Neuronal Roles of the Integrin-associated Protein (IAP/ CD47) in Developing Cortical Neurons*§

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§ The on-line version of this article (available at http://www.jbc.org) contains supplementary Figs. 1–4, which present further data on IAP-mediated effects on cortical cultures.

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**The abbreviations used are: IAP, integrin-associated protein; BDNF, brain-derived neurotrophic factor; DIV, days in vitro; ERK, extracellular signal-regulated kinase; GABA, γ-aminobutyric acid; GFP, green fluorescent protein; MAP2, microtubule-associated protein 2; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK kinase; PI, phosphatidylinositol; siRNA, small interfering RNA; SNAP25, 25-kDa synaptosomal associated protein; TUJ1, class III β-tubulin.

Little is known about the role of the integrin-associated protein (IAP, or CD47) in neuronal development and its function in the central nervous system. We investigated neuronal responses in IAP-overexpressing cortical neurons using a virus-gene transfer system. We found that dendritic outgrowth was significantly enhanced in IAP (form 4)-transfected neurons. Furthermore, synaptic proteins including synaptotagmin, syntaxin, synapsin I, and SNAP25 (25-kDa synaptosomal associated protein) were up-regulated. In accordance with this finding, the release of the excitatory transmitter glutamate and the frequencies of Ca\(^{2+}\) oscillations (glutamate-mediated synaptic transmission) were increased. Interestingly, the overexpression of IAP activated mitogen-activated protein kinase (MAPK), and this activation was required for the IAP-dependent biological effects. After down-regulation of the endogenous IAP by small interfering RNA, MAPK activity, synaptic protein levels, and glutamate release decreased. These observations suggest that the IAP plays important roles in dendritic outgrowth and synaptic transmission in developing cortical neurons through the activation of MAPK.

Growing evidence has suggested that cell adhesion proteins and associated proteins such as the neural cell adhesion molecule, cadherin, and integrins play an important role in the formation, maintenance, and function of synapses (1, 2). Integrin forms heterodimers of α- and β-subunits linked to the actin cytoskeleton (3, 4). Recently, Chan et al. (5) showed that many different integrins, including α\(_5\) and α\(_6\), are expressed in the brain and are associated with synapses. In contrast, little is known about integrin-associated protein (IAP\(^1\))-dependent regulation in neuronal function, although IAP physically associates with integrins including α\(_{3}\)β\(_{1}\), α\(_{5}\)β\(_{1}\), and α\(_{6}\)β\(_{3}\) (6–8).

The function of IAP in non-neuronal cell populations has been well studied. For example, IAP plays an essential role in host defense by participating in the migration and activation of leukocytes in response to bacterial infection (9). IAP is a receptor for the C-terminal cell-binding domain of thrombospondin (10). Thrombospondin stimulates the integrin-dependent adhesion, spreading, and motility of endothelial cells, leukocytes, and smooth muscle cells (10, 11). Furthermore, IAP is a ligand for the transmembrane signal-regulatory protein (12) and is involved in macrophage function. In platelets, a peptide from the C-terminal cell-binding domain of thrombospondin induces aggregation (13) and spreading on immobilized fibrinogen or collagen (7). There is some evidence that IAP is involved in neuronal function. Memory retention and long-term potentiation were reduced in IAP-deficient mice (14). Monoclonal antibody to IAP significantly impaired memory retention and reduced the amplitude of long-term potentiation in dentate gyrus neurons (15). However, little is known as to the possible effects of IAP on neuronal development and function at the molecular level.

In the present study, we investigated the roles of IAP in cultured cortical neurons with respect to dendritic outgrowth, neurotransmitter release, and the underlying intracellular signal transduction. There are four alternatively spliced forms of IAP that differ only at their intracytoplasmic carboxyl termini (16). Here, we focused on the effects of IAP form 4 because it is an abundant isoform expressed primarily in neurons (17). By using overexpressing IAP through virus-mediated gene transfer, we found that dendritic outgrowth was significantly enhanced. Synaptic proteins including synaptotagmin, synapsin I, SNAP25, and syntaxin were up-regulated. Glutamate release and Ca\(^{2+}\) oscillations, which are glutamate-mediated spontaneous synaptic transmissions (18) in cortical neurons, were reinforced by overexpression. These IAP-induced effects required activation of the MAPK pathway. Furthermore, MAPK activity, synaptic protein expression, and glutamate release were decreased after down-regulation of endogenous IAP by small interfering RNA (siRNA), suggesting that IAP is important to synaptic function in developing cortical neurons.

EXPERIMENTAL PROCEDURES

Cell Culture—Disassociated cortical cultures were prepared from a postnatal 2- or 3-day-old rat (SLC, Sizuoka, Japan) cortex as described previously (18). The cortical neurons were plated at a density of 5 × 10^5 cells per 24-mm dish on poly-L-lysine-coated cover slips in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mm glutamine, and 50 U/ml penicillin, 50 μg/ml streptomycin at a final volume of 0.5 ml. Coverslips were then placed in 12-mm NeuroMax II Culture Chambers (Lab-Tek) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO\(_2\).
from the 0 to 5% interface. After centrifugation using Ficoll gradients, fractions were collected and the bottom (natal 15-day-old and adult rats). Section b of protein from each fraction was probed with the indicated antibodies. A discontinuous gradient of 15 and 5% Ficoll in buffer A. After centrifugation, fractions were collected from the 0 to 5% interface (L1, 5 to 15% interface (L2; synaptosomes), and the bottom (P3; pellet). HG indicates homogenate. An equal amount of protein from each fraction was probed with the indicated antibodies. Section b, expression of IAP and syntaxin in synaptosomes from postnatal 15-day-old and adult rats.

107/cm2 on polyethyleneimine-coated plates (Corning) or cover glasses (Matsunami, Osaka, Japan) attached to flexiPERM (In Vitro Systems and Services, Osterode, Germany). The culture medium consisted of 5% precolostrum newborn calf serum, 5% heated-inactivated horse serum, and a 90% mixture of Dulbecco’s modified Eagle’s medium and Ham’s F-12 medium (1:1) containing 15 mM HEPES buffer, pH 7.4, 30 mM Na2SeO3, and 1.9 mg/ml NaHCO3. Preparation of Synaptosomes—Synaptosomes were prepared according to a previous report (19). The cerebral cortex was removed under anesthesia. Cortex sections were rinsed briefly in phosphate-buffered saline and then homogenized in ice-cold buffer A (0.35 mM sucrose, 1 mM MgCl2, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4) using a loose fitting Potter homogenizer. Homogenate was centrifuged for 1 min at 2,000 × g, and the supernatant was centrifuged for 5 min at 23,000 × g (P2 fraction). This pellet was resuspended with buffer A and transferred to a discontinuous gradient of 15 and 5% Ficoll in buffer A. After centrifugation at 43,000 × g, synaptosomes were collected from the 5 to 15% interface. All of these procedures were carried out at 4 °C.

Viral Constructs—The Sindbis virus, a bicistronic vector plasmid (pSinEGds), was provided by Dr. Kawamura (Niigata University, Niigata, Japan). The plasmid was derived from pSinRep5 (Invitrogen) and had two subgenomic promoters followed by a multiple cloning site for an arbitrary gene insertion and an enhanced GFP open reading frame; thus, the virus can produce arbitrary protein and enhanced GFP independently in the infected cell (20). IAP form 4 cDNA, amplified by reverse transcription PCR with specific primers (5’-AATTTCCTA-GAATGCTGCCCCCTTGGCAGGGCCG-3’ and 5’-TATATGCTACTTTAT-TGTCATTACATCATCCTCC-3’), was inserted into the XbaI and SphI sites of the multiple cloning site. The plasmid was linearized by PacI, and mRNA was synthesized in vitro using an mMESSAGE mMachine kit (Ambion). Subsequently, baby hamster kidney cells were transfected with the mRNA and 20 S helper mRNA by electroporation (1250 V/cm, 50 μF, single pulse) using Gene Pulser2 (Bio-Rad). The cells were incubated with Dulbecco’s modified Eagle’s medium supplemented with 5% fetal calf serum for 24 h at 37 °C, and the supernatant was collected as the pseudovirion-containing solution.

Adenovirus—cDNAs of nuclear localization signal–possessing LacZ, MyoH/His-tagged rat IAP form 4 were subcloned into the SwaI site of the cosmid vector pAXCAwt under the control of the CAG promoter (21, 22). These cosmids vectors and the EcoT221-digested DNA-terminal protein complex of Ads5-dlx, which is a human type 5 adenovirus lacking the E3 region, were cotransfected into HEK 293 cells by the calcium phosphate method. The recombinant adenoviruses AdsCAANL5LacZ and AdsCAA-IAP-MyoH/His, generated by homologous recombination in HEK 293 cells, were isolated. As the recombinant adenoviruses did not contain the E1A region, PCR amplification of this region was performed to ensure no contamination by the wild-type adenovirus (Ads5-dlx). The recombinant adenoviruses, which showed no PCR amplification of E1A, were used. These adenoviruses were propagated in HEK 293 cells and purified by cesium gradient centrifugation. The viral titer was determined by a plaque-forming assay on HEK 293 cells. The viral infection was carried out at a multiplicity of infection of 10.

Immunocytochemistry— Cultured neurons were fixed for 4% paraformaldehyde for 20 min and then rinsed three times with phosphate-buffered saline for 5 min. Neurons were permeabilized with 0.2% Triton-X in phosphate-buffered saline for 5 min at room temperature. Then, primary antibodies (anti-MAP2 (1:2000) from Sigma and anti-synaptotagmin (1:1500) from Calbiochem) with 1% skin milk in phosphate-buffered saline were applied overnight at 4 °C. Secondary antibodies (Alexa Fluor, Molecular Probes) were applied for 1 h at 4 °C. Fluorescent images were captured by an inverted microscope (TE300, Nikon) with a monochrome charge-coupled device camera (ORCA-ERR, Hamamatsu) or a microscope (Axiovert 200, Zeiss) with a charge-coupled device (cool SNAPs, Zeiss). Monochrome images were turned into grayscale.
color and analyzed using software (AQUACOSMOS from Hamamatsu or Slide Book™ 3.0 from Intelligent Imaging Innovations, Inc.). The images of GFP were analyzed with the same software.

Detection of Amino Acid Neurotransmitters—The amount of amino acids released from neurons was measured by high performance liquid chromatography (Shimadzu, Kyoto, Japan) as reported (23, 24). To induce exocytosis in neurons, we used a high KCl (HK/H11001) solution consisting of 85 mM NaCl, 50 mM KCl, 1.2 mM NaH₂PO₄, 1.8 mM CaCl₂, 10 mM glucose, 1% bovine serum albumin, and 25 mM HEPES, pH 7.4. Before HK/H11001 (1 min) stimulation, basal fractions (1 min) were collected. U0126, an inhibitor of MEK (upstream of MAPK), was purchased from Promega. The 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 Figs. 1 and 5.

Immunoblotting—Cells were lysed in SDS lysis buffer containing 1% SDS, 20 mM Tris-HCl (pH 7.4), 5 mM EDTA (pH 8), 10 mM NaF, 2 mM Na₃VO₄, 0.5 mM phenylarsine oxide, and 1 mM phenylmethylsulfonyl fluoride. Immunoblotting was carried out as described previously (23). For the measurement of exogenous IAP on adenovirus gene transfer, tagged Myc protein was detected. Incubation with 0.2 µg/ml anti-Myc monoclonal antibody diluted with Tris-buffered saline/Tween 20 containing 1% (w/v) skim milk was performed for 12 h at 4 °C. Furthermore, immunoblottings were carried out with the anti-synaptotagmin antibody (1:1000, mouse monoclonal; BD Transduction Laboratories), the anti-synapsin I antibody (1:1000, rabbit anti-serum; Chemicon), the anti-syntaxin antibody (1:3000, mouse monoclonal; Sigma), the anti-SNAP25 antibody (1:3000, mouse monoclonal; Synaptic System), the anti-GFP antibody (1:1000, goat polyclonal; Santa Cruz Biotechnology), the anti-Bad antibody (1:500, mouse monoclonal; BD Transduction Laboratories), the anti-Bcl-2 antibody (1:500, mouse monoclonal; BD Transduction Laboratories), and the anti-TUJ1 antibody (1:5000, mouse monoclonal; Berkeley Antibody Company). In addition, the anti-
phospho-Trk antibody (1:1000) was purchased from New England Biolabs Inc., whereas the anti-Akt (1:1000) and anti-phospho-Akt (1:1000) antibodies were obtained from Cell Signaling. Anti-Trk (1:1000), anti-MAPK (1:1000), and anti-phospho-MAPK (1:1000) antibodies were purchased from Santa Cruz Biotechnology Inc. To quantify the amount of MAPK after Western blotting, we measured the density of immunoblots with image analysis software (Science Lab 98 Image Gauge, Fuji Photo Film Co. Ltd., Tokyo, Japan).

**siRNA Transfection**—The siRNA transfections were performed as reported (25). We used 23-nucleotide siRNA duplexes with two 3′-overhanging nucleotides of the rat IAP mRNA coding region (5′-AAT-GACACTGGTGTACCATCCCTTG-3′). Both sense and antisense strands were chemically synthesized by Dharmacon Research Inc. The siRNA (5′-GGACCCGGCGCCGAUUCG-3′, named Scramble II (Dharmacon Research Inc.) was used as a control. Transfection of both siRNAs (final 2 mg/ml) was performed using NeuroPORTER™ (Gene Therapy Systems, Inc.). We carried out the siRNA transfer 48 or 72 h prior to the glutamate release assay or the collecting of samples for immunoblotting. The transfection was performed at 3 or 4 days after the initiation of cortical cultures.

**RESULTS**

_**Endogenous IAP Increases during Neuronal Maturation and Exists in Synapses**_—We first examined the change in the expression of endogenous IAP during neuronal development. We determined IAP levels in cultured cortical neurons prepared from postnatal 2–3-day-old rats during maturation in vitro from DIV 3 (i.e. 3 days in vitro) to DIV 11. To estimate synaptic maturation, the synaptic proteins synapsin I, SNAP25, and syntaxin were also quantified. There was a large increase in IAP, synapsin I, and SNAP25 expression from DIV 3 to DIV 5, followed by increase until DIV 11 (Fig. 1A). Syntaxin also increased (Fig. 1A). TUJ1 (class III β-tubulin) was examined as a neuronal marker (Fig. 1A). Synaptosomes from the adult rat cortex contained IAP (section a in Fig. 1B). The expression of IAP in the synaptosomes increased during brain maturation (postnatal days 15 to adulthood) (section b in Fig. 1B).

_**IAP Enhances Dendritic Outgrowth in Developing Cortical Neurons**_—We examined whether IAP was involved in the dendritic outgrowth of cultured cortical neurons using a Sindbis virus-mediated IAP overexpression system. The transfection of IAP was performed 1 h after the plating of the cells. Eighteen hours later, we observed dendritic outgrowth (Fig. 2A). The outgrowth was markedly enhanced in IAP-infected neurons (section b in Fig. 2A), but no such effect was observed in GFP-infected neurons (control) (section a in Fig. 2A). A Sindbis virus vector that had the GFP gene only was used as a control. The percentage of cells that had dendrites twice the length of the diameter of the cell body was determined (section c in Fig. 2A). The result indicated that IAP markedly enhances dendritic outgrowth in developing cortical neurons. Furthermore, we assessed the number of dendrites from IAP- or control virus (GFP only)-infected neurons. At 24 h (DIV 2) after the viral infection (DIV 1), the IAP-infected neurons had many more dendrites than did the control neurons (sections a and b in Fig. 2B). The number of primary dendrites (from the cell body) that were four times the length of the cell diameter or longer was significantly increased (section c in Fig. 2B). A similar difference was recognized in the number of intersections of dendrites and a circle with a radius twice as long as the shortest diameter of the cell body (section d in Fig. 2B). In contrast, the dendritic outgrowth of IAP-overexpressing mature neurons infected at DIV 14 was not enhanced (the primary dendrite number 48 h after infection for GFP (control) was 7.40 ± 1, and for IAP it was 7.32 ± 1.34, n = 78, where n indicates the cell number selected from three independent cultures). In this assay, the number of axons was excluded (see supplementary Fig. 1 in the on-line version of this article).

_**Presynaptic Sites Are Increased by IAP Overexpression**_—To determine the presynaptic sites, we performed immunostaining with an anti-synaptotagmin antibody (shown as a red presynaptic marker in Fig. 3, A and B, section a), an anti-MAP2 antibody (shown as a blue dendritic marker in Fig. 3, A and B, section b), and a GFP (shown as green axons in Fig. 3, A and B, section c). The number of terminals of presynapses (synaptotagmin and GFP double-positive vesicle-like buttons on MAP2-positive neurons) was increased by IAP overexpression (see sections c in Fig.
**Fig. 5.** Activation of the MAPK pathway in cultured neurons with IAP overexpression. A, basal activity of MAPK/ERK (p44 or p42) was significantly increased by IAP overexpression. Section a, the time course of MAPK activation (pERK, phosphorylated ERK) in IAP/GFP-infected cultures. Sections b and c, MAPK activation in GFP-infected (Con, control) (b) and non-infected (c) sister cultures. The infection was performed at DIV 4. Upper band, p44 MAPK, lower band, p42. Sections d and e, quantification of the levels of activated MAPK (d) and non-activated MAPK (e) at 24 h after viral infection. The amount of each protein was quantified by densitometry after Western blotting. Data represent mean ± S.D. (n = 4, from four separate cultures). ***, p < 0.001 versus none (t test). B, no change in Akt activation (pAkt) was observed in IAP-overexpressing cultures. Furthermore, IAP overexpression did not activate the BDNF receptor TrkB (pTrkB). The expression of TUJ1 was not altered. The data from 24 h after infection are shown.

3, A (control) and B (IAP/GFP-infected). Plots of the data obtained from 46 sections (per 50-μm section of dendrites) are shown in Fig. 3C. In this experiment, the glutamatergic terminals were predominant (see supplementary Fig. 2 in the on-line version of this article). The MAP2-positive, GFP-non-positive neurons were chosen as postsynapse neurons.

**Presynaptic Function Is Increased by IAP Overexpression—**To see presynaptic function, we performed a highly concentrated viral infection and confirmed that 80% of all cells were GFP-positive (Fig. 4A). The proportion of GFP-positive neurons in control- and IAP-infected cultures was similar, that is, GFP-positive/MAP2-positive cells per field was 80 ± 16% for controls and 78.4 ± 14% for IAP overexpression (n = 9 for both cell groups). After infection, we determined the expression of presynaptic proteins, because such proteins are essential for exocytotic transmitter release. As expected, the levels of synaptotagmin, synapsin I, syntaxin, and SNAP25 were significantly increased in IAP-overexpressing cells as compared with the control cells (Fig. 4B), suggesting that IAP enhances the presynaptic machinery. The infection efficiency was determined by immunoblotting with an anti-GFP antibody (Fig. 4B). It was also confirmed that IAP was overexpressed (Fig. 4B). No significant change in TUJ1 (negative control) and GFP expression was observed following control or IAP infection (Fig. 4B).

The up-regulation of synaptic proteins suggested that neurotransmitter release increases with IAP. We went on to measure glutamate, an excitatory transmitter, in cortical cultures. The amount of basal glutamate from IAP-infected neurons was increased at 24 h after infection at DIV 4 compared with the uninfected cells (section a in Fig. 4C). In contrast, the basal release of glutamate in control-infected cultures was no different from that in uninfected cultures, indicating that IAP overexpression results in an elevation of glutamate release (section a in Fig. 4C). We also measured HK− (high potassium, 50 mM KCl)-evoked glutamate release and found that the release was enhanced in IAP-infected cultures at 24 h after infection (section a in Fig. 4C). Both the basal release and the HK−-evoked release were also enhanced by IAP at 48 h after infection (section b in Fig. 4C). In contrast, an inhibitory transmitter, GABA, was unchanged (section c in Fig. 4C), suggesting that IAP might be specifically involved in the excitatory transmission. In these experiments, the highly concentrated viral infection did not influence the survival of cultured cortical neurons (see supplementary Fig. 3 in the on-line version of this article).

**IAP Activates the MAPK Pathway—**We then investigated the intracellular signal transduction necessary for the effects of IAP. We examined the possible involvement of ERK (p44/42 MAPK) and phosphatidylinositol (PI) 3-kinase pathways, because these pathways have important roles in synaptic transmission (23, 26–29). We found that the basal activity of MAPK (phosphorylated ERK, activated MAPK form) was significantly increased in IAP-infected cultures at 24 and 36 h after infection (section a in Fig. 5A). In contrast, the activation of MAPK in control-infected cultures was not different from that in uninfected cultures (sections b and c in Fig. 5A). Quantification of the level of activated MAPK at 24 h after viral infection was
carried out (section d in Fig. 5A). No significant change in the level of non-activated MAPK was observed following control or IAP infection (section e in Fig. 5A). The PI 3-kinase pathway (pAkt and Akt) was not activated (Fig. 5B). Neurotrophins play a fundamental role in the support of survival, development and the regulation of neuronal transmission and plasticity (30–34). However, when the activation of the BDNF-specific TrkB receptor was examined, the overexpression of IAP did not activate TrkB (Fig. 5B). Thus, the involvement of BDNF in the IAP-dependent effect was unlikely. These results suggested that the MAPK pathway is important for IAP-induced biological effects.

We tested the effect of U0126 (an inhibitor of MEK upstream of MAPK) on the IAP-enhanced glutamate release. The IAP-enhanced release was completely suppressed (section a in Fig. 6A). The inhibitory effect of U0126 on MAPK activation was confirmed (section b in Fig. 6A). IAP-induced dendritic outgrowth was also blocked by U0126 (Fig. 6B). These results indicated that the IAP-enhanced glutamate release and dendritic outgrowth require the activation of the MAPK pathway.

Spontaneous Glutamatergic Transmission Is Reinforced in IAP-infected Cortical Neurons—As described above, overexpression of IAP enhanced the outgrowth of dendrites, the expression of synaptic proteins, and the release of glutamate, suggesting that IAP influences glutamatergic transmission. Then, we monitored Ca^{2+} oscillations in developing cortical neurons. Spontaneous Ca^{2+} oscillations appear to affect processes that are central to the development and plasticity of the central nervous system, and several patterns of Ca^{2+} dynamics are known (35–37). We reported previously that, during the maturation of cultured cortical neurons, networks among neurons develop that can be detected as synchronized spontaneous Ca^{2+} oscillations (18). Cultured cortical neurons displayed oscillations on DIV 5 or later, and such oscillations were glutamate-mediated neuronal transmissions (18). To detect the intracellular Ca^{2+} in the present study, an IAP transfection was performed with an adenovirus-mediated gene transfer system because the GFP (by the Sindbis virus) signal overlaps with the fluo-3 (a Ca^{2+} indicator dye) signal. We confirmed that the adenovirus infection did not influence neuronal survival (see supplementary Fig. 3 in the online version of this article). The exogenous IAP was detected by Western blotting with the anti-Myc antibody (Fig. 7A). Images of fluo-3-filled cells acquired every 4 s in IAP-infected cultures were obtained (sections a–c in Fig. 7B). The Ca^{2+} oscillations did not occur in immature cortical neurons at DIV 4 (before infection) (section a in Fig. 7C). Twenty-four hours after infection, IAP-overexpressing cells showed a significant increase in the frequency of oscillations (section c in Fig. 7C) compared with the controls (LacZ-adenovirus; section b in Fig. 7C). Plots of the summarized data
of oscillation frequency obtained from four independent series of cultures are shown (section d in Fig. 7C). These results suggested that IAP has an important role in synaptic transmission. Furthermore, the IAP-enhanced oscillations were suppressed by U0126 (section d in Fig. 7C), suggesting that IAP enhances synaptic transmission through the MAPK pathway.

We confirmed that the glutamate release enhanced by IAP occurred in a MAPK activation-dependent manner (Fig. 7D).

**Glutamate Release Decreases after Down-regulation of Endogenous IAP by siRNA**—To further consolidate the roles of IAP, we examined the effect of IAP-siRNA. Endogenous protein expression in neurons is down-regulated by siRNA (38, 39). We reported previously the siRNA-dependent down-regulation of neurotrophin receptor p75 (25), demonstrating that siRNA is useful for the knockdown of endogenous protein. As shown in Fig. 8A, SNAP25, synapsin I, and phosphorylated ERK (activated MAPK form) decreased after IAP-siRNA transfection. The endogenous IAP was not detected after the application of siRNA, suggesting that endogenous IAP is involved in MAPK activation and synaptic protein expression. In contrast, the PI3-kinase pathway (pAkt and Akt) was unchanged (Fig. 8A), suggesting that IAP specifically regulates the MAPK pathway. Bad or Bcl-2 and apoptosis- and survival-associated proteins were detected as a negative control (Fig. 8A); TUJ1 was detected as a neuronal marker. Furthermore, the glutamate release in IAP-siRNA-transfected cultures was significantly reduced (Fig. 8B). The release in Scramble (control) siRNA-transfected cells was unchanged (Fig. 8B).

**DISCUSSION**

The present study has analyzed, for the first time, IAP-mediated neuronal responses including outgrowth of dendrites, presynaptic machinery, and Ca2+ oscillations in developing cortical neurons. We found that the overexpression of IAP significantly enhanced these neuronal responses. Furthermore, the IAP-mediated neuronal responses were regulated through the activation of the MAPK pathway. Consistent with these findings, MAPK activation, synaptic protein expression, and glutamate release were decreased by the knockdown of endogenous IAP by siRNA. The results suggest that IAP plays a role in functional development in cortical neurons.

The significant enhancement of the outgrowth and branching of dendrites was induced by IAP overexpression in early developing cortical neurons (Fig. 2), implying that postsynaptic structural development may be accelerated. In contrast, dendritic outgrowth of IAP-overexpressing mature neurons was not enhanced, implying that IAP may be important early in neuronal development.

We observed an up-regulation of the presynaptic machinery in IAP-overexpressing cultures, that is, SNAP25, syntaxin,
synaptotagmin, and synapsin I (Fig. 4). Furthermore, after the suppression of endogenous IAP by siRNA, the expression of these synaptic proteins decreased significantly (Fig. 8). We chose DIV 4 for viral infection because there was a large increase in endogenous IAP, synapsin I, and SNAP25 expression from DIV 3 to DIV 5 followed by a gradual increase after that; therefore, there was a possibility that DIV 4 is a critical point for synaptic formation in cortical cultures, which is consistent with our previous report (18), although further synaptic maturation gradually continues. SNAP25, along with syntaxin, exists as a plasma membrane protein and is one of the components essential for exocytosis and synaptogenesis (40). Synaptotagmins are synaptic vesicle proteins of which several isoforms have been identified as Ca\(^{2+}\) sensor proteins with different affinities (41, 42). Synapsin I is highly concentrated at presynaptic nerve terminals in central nervous system neurons and plays major roles in axon elongation and branching (43).

Thus, the present results supported the view that the presynaptic machinery is up-regulated by the overexpression of IAP. In fact, both basal and depolarization-evoked exocytotic glutamate release was reinforced by the overexpression (Fig. 4C). In our experiments, only neuronal, but not glial, cells were infected with the Sindbis virus. In addition, it was confirmed that the glutamate release in cultured astroglial cells (95% pure, judging from immunostaining with anti-GFAP antibody) was not changed via IAP overexpression by the adenovirus system (none (basal), 22.1 ± 1 and HK\(^{+}\), 21.6 ± 1.6; LacZ (control) (basal), 19.5 ± 1.3 and HK\(^{+}\), 19.4 ± 1.4; and IAP (basal) 19.1 ± 1.0 and HK\(^{+}\), 20.0 ± 0.7 (×10\(^{-10}\) mol/well), n = 6) because the adenovirus system was effective in both neurons and glial cells. These results suggest that the IAP-induced enhancements of neuronal responses are unlikely to be ascribed to the effects of glial cells.

We then tried to determine the intracellular signal transduction necessary for the IAP-mediated neuronal function. MAPK/ERK is known to be an important component of the cellular signaling system during mitogenesis and differentiation. Recently, activation of the MAPK pathway in post-mitotic neuronal cells has been implicated in neuronal function and the consolidation of learning. Wu et al. (26) reported that the activation of MAPK was critical for the formation of stable dendritic filopodial extensions of the kind associated with the enduring remodeling of synapses and the induction of long-term synaptic changes. It was also reported that MAPK activation increased in the rat hippocampus after an associative learning task, i.e. contextual fear conditioning (44). Previously, we reported that the fibroblast growth factor induced glutamate release through activation of the MAPK pathway in the short-term (23). These studies indicated that the MAPK pathway plays important roles in both short- and long-term synaptic functions. Therefore, we examined whether the MAPK pathway was involved in the IAP-enhanced neuronal responses. The basal activity of MAPK in IAP-overexpressing neurons was significantly increased (Fig. 5). The enhanced outgrowth of dendrites (Fig. 6B), the release of glutamate (Figs. 6A and 7D), and the glutamate-mediated spontaneous Ca\(^{2+}\) oscillations (Fig. 7) caused by overexpression of IAP were suppressed by a MAPK pathway inhibitor, U0126, indicating that activation of MAPK is required for the IAP-mediated neuronal effects. Notably, after the down-regulation of endogenous IAP expression by siRNA, the activity of MAPK and the level of synaptic proteins were significantly reduced (Fig. 8A). Consistent with this finding, the glutamate release in IAP-siRNA transfected cultures was decreased (Fig. 8B), strongly suggesting that MAPK/ERK is important to the IAP-mediated synaptic function. Wang et al. (45) showed that IAP stimulates \(a_{\beta}\) integrin-mediated smooth muscle cell migration through inhibition of ERK (MAPK) activity. In contrast, IAP can transduce signals following interaction with thrombospondin, that is, the binding of thrombospondin to IAP stimulated the phosphorylation of ERK (pERK) were decreased after IAP-siRNA transfection. The endogenous IAP was down-regulated by IAP-siRNA. Scramble (control) siRNA had no effect. In contrast, the basal pAkt and Akt (both included in a PI 3-kinase pathway) were unchanged by IAP-siRNA. Bad and Bcl-2 (apoptosis or survival-associated proteins) were shown as a negative control. TUJ1 was detected as a neuronal marker. B, the glutamate release in IAP-siRNA-transfected cultures was significantly reduced. The glutamate release in Scramble siRNA-transfected cells was not influenced. Data represent mean ± S.D. (n = 4). (t test).

**FIG. 8.** Synaptic proteins and glutamate release were decreased after down-regulation of endogenous IAP by IAP-siRNA. A, the levels of SNAP25, synapsin I, and phosphorylated ERK (pERK) were decreased after IAP-siRNA transfection. The endogenous IAP was down-regulated by IAP-siRNA. Scramble (control) siRNA had no effect. In contrast, the basal pAkt and Akt (both included in a PI 3-kinase pathway) were unchanged by IAP-siRNA. B, the glutamate release in IAP-siRNA-transfected cultures was significantly reduced. The glutamate release in Scramble siRNA-transfected cells was not influenced. Data represent mean ± S.D. (n = 4). (t test).
IAP Increased Synaptic Function

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