingomyelinase, which enzymatically produces ceramide, was examined, and significant glutamate release was observed within 3 min after the addition (0.1 unit/ml, basal; 47.2 ± 3.4, stimulated; 103.6 ± 12.5 × 10⁻³⁰ mol/well, p < 0.01 (n = 6), respec-
tively), indicating that the ceramide generation resulted in glutamate release.

Next, the change in the intracellular Ca$^{2+}$ concentration induced by ceramide was monitored. C6-ceramide increased intracellular Ca$^{2+}$ significantly (Fig. 5C). The application of C6-ceramide evoked an acute increase in the intracellular Ca$^{2+}$, and the level of Ca$^{2+}$ was maintained for 1–2 min (Fig. 5C, c). Almost all of the neurons responded to C6-ceramide (Fig. 5C, a and b). However, dihydroceramide did not increase Ca$^{2+}$ (Fig. 5C, d), indicating that this neuronal response to ceramide was specific. The C6-ceramide-evoked increase in Ca$^{2+}$ was dantrolene-sensitive (Fig. 5C, e). These results suggested that the new Ca$^{2+}$ was derived from the RyR and that the Ca$^{2+}$ increase is essential for the C6-ceramide-induced release of glutamate.

NGF-induced glutamate release was through p75 receptor. A, release of glutamate stimulated by NGF was enhanced in p75-transfected neurons. The enhancement was not observed in lacZ-transfected cells. Data represent the means (ratio; NGF-induced/basal) ± S.D. (n = 6). *, p < 0.1; **, p < 0.01 versus lacZ-transfected (t test). NGF was applied at 100 ng/ml (DIV6). B, a–f, immunocytochemical staining of cultured neurons overexpressing p75 with two anti-p75 antibodies. Cerebellar neurons were infected with p75 and NLS-lacZ adenovirus after 7 days in culture. At 24 h after infection, p75 expression was measured using two anti-p75 antibodies. Left, p75 staining with anti-p75 antibody (Promega) (a, p75-transfected, m.o.i. 10; c, p75, m.o.i. 3; e, LacZ-transfected, m.o.i. 10, respectively). Right, p75 staining with MC-192 anti-p75 antibody (b, p75, m.o.i. 10; d, p75, m.o.i. 3; f, LacZ, m.o.i. 10). Bar = 100 μm. g, the level of p75 overexpression in the adenovirus-infected cultures was determined by Western analysis. The assay was performed 24 h after infection at DIV7 (p75 or lacZ at m.o.i. 10, respectively). TrkB expression was not altered by p75 adenovirus infection. To confirm that equal amounts of total protein were applied in each lane, immunoblotting with anti-class III β-tubulin (TUJ1) antibody was also performed. C, BDNF-induced glutamate release was not increased by p75 overexpression. The assay was performed 24 h after infection at DIV7 (p75 or lacZ at m.o.i. 10, respectively). NGF or BDNF was applied at 100 ng/ml. t test, **, p < 0.01 versus lacZ-transfected (n = 6). D, MC-192 anti-p75 antibody completely blocked the NGF-induced glutamate release in untransfected cells. In contrast, BDNF still induced the release with MC-192. A heat-inactivated NGF (boiled for 3 min at 100 °C) did not trigger the glutamate release. t test, **, p < 0.01 versus basal (n = 6) (DIV7). E, effect of p75-siRNA on the NGF-induced glutamate release. a, after transfection of p75-siRNA (2.0 μg/ml), the NGF-induced release was significantly inhibited. Scramble (control, 2.0 μg/ml) siRNA was not effective. siRNAs transfection was carried out at DIV5. t test, **, p < 0.01 versus basal (n = 6) (DIV7). b, the expression of endogenous p75 was down-regulated by p75-siRNA. p75-siRNA did not change the expression of endogenous TrkB. Immunoblotting with anti-TUJ1 antibody was also performed.
NGF-induced p75-dependent Glutamate Release

Fig. 3. NGF increased the intracellular Ca\(^{2+}\) concentration in cultured cerebellar neurons. A, the increase in Ca\(^{2+}\) induced by NGF in a selected area (DIV6). Images of Fluo-3-filled cells are shown. a, monochrome; b, pseudocolored basal image; and c, 10 s after NGF application. Bar = 50 \(\mu\)m. B, time course of the NGF-induced Ca\(^{2+}\) increase. a, NGF increased the Ca\(^{2+}\) levels in the presence of 1.8 mM Ca\(^{2+}\) and in the presence of thapsigargin (1.0 \(\mu\)M). The trace indicates three cells, which were selected from the same well. C, characterization of the NGF-induced Ca\(^{2+}\). Plots summarize the data from 18 selected cells in the same culture. F, intensity of the NGF-induced maximum Ca\(^{2+}\) transient. FIM, intensity of baseline before exposure to NGF. Both BAPTA-AM and thapsigargin blocked the NGF-induced Ca\(^{2+}\) increase whereas extracellular Ca\(^{2+}\) free did not. D, dantrolene (1.0 \(\mu\)M), a ryosine receptor blocker, blocked the Ca\(^{2+}\) increase by NGF. Xestospongin C (10 \(\mu\)M), an IP\(_3\) receptor blocker, did not inhibit the NGF-induced Ca\(^{2+}\) increase, \(n = 20\) (\(n\) indicates the number of selected cells in the same culture). **, \(p < 0.01\) versus control (BSA, \(t\) test).

Fig. 4. Ca\(^{2+}\) dependence of NGF-induced glutamate release. A, extracellular Ca\(^{2+}\) free did not block the NGF-induced release. In contrast, BAPTA-AM (100 \(\mu\)M) or thapsigargin (1.0 \(\mu\)M) completely blocked the release. Data represent the means \(\pm\) S.D. (\(n = 4\)). **, \(p < 0.01\) versus basal (\(t\) test) (DIV6). B, dantrolene blocked the NGF-induced glutamate release, but xestospongin C (10 \(\mu\)M), an IP\(_3\) receptor blocker, did not. \(t\) test, **, \(p < 0.01\) versus basal (\(n = 6\)).

Glutamate. As shown in Table I, we investigated the Ca\(^{2+}\) dependence of the C\(_2\)-ceramide-induced glutamate release. C\(_2\)-ceramide still induced the release under extracellular Ca\(^{2+}\)-free conditions. BAPTA-AM or thapsigargin blocked the C\(_2\)-ceramide-induced release. Dantrolene also significantly inhibited the C\(_2\)-ceramide-induced glutamate release. Furthermore, we examined the Ca\(^{2+}\) increase induced by C\(_2\)-ceramide (Table II). These results indicated that the Ca\(^{2+}\) dependence of the C\(_2\)-ceramide-induced glutamate release showed the same properties as that of the NGF-induced release. However, the exogenous C\(_2\)-ceramide had much stronger effects on the Ca\(^{2+}\) increase and glutamate release than NGF. The Ca\(^{2+}\)-free condition slightly reduced the C\(_2\)-ceramide-induced Ca\(^{2+}\) increase and glutamate release, but the reductions were detectable. On the other hand, the Ca\(^{2+}\)-free condition had no effect on the Ca\(^{2+}\) increase and glutamate release induced by NGF. Therefore, it is possible that the excess C\(_2\)-ceramide may be affecting an influx of extracellular Ca\(^{2+}\) through voltage-dependent Ca\(^{2+}\) channels, although RyR receptors were dominant.

To further investigate the possibility that ceramide acts downstream of p75 stimulated by NGF, sphingomyelinase activity and the amount of ceramide after NGF stimulation were determined. NGF activated the sphingomyelinase within 1 min in cultured cerebellar neurons (Fig. 6A, a), and this activation was sustained for at least 10–15 min. Interestingly, another neurotrophin, BDNF, produced no activation. Furthermore, NGF significantly increased the amount of ceramide (Fig. 6A, b).

Next, we measured the glutamate release induced by NGF after treatment with ISP-1, which is known to inhibit the generation of ceramide (47, 48). ISP-1 (0.1, 1.0, and 10 \(\mu\)M for 24 h) efficiently blocked the NGF-induced glutamate release in a dose-dependent manner (Fig. 6B). Chronic treatment with ISP-1 did not influence the neuronal survival (none; 329 \(\pm\) 13.2, ISP-1; 339 \(\pm\) 16.5, numbers of MAP2-positive cells/field, \(n = 5\)). In contrast, C\(_2\)-ceramide induced the release after intracellular ceramide deprivation by ISP-1 (Fig. 6B). Another inhibitor of ceramide synthesis, fumonisin B1 (treatment for 18 h, at 50 \(\mu\)M), also blocked the glutamate release (basal; 53.21 \(\pm\) 7.33, NGF; 53.05 \(\pm\) 9.68 \(\times\) 10\(^{-10}\) mol/well, \(n = 6\), respectively). As both these inhibitors required an extended application, there is a possibility that they have an indirect effect on the cell mem-
NGF-induced Glutamate Release Occurred in Developing Cerebellar Neurons—There is accumulating evidence for the involvement of neurotrophin/Trk-mediated regulation in the synaptic function. In this study, it was found that NGF induced the release of glutamate through a p75 receptor-mediated signal in cerebellar neurons that did not express TrkA. Therefore, to identify the physiological role of the NGF-induced release, we focused on the action of NGF during neuronal development (see Figs. 7 and 8). As shown in Fig. 7A, both NGF and BDNF were unable to induce glutamate release at 3 days in vitro (DIV3). On the other hand, at DIV5 or DIV7, NGF and BDNF both induced significant glutamate release. Interestingly, NGF induced no release in more mature neurons (at DIV9). In contrast, the BDNF-induced release was increased in mature culture compared with the NGF-induced release, indicating that the glutamate release induced by the two neurotrophins occurs at different periods of in vitro maturation. High KCl (50 mM; HK⁺)-stimulated depolarization was also examined for the positive control in the release of glutamate. The HK⁺-evoked glutamate release was also observed at DIV5 and later, the extent of which was greater than that of the neurotrophin-induced release at DIV9, implying that synaptic maturation had occurred. The Ca²⁺ response of cultured cerebellar neurons to the two neurotrophins was analyzed. The percentage of cells showing a Ca²⁺ response to NGF was increased at DIV3, DIV5, and DIV7, but not at DIV14, indicating that immature neurons responded to NGF (Fig. 7B, a). However, BDNF was only capable of inducing a Ca²⁺ response in the mature culture (Fig. 7B, a). The same tendency was displayed for the intensity of the Ca²⁺ increase elicited by NGF or BDNF (Fig. 7B, b, activated cells only), suggesting that NGF exerted its effects in developing cerebellar neurons.

Finally, we examined the change in the p75-dependent glutamate release from cerebellar (CBL) slices during neuronal maturation (Fig. 8). First, we confirmed that the expression of p75 increased during the immature stage (postnatal days 2 to 10), and the level of p75 protein was reduced slightly at postnatal day 17 (Fig. 8A). CBL prepared from adult rats (over four months) expressed p75 at a lower level than that from postnatal day 2 rats (data not shown). In contrast, TrkB expression increased with neuronal maturation in CBL (Fig. 8A). To indicate the singularity of the CBL response to NGF, we showed the p75 expression in the cortex, which was much lower than that in the CBL, because NGF had no effect on glutamate release in cultured cortical neurons (11). Furthermore, the NGF-induced glutamate release in cultured cortical neurons overexpressing p75 was analyzed. The p75-overexpressing cortical cells began to respond to NGF (lacZ (m.o.i. 10); 1.04 ± 0.09, p75 (m.o.i. 10); 1.31 ± 0.08, ratio (NGF-induced/basal), p < 0.01 (n = 6), respectively), indicating that cortical neurons (with normal levels of p75) did not respond to NGF. Furthermore, we performed a release analysis with acute CBL slices. Marked glutamate release was observed with the NGF (200 ng/ml) application in slices prepared at postnatal day 12 (Fig. 8B). However, CBL slices from postnatal day 17 responded to BDNF (200 ng/ml) but not to NGF, indicating that the p75-dependent glutamate release induced by NGF occurred in only the early developing stage.

**DISCUSSION**

In this report, we have shown that NGF induced a rapid release of glutamate through the p75 receptor-mediated activation of sphingomyelinase and generation of ceramide. We found that the p75-mediated ceramide generation pathway resulted in an intracellular Ca²⁺ increase through RyR and that the Ca²⁺ was required for the glutamate release. Specifically, the NGF-induced release was only observed in immature
neurons; that is, matured cerebellar neurons did not respond. These results suggest that the action of NGF via a p75-dependent pathway is involved in the release of neurotransmitters in developing cerebellar neurons.

The p75-transfected neuronal culture showed a higher responsiveness to NGF than the lacZ-transfected culture (Fig. 2, A and C). However, no enhanced release of glutamate was observed on stimulation with BDNF (Fig. 2C) in addition to the lack of Trks phosphorylation, the effectiveness of NGF in the presence of the Trk kinase inhibitor, K252a (Fig. 1), suggested that p75 was involved in the NGF-dependent release. Further, a neutralizing effect of MC192, a p75 antibody, was observed.

**TABLE I**

|                | Ca²⁺-free | BAPTA-AM | Thapsigargin | Dantrolene |
|----------------|-----------|----------|--------------|------------|
| Basal          | 17.1 ± 1.83 | 16.5 ± 2.42 | 13.5 ± 1.87 | 14.5 ± 0.98 |
| C₂-ceramide    | 69.1 ± 7.37° | 51.8 ± 5.48° | 17.3 ± 2.86 | 15.2 ± 1.96 × 10⁻¹⁰ mol/well |

° p < 0.01 vs. basal (t-test).

**TABLE II**

|                | Ca²⁺-free | BAPTA-AM | Thapsigargin | Dantrolene |
|----------------|-----------|----------|--------------|------------|
| Ca increase (F/F₀) | 2.02 ± 0.14 | 1.55 ± 0.09 | 0.97 ± 0.05 | 1.13 ± 0.14 |

**FIG. 6.** Sphingomyelinase activation and ceramide generation were involved in the NGF-induced glutamate release. A, a, neutral sphingomyelinase activity after NGF (100 ng/ml) stimulation was determined. NGF activated sphingomyelinase activity within 1 min, and the effect was sustained for at least 10–15 min. BDNF (100 ng/ml) had no effect. Data represent the means ± S.D. (n = 4). ***, p < 0.01 versus basal (0 min) (t test). b, ceramide production on NGF application. NGF significantly increased the amount of ceramide. NGF was applied at 100 ng/ml. Data represent the means ± S.D. (n = 4). **, p < 0.01 versus basal (0 min) (t test). B, ISP-1, a ceramide synthesis inhibitor (0.1, 1.0, and 10 µM for 24 h), inhibited the NGF-induced glutamate release in a dose-dependent manner. C₂-ceramide (1.0 µM) reversed this effect. C, sphingomyelinase inhibitor, scyphostatin (0.1 or 1.0 µM for 20 min), significantly blocked the NGF-dependent glutamate release.
Furthermore, it was revealed that the NGF-induced release was significantly reduced after transfection of p75-siRNA (Fig. 2E). These results strongly suggested that the NGF-induced glutamate release was p75-dependent. Blochl and Sirrenberg (33) reported that BDNF stimulated the release of dopamine by the activation of TrkB, whereas NGF affected the release via p75 in cultured mesencephalic dopaminergic neurons. The dopaminergic neurons expressed TrkB and p75 but not TrkA, suggesting that p75 mediated the dopamine release. Here, we attempted to clarify the p75-mediated glutamate release, because it is not known how NGF exerts an acute effect on the glutamatergic neuronal system.

TrkA is primarily activated by NGF, whereas TrkB is the primary receptor for BDNF (49). In contrast, various neurotrophins are known to bind to p75 with the same affinity. If glutamate release is induced through the p75 pathway, not only NGF but also BDNF should trigger the release even in the presence of the Trk inhibitor, K252a. However, BDNF was incapable of releasing glutamate under those conditions (Fig. 1B). It was reported that NGF specifically activated p75 leading to apoptosis under certain conditions (50). Dobrowsky et al. (51) reported differences in sphingomyelin hydrolysis stimulated by a variety of neurotrophins including NGF, BDNF, NT-3, and NT-4/5 in NIH-3T3 and PC12 cells. They showed that NGF, BDNF, NT-3, and NT-4/5 stimulated sphingomyelin hydrolysis with similar kinetics in NIH-3T3 cells expressing solely p75. By contrast, PC12 cells expressing TrkA and p75 did not respond to NGF, suggesting that a cross-talk exists between the Trks- and p75-dependent pathways. Therefore, to elucidate the involvement of any Trks and p75 interaction in cerebellar neurons that expresses TrkB, TrkC, and p75 but not TrkA, the effect of BDNF and NGF together was examined. Co-application resulted in a slight decrease in glutamate release compared with a single application of BDNF or NGF, respectively (none; 36.2 ± 3.62, NGF; 129 ± 16.5, BDNF; 113 ± 13.8, NGF and BDNF; 91.2 ± 11.6 × 10^{-10} \text{ mol/well}, p < 0.01 \text{ NGF versus NGF and BDNF (n = 6), both applied at 100 ng/ml}). As BDNF (not NGF) specifically phosphorylated Trks (Fig. 1C), this implied that the Trk (likely TrkB) pathway reduced the p75-dependent signaling. However, the amount of glutamate released by co-application of both neurotrophins...
In the present study, dantrolene, a RyR blocker, blocked the release of glutamate via activation of the MAP kinase pathway. Factor and that basic fibroblast growth factor promotes the various growth factors. It seems to be stimulated through different mechanisms among neurons. An influx of extracellular Ca\(^{2+}\) of glutamate (Fig. 4). Surprisingly, it was found that the p75-dependent pathway; that is, TrkA has specific blocking effects on the NGF-activated p75 pathway. Second, because Trks signaling can inhibit signaling through p75, the proportion of Trks and p75 expression was critical to blocking of the p75 pathway. It was speculated that the Trks expression was too low to decrease the p75-dependent signal in the cerebellar neurons. Previously we reported (11) that cultured cortical neurons did not respond to NGF. In the present study, the effect of NGF on cortical neurons overexpressing p75 was analyzed. Cortical neurons are known to express TrkB, TrkC, and a low level of p75 (see Fig. 8A). The p75-overexpressing cortical cells began to respond to NGF, suggesting that the ratio of p75 to Trks expression was critical (lacZ (m.o.i. 10); 1.04 ± 0.09, p75 (m.o.i. 10); 1.31 ± 0.08, ratio (NGF-induced/basal), p < 0.01 (n = 6), respectively). Furthermore, it was found that the inhibitory effect of BDNF co-application was blocked by TrkB-IgG (data not shown), implying that Trks can inhibit signaling through p75 via a K252a-insensitive pathway although we cannot exclude the possibility that specificity in the neuronal cell population and ligand-specific p75-mediated signal transduction is important. Specific interaction between p75 and tumor necrosis factor receptor-associated factor-6 in HEK 293 cells after transient transfection was reported (52). In that report, NGF exerted the maximum effect on the interaction whereas BDNF, NT-3, and NT-4/5 promoted the association to a lesser extent. Interestingly, DeFreitas et al. (53) reported that the cell survival of neocortical subplate neurons was dependent on a p75-, ceramide-mediated signaling pathway. In their study, BDNF promoted neuronal survival with K252a treatment, indicating that Trk signaling was not necessary. These results, including our study, suggest that it is important to consider not only the expression pattern of neurotrophin receptors but also cell specificity.

Our results show a novel phenomenon in that the NGF-induced Ca\(^{2+}\) accumulation was through the p75 pathway and not Trks. We found that Ca\(^{2+}\) accumulation was stimulated by NGF (Fig. 3) and that the Ca\(^{2+}\) increase was necessary for the glutamate release (Fig. 4). Surprisingly, it was found that the Ca\(^{2+}\) was derived from RyR (Fig. 3D). Growth factors increase intracellular Ca\(^{2+}\) through its release from intracellular stores (9, 54, 55). We showed previously (10, 11) that BDNF increased Ca\(^{2+}\) in a TrkB/PLC-\(\gamma\) pathway-dependent manner in cultured cortical and cerebellar neurons. An influx of extracellular Ca\(^{2+}\) is also involved in growth factor-mediated Ca\(^{2+}\) increase. Drosophila transient receptor potential membrane proteins and some mammalian homologs (transient receptor potential channel 1–7 proteins) have been thought to mediate capacitative Ca\(^{2+}\) entry (56, 57). Li et al. (7) reported that transient receptor potential channel 3 regulates Ca\(^{2+}\) and Na\(^+\) influx through the TrkB/PLC/IP\(_3\) pathway in pontine neurons. We reported previously (38) that Ca\(^{2+}\) influx through voltage-dependent Ca\(^{2+}\) channels was elicited by basic fibroblast growth factor and that basic fibroblast growth factor promotes the release of glutamate via activation of the MAP kinase pathway. In the present study, dantrolene, a RyR blocker, blocked the increase in intracellular Ca\(^{2+}\) induced by NGF (Fig. 3D), suggesting that RyR was involved in the NGF-induced events. These findings indicate that the increase in Ca\(^{2+}\) concentration seems to be stimulated through different mechanisms among the various growth factors.

It was necessary to identify the kind of signal pathway (not including Trks phosphorylation) involved in the NGF-induced effects. To investigate this, we examined the involvement of ceramide, because the generation of ceramide following the activation of p75 regulates many aspects of neuronal response, including cell survival and apoptotic cell death by neurotrophins (24, 28). Bloch and Sirrenberg (33) suggested the possibility that NGF affected dopamine release through the sphingomyelin pathway. In the present study, we showed that the NGF-induced glutamate release required ceramide production via activation of the neutral sphingomyelin pathway (Fig. 6). Furthermore, we found that sphingomyelinase activation was essential for the increase of intracellular Ca\(^{2+}\) and the release of glutamate after NGF stimulation, suggesting that the p75/ceramide/Ca\(^{2+}\) pathway stimulated by NGF is involved in neurotransmission in CNS neurons.

Interestingly, the NGF-induced release of glutamate and Ca\(^{2+}\) was only observed at the immature neuronal stage, whereas the BDNF-induced release significantly increased with maturation (Fig. 7, A and B). p75 receptor expression increased during the postnatal period between 2 and 10 days but slightly decreased at 17 days in CBL slices (Fig. 8A). In addition, CBL slice responsiveness (NGF-induced glutamate release) occurred only at the immature stage (Fig. 8B), implying that the p75-dependent glutamate release had a different physiological role from the Trk-dependent release. Maisonnier et al. (58) reported that BDNF expression was low in the immature regions of the brain and that it increases as these regions mature. In contrast, NT-3 is by far the most highly expressed neurotrophin in the immature regions of the CNS, and it decreases dramatically with neuronal maturation. Furthermore, they indicated that NGF expression varied during CNS development but was not consistent. Therefore, the physiological role of the NGF-dependent action in CNS neurons needed to be studied carefully. Korkotian and Segal (59) found that Ca\(^{2+}\) from RyR alters the morphology of the dendritic spines of young hippocampal neurons within minutes, whereas the dendritic spines of mature neurons are stable. In addition, it has been reported that glutamate regulates the collapse of dendritic spines (60). In the cerebellum, Purkinje cell is innervated by parallel fibers, bifurcated axons of granule cells. During the second and third postnatal weeks, synapses are actively generated. Glutamate is released from terminals of parallel fibers (granule cells) and activates glutamate receptors on Purkinje cells. Kurihara et al. (61) showed that glutamate receptor \(\Delta 2\) is involved in the stabilization and strengthening of synaptic connectivity between parallel fibers and Purkinje cells, leading to the association of Purkinje cell spines with parallel fiber terminals to form functionally mature synapses. Taken together, Ca\(^{2+}\) or glutamate stimulated by NGF in the early developing stage may have an important role in the elongation of neurites, stability of dendritic spines, or formation of synaptic connections. It is well known that NGF secretion and expression are regulated by various stimuli (including glutamate) in astrocytes (62, 63). Therefore, it is highly possible that NGF acts as a physiological mediator in the growing CNS neurons that express p75. This study suggests that NGF and BDNF regulate each other in excitatory neurotransmitter release at an early stage in the developing cerebellar, and it is possible that a transfer from NGF- and p75-dependent signaling to BDNF-mediated Trk signaling is completed during cerebellar synaptic maturation.

High affinity interactions of pro-neurotrophins with p75 have contributed to neuronal apoptosis through the p75/sphingomyelinase pathway (64). A study of p75 knockout mice by von Schack et al. (65) demonstrated the importance of the p75 neurotrophin receptor. Thus it is valuable to study the acute...
NGF-induced p75-dependent Glutamate Release

41269

effects of neurotrophins on neurotransmitter release, not only with respect to the ligand/receptor and signal transduction but also in terms of the pro-neurotrophin forms and changes in biological effects during neuronal development.

Acknowledgments—We are grateful to Regeneron Pharmaceutical Co. for donating TrkB-IgG. We thank Regeneron Pharmaceutical Co. and Takeda Chemical Industries, LTD. for generously donating BDNF. We thank Dr. Yamada for generously donating NGF. We thank Dr. H. Hohi (National Institute of Neuroscience, Japan) for critical suggestions concerning effects of siRNA.

REFERENCES

1. Lindholm, D., Dechant, G., Heisenberg, C. P., and Thoenen, H. (1993) Eur. J. Neurosci. 5, 1455–1464

2. Minichiello, L., and Klein, R. (1996) Genes Dev. 10, 2849–2858

3. Schwartz, P. M., Borghesani, P. R., Levy, R. L., Pomeroy, S. L., and Segal, R. A. (1994) J. Neurosci. Res. 36, 21–25

7. Li, H. S., Xu, X. Z., and Mentelli, C. (1999) Neuron 24, 261–273

16. Wang, S., Bray, P., McCaffrey, T., March, K., Hempstead, B. L., and Kraemer, R. D. (1995) Neuron 15, 565–574

22. Casaccia-Bonnefil, P., Carter, B. D., Dobrowsky, R. T., and Chao, M. V. (1996) J. Neurosci. Res. 43, 261–273

40. Veldhoven, P. P. V., and Bell, R. M. (1988) Biochim. Biophys. Acta

41. Veldhoven, P. P. V., Bolognesi, D. P., and Bell, R. M. (1992) J. Biol. Chem. 267, 157–166

45. Chandler, C. E., Parsons, L. M., Hosang, M., and Shooter, E. M. (1984) J. Biol. Chem. 259, 501–509

52. Khursigara, G., Orlinick, J. R., and Chao, M. V. (1999) J. Biol. Chem. 274, 594–605