et al., 2000). As described in the introduction, learning/memory and the development of drug dependence share similar mechanisms. Kyosseva et al. (2001) have reported that chronic administration of phencyclidine in rats produces a specific activation of ERK1/2, indicating a role for the ERK signaling pathway in phencyclidine abuse and perhaps in schizophrenia. Valjent et al. (2000) have reported that ERK activation was induced 10 min after acute administration of cocaine in the NAc and St of rat, and systemic administration of SL327, a selective MEK inhibitor, inhibits hyperlocomotion and acquisition of CPP induced by cocaine. Taken together, these reports strongly indicate that MAP and other psychostimulants activate the ERK1/2 signaling cascade in the brain.

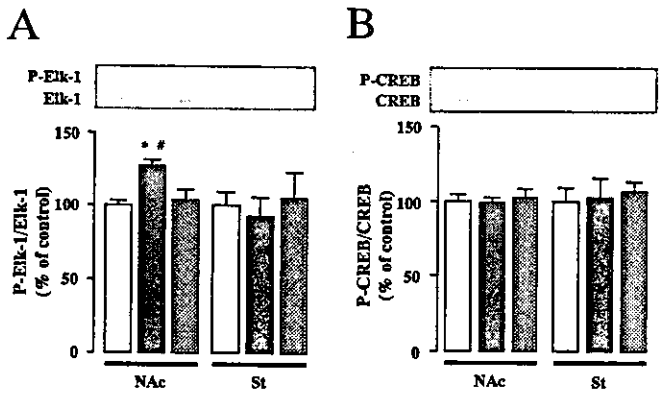


**Fig. 4.** A, Elk-1 activation associated with the expression of MAP-induced CPP. B, no changes in CREB phosphorylation on MAP-induced CPP. Rats were given MAP (2 mg/kg) or saline for the conditioning to the CPP test and were killed immediately after the test. Open column, saline-treated $CPP^+$ rats; closed column, MAP-treated $CPP^+$ rats; hatched column, MAP-treated $CPP^-$ rats. Data are presented as the mean ± S.E. ($n$ = 5). *, $P < 0.05$ vs. saline-treated $CPP^-$ rats; #, $P < 0.05$ vs. MAP-treated $CPP^-$.

In the present study, we found that ERK1/2 phosphorylation was activated in the NAc and St, but not in other brain areas of MAP-treated $CPP^+$ animals, whereas no such phosphorylation was seen in the control and the MAP-treated $CPP^-$ animals. Therefore, it is highly likely that ERK1/2 activation was induced by exposure to the context in which the animals had previously received MAP, not the MAP treatment itself. The microinjection of PD98059 into the NAc significantly inhibited the expression of MAP-induced CPP and abolished the ERK1/2 activation evoked by MAP-induced CPP, suggesting a critical involvement of the ERK signaling cascade in the expression of CPP induced by MAP. Our hypothesis is that the activation of ERK1/2 and Elk-1 in the

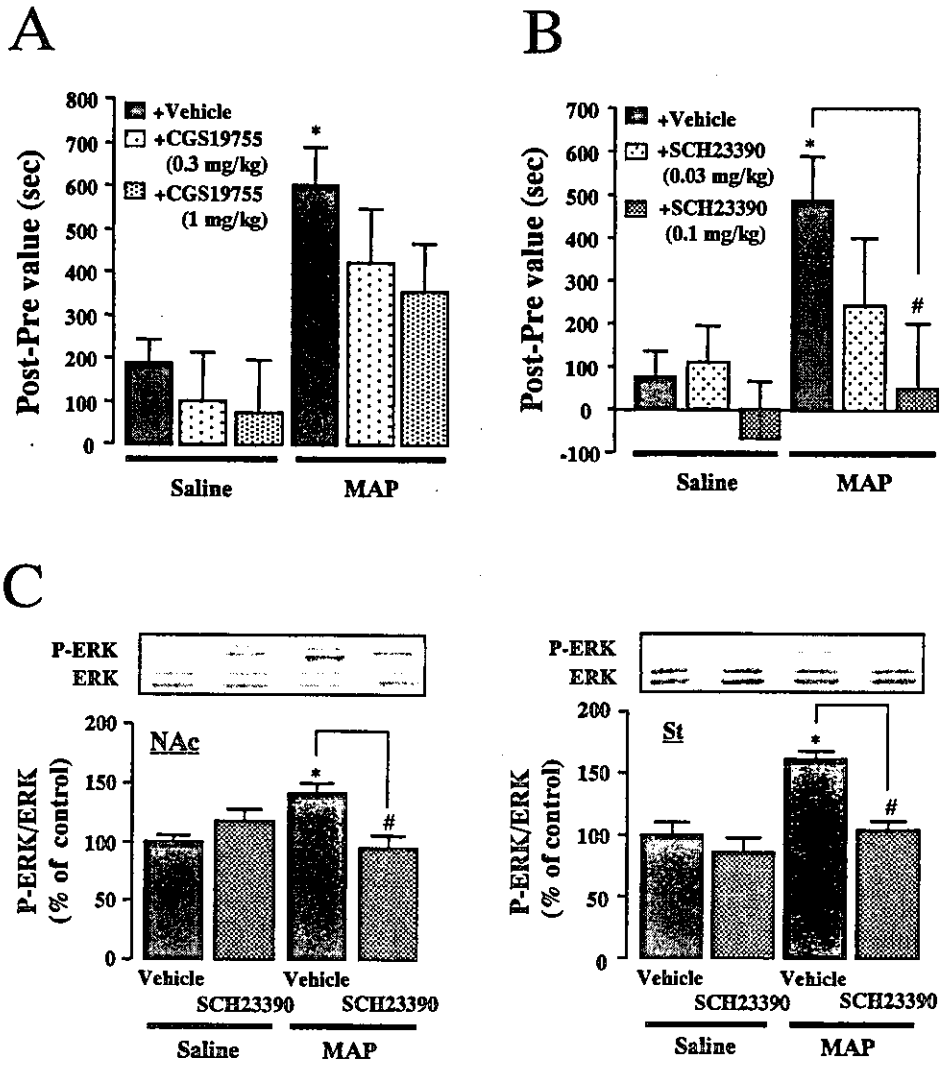


**Fig. 5.** Effect of NMDA receptor antagonist (A) and dopamine D1 receptor antagonist (B) on the expression of MAP-induced CPP; and effect of SCH23390 (0.1 mg/kg) on the hyperphosphorylation of ERK1/2 evoked by MAP (C). Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed immediately after the CPP test. SCH23390 was administered i.p. 30 min before the CPP test. Data are presented as the mean ± S.E. ($n$ = 7~16 for A; $n$ = 7~16 for B; $n$ = 5~7 for C). *, $P < 0.05$ vs. vehicle-treated saline group; #, $P < 0.05$ vs. vehicle-treated MAP group.

NAc represents the neuronal response related to the contextual memory of the rewarding effects of MAP, and is thereby associated with long-lasting neuroadaptation in MAP dependence.

The dopamine D1 receptor antagonist SCH23390 at 0.1 mg/kg significantly reversed the hyperphosphorylation of ERK1/2 in the NAc and St evoked by MAP-induced CPP as well as the expression of CPP in MAP-treated animals. These results suggest that the expression of CPP induced by MAP may be related to the ERK1/2 activation via dopamine D1 receptors. It is well known that stimulation of dopamine D1 receptors results in the activation of ERK1/2 (Valjent et al., 2000; Zanassi et al., 2001). Several molecules could be responsible for the link between dopamine D1 receptors and ERK, such as the small Ras-related G protein Rap1, activated by PKA, and the subsequent activation of the B Raf isoform (Vossler et al., 1997; Zanassi et al., 2001). Another possible intermediate between the D1 receptor and ERK activation could be calcyon, a dopamine D1 receptor-interacting protein, expressed in the St (Lezcano et al., 2000). Calcyon stimulates intracellular calcium release, which is known to activate the Ras/ERK pathway (Lev et al., 1995). It should be investigated whether these intermediates are responsible or not for MAP-induced CPP in the near future. The dopamine D2 receptor antagonist raclopride significantly abolished the expression of MAP-induced CPP. However, ERK1/2 hyperphosphorylation evoked by MAP-induced CPP was blocked in the St but not the NAc. Dopamine D1 and D2 receptors are coupled with $G_s$ and $G_{i/o}$ protein, respectively, and thus they have opposite effects on intracellular signaling, such as ERK1/2 activation via the cAMP/PKA pathway (Zanassi et al., 2001). However, a recent study demonstrated that the dopamine D2 receptor agonist quinpirole induces ERK1/2 and CREB phosphorylation in neurons via protein kinase C/Ras/Raf/MEK and DARPP-32, a dopamine and cAMP-regulated phosphoprotein (Yan et al., 1999). Furthermore, it is shown that dopamine D1 and D2 receptors synergistically activate immediate early gene expression and locomotion in dopamine-depleted rats (Paul et al., 1992; Keefe and Gerfen, 1995) and are required to evoke neural and behavioral phenotypes of cocaine sensitization (Capper-Loup et al., 2002). Thus, it is possible that raclopride inhibits the expression of MAP-induced CPP by inhibiting the ERK1/2 hyperphosphorylation through the blockade of dopamine D2 receptors in the St.

Activation of tyrosine kinase receptors or NMDA receptors results in an activation of ERK1/2. The neurotrophins, which play an important role in several forms of synaptic plasticity, such as learning and memory (Thoenen, 1995; Yamada et al., 2002), are expressed by dopamine neurons in the ventral midbrain (Davies, 1994). The members of the nerve growth factor family of neurotrophins that are active in the brain include nerve growth factor, brain-derived neurotrophic factor (BDNF), neurotrophin-3, and neurotrophin-4/5 (Davies,

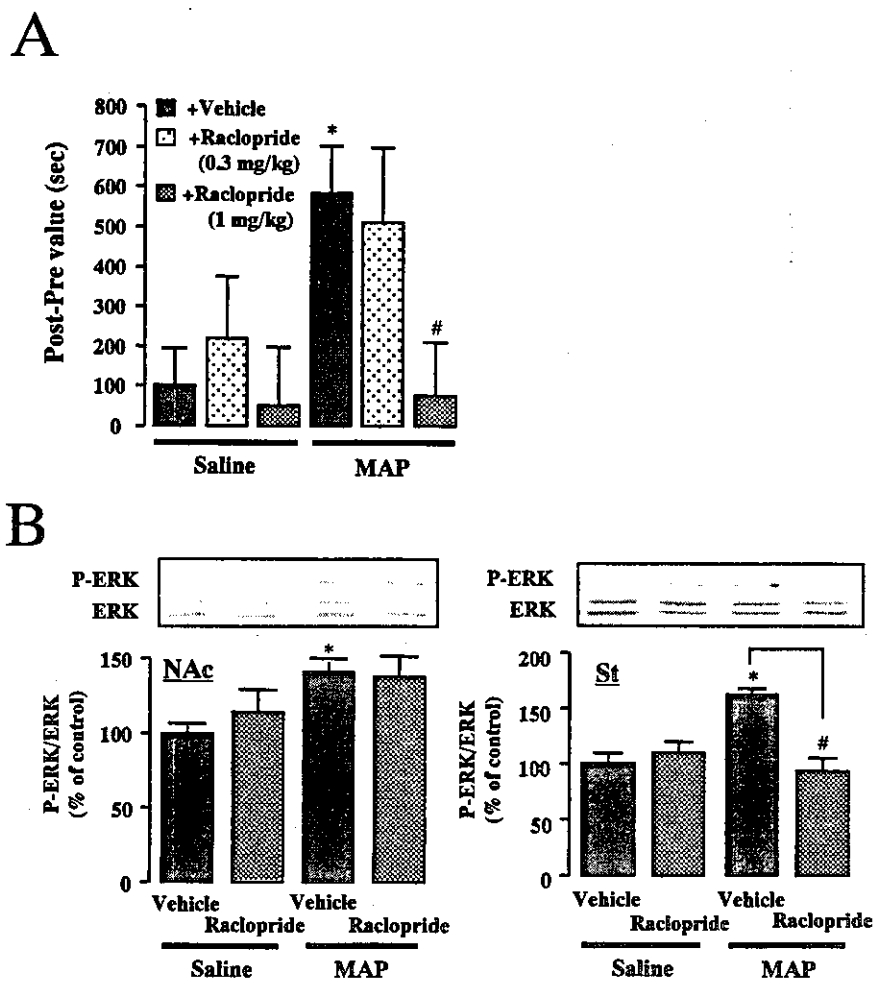


**Fig. 6.** Effect of dopamine D2 receptor antagonist on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the test and were killed immediately after the test. Raclopride was administered i.p. 30 min before the CPP test. Data are presented as the mean ± S.E. ($n$ = 8~14 for A; $n$ = 4~7 for B). *, $P < 0.05$ vs. vehicle-treated saline group; #, $P < 0.05$ vs. vehicle-treated MAP group.

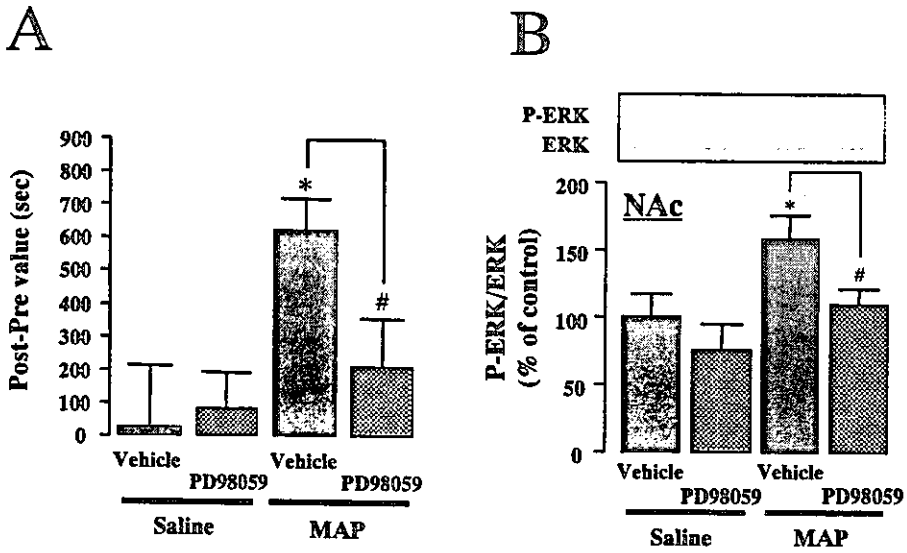


**Fig. 7.** Effects of microinjection of PD98059 into the NAc on the expression of CPP (A) and hyperphosphorylation of ERK1/2 (B) evoked by MAP. Rats were given MAP (2 mg/kg) or saline for conditioning to the CPP test. PD98059 (2 μg/side) or vehicle (60% dimethyl sulfoxide) was injected bilaterally into the NAc in a volume of 1.5 μl, 20 min before the CPP test. Data are presented as the mean ± S.E. ($n$ = 9~13 for A; $n$ = 4~8 for B). *, $P < 0.05$ vs. vehicle-treated saline group; #, $P < 0.05$ vs. vehicle-treated MAP group.

1994; Pierce et al., 1999). The effects of neurotrophin are initiated by binding to their receptor tyrosine kinase, TrkA, TrkB, and TrkC, respectively (Segal and Greenberg, 1996). The MAP kinase pathway is one of the major signaling cascades activated downstream of neurotrophin stimulation of Trk receptors (Segal and Greenberg, 1996). For instance, chronic morphine treatment or chronic infusion of BDNF induces ERK activation in the VTA, and the morphine-induced increase in ERK activity is blocked by local infusion of NMDA receptor antagonist into the VTA (Berhow et al., 1996). Moreover, neurotrophin-3 contributes to the initiation of behavioral sensitization to cocaine by activating the Ras/ERK kinase signal transduction cascade through TrkC (Pierce et al., 1999). However, no phosphorylation of Trk receptors was seen in any brain regions of MAP-treated CPP$^+$ animals. Moreover, CGS19755 did not inhibit the expression of CPP evoked by MAP. Therefore, it is unlikely that tyrosine kinase receptors and NMDA receptors play an important role in the activation of ERK1/2 for the expression of MAP-induced CPP. However, in the present study, Trk phosphorylation was analyzed at the same time point as ERK1/2 immediately after the CPP. Because this is an early event in the Ras/ERK1/2 cascade, it remains possible that Trk phosphorylation occurs before this event.

Neither JNK nor p38 was phosphorylated by the expression of CPP evoked by MAP. This result is consistent with a report that neither dopamine nor forskolin activates JNK in striatal primary neuronal cells (Schwarzschild et al., 1997). Therefore, it seems likely that among MAP kinases, ERK1/2 is related and important to the expression of CPP induced by MAP.

How does ERK1/2 activation contribute to the expression of CPP induced by MAP? ERK1/2 is localized to both pre- and postsynaptic neurons in the hippocampus and cerebral cortices (Atkins et al., 1998; Jovanovic et al., 2000) and regulates synaptic vesicle proteins such as synapsin, voltage-gated ion channels, and transcription factors, including CREB and Elk-1. In postsynaptic neurons, ERK1/2 activation through both dopamine D1 and D2 receptors results in its translocation to the nucleus, resulting in phosphorylation of Elk-1. These signalings to the nucleus would initiate cell-specific gene expression programs necessary for synaptic remodeling in the expression of CPP induced by MAP. Moreover, BDNF-induced glutamate and GABA release is linked to the phosphorylation of synapsin via the activation of TrkB and ERK1/2 (Jovanovic et al., 2000). Depolarization-induced activation of ERK1/2 stimulates tyrosine hydroxylase phosphorylation and dopamine synthesis in rat striatal slices (Lindgren et al., 2002), suggesting that ERK1/2, which has been extensively studied in relation to postsynaptic changes such as gene expression, is also able to regulate presynaptic function transiently in the brain. Therefore, it is possible that activated ERK1/2 in presynaptic neurons plays a role in increasing dopamine biosynthesis and release for the expression of CPP induced by MAP. These events in pre- and postsynaptic neurons in the NAc and St might be concerned with the expression of MAP-induced CPP when MAP-treated rats are exposed to the environment in which they had previously received drug treatment.

In conclusion, our study indicates that ERK1/2 activation can lead to the expression of CPP induced by MAP through both dopamine D1 and D2 receptors. ERK1/2 activation observed in both NAc and St may play a role, at least in part, in the learning/memory mechanisms of drug dependence induced by MAP.

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Research report

# Molecular mechanisms in dizocilpine-induced attenuation of development of morphine dependence: an association with cortical $Ca^{2+}$/calmodulin-dependent signal cascade

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**Abstract**

We investigated how dizocilpine, a non-competitive *N*-methyl-D-aspartate (NMDA) receptor antagonist, affects the development of morphine dependence in mice. Co-administration of dizocilpine (0.25 mg/kg) and morphine (10 mg/kg) for 5 days attenuated the development of tolerance to the antinociceptive effects of morphine. The withdrawal manifestation induced by the naloxone-challenge (5 mg/kg) was significantly reduced in mice that were treated with a combination of dizocilpine and morphine, compared to the mice treated with morphine and saline. The present study revealed a significant increase in c-Fos protein expression in the cortex and thalamus of mice showing naloxone-precipitated withdrawal syndrome. The combination of dizocilpine and morphine prevented the increase of c-Fos protein expression in the cortex and thalamus. Interestingly, repeated co-administration of dizocilpine and morphine prevented the withdrawal-induced phosphorylation of $Ca^{2+}$/calmodulin kinase II (p-CaMK II) in the cortex, but not in the thalamus. Acute dizocilpine treatment prior to the naloxone-challenge and repeated treatment with dizocilpine alone had no effect on analgesia, withdrawal manifestations, p-CaMK II levels or c-Fos protein levels. These results showed that co-administration of dizocilpine and morphine prevented the development of morphine tolerance and dependence and suggested that the preventive effect of dizocilpine results from the regulation of c-Fos protein expression, which is possibly involved in the activation of the $Ca^{2+}$/calmodulin-dependent signal cascade in the cortex.


*Keywords:* Morphine; Withdrawal manifestations; Dizocilpine; c-Fos protein; $Ca^{2+}$/calmodulin kinase

## 1. Introduction

Opioid analgesics such as morphine are widely used in the management of pain. Repeated use may lead to the development of tolerance and dependence. Tolerance is indicated by a decreased efficacy of the drug after chronic use leading to the requirement for a higher dose to get the desired analgesic effect. Dependence is a continued need for the drug to maintain a state of physiological equilibrium, following repeated administration, and is evidenced by withdrawal manifestations when drug administration is terminated [7]. The mechanisms underlying the development of morphine tolerance, dependence and withdrawal manifestations are not fully understood.

The *N*-methyl-D-aspartate (NMDA) receptor is one of the glutamate receptors that plays a key role in synaptic plasticity and neuronal development [19]. Recent studies proved that excitatory amino acids (EAA) and their receptors have a role in the chronic actions of opioids [13,15] and the NMDA receptor plays an important role in opiate tolerance [23] and dependence [8,33]. The activation of NMDA receptors leads to the opening of receptor-gated ion channels, which allow $Ca^{2+}$ to enter the neuron, where it participates in numerous processes, including the activation of protein kinases [35]. Protein kinases such as $Ca^{2+}$/calmodulin kinase II (CaMK II) have been reported to play an important role in various neuronal adaptive processes such as long-term potentiation [21], drug addiction [24] and the induction of

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immediate early gene expression such as c-*fos* and c-*jun*, etc. [18]. Chronic morphine treatment increases the expression of CaMK II [20], and causes adaptive increases in $Ca^{2+}$ accumulation [35]. Further, opioid withdrawal has been demonstrated to lead to an induction of the c-*fos* mRNA and its c-Fos protein product in several regions of the rat brain [10,12,30]. Dizocilpine, a non-competitive NMDA receptor antagonist, has been shown to prevent the morphine dependence in rodents [8,34]. However, the pathway by which dizocilpine could modulate opioid dependence and tolerance, and the effects of dizocilpine on opiate-induced changes in neuronal activity remain unclear.

In the present study, we here investigated how the dizocilpine affects the development of morphine dependence and tolerance in mice: (1) whether dizocilpine could attenuate the development of opioid dependence and tolerance according to previous reports [8,30]. (2) The effects of dizocilpine on withdrawal-induced changes in neuronal activity by using expression of c-Fos protein as a marker in the cortex and thalamus. (3) The possible role of the $Ca^{2+}$/calmodulin-dependent signal cascade in the symptoms of morphine withdrawal [16,22,27] by measuring phosphorylation of CaMK II (p-CaMK II) in the cortex and thalamus. We have previously demonstrated that both brain regions are involved in the development morphine dependence, since ERK activation and/or the increased cAMP levels are observed at the cortex and/or thalamus of morphine-dependent mice showing naloxone-precipitated withdrawal syndrome [10,22,28]. Thus, we used these both regions in the present study.

## 2. Materials and methods

### 2.1. Animals

Male C57BL/6J mice, 7 weeks old, weighing 20–25 g were used. The animals were handled in accordance with the guidelines established by the Institute for Laboratory Animal Research of Nagoya University. They were housed in groups of five in a temperature and light controlled room (23±1 °C, a 12-h light/dark cycle, with lights on at 7.30 a.m.) with free access to food and water.

### 2.2. Drug treatment

Morphine hydrochloride (Shionogi Pharmaceutical, Co. Ltd., Osaka, Japan), dizocilpine hydrogen maleate (Sigma, St. Louis, MO, USA) and naloxone hydrochloride (Sigma) were used.

To develop morphine tolerance and dependence, mice received morphine (10 mg/kg s.c.) with or without dizocilpine twice daily for 5 days. Dizocilpine was administered 30 min before every morphine treatment. In our preliminary experiments, we found that the higher doses (0.5 and 1 mg/kg i.p.) of dizocilpine markedly induced motor dysfunction such as ataxic behavior and motor in-coordination in mice. A dose of 0.25 mg/kg i.p. of dizocilpine was chosen for subsequent experimental steps. This dose has a significant effect on the attenuation of withdrawal manifestations and avoids the toxic interaction between higher doses of dizocilpine and morphine during induction of tolerance and dependence.

### 2.3. Hot plate test

The temperature of the hot plate was kept at 52 ± 0.2 °C. The analgesic threshold was considered to be the latency between the moment an animal was placed on the plate and the time when it started licking its hind paws or jumping with all four feet. Prior to drug administration, all mice were tested on the hot plate for 4 days in order to obtain a stable control response level. The animals were removed from the hot plate if they did not respond within 30 s in order to avoid tissue damage. Any animal, which failed to respond within 30 s was excluded immediately and re-tested again after 30 min. The antinociceptive effect of morphine was determined 60 min after the first injection on the first, third and fifth day.

### 2.4. Induction of withdrawal syndrome

All groups received naloxone (5 mg/kg i.p.) 2 h after the last injection of morphine on the sixth day of treatment. Immediately after the naloxone injection, each animal was placed in a transparent acryl cylinder (20 cm in diameter, 35 cm in height) to observe withdrawal manifestations (jumping, rearing, teeth chatter, paw tremors and diarrhea) for 30 min. The withdrawal manifestations were manually evaluated by co-workers blind to the treatment protocol. Diarrhea was scored as 0: no, 1: mild, 2: moderate, and 3: severe diarrhea.

### 2.5. Immunocytochemistry

#### 2.5.1. Brain fixation

One hour after naloxone treatment according to our previous report [10], the animal was anesthetized with sodium pentobarbital (50 mg/kg i.p.), and perfused transcardially with 60 ml of heparinized saline, followed by 60 ml of 10% formalin in 0.1 M phosphate-buffered saline (PBS) (pH 7.4). The brain was removed, post-fixed at 4 °C overnight in the same fixative and then cryoprotected in 30% sucrose in the same buffer for one night, and kept at −80 °C until the assay.

#### 2.5.2. Staining

The brains were cut into 50 μm thick coronal sections by a cryostat, extensively rinsed with PBS, and processed for immunocytochemistry as described previously [11]. Briefly, the sections were incubated in 1.5% normal goat serum in PBS for 1 h at room temperature. Then, they were incubated with primary antibody diluted (1:1000) in PBS for 48 h at 4 °C, washed three times in PBS (for 10 min each) and incubated with biotylinated secondary antibody (1:200)

in PBS for 2 h at room temperature. Next, they were washed as above and then incubated with avidin–biotin-peroxidase complex (1:100) for 1 h at room temperature. After another wash the sections were incubated in 0.04% diaminobenzidine with 0.0075% hydrogen peroxide and 10 μl of imidazole in PBS for 4–5 min. The reaction was stopped in PBS, and the sections were mounted on gelatin-subbed slides. The slides were allowed to dry prior to being dehydrated in gradually concentrated ethanol, cleared in xylene and cover-slipped with mounting medium.

#### 2.5.3. Counting of c-Fos protein-positive cells

Tissue sections were examined at low power (50×) by light microscopy, to determine the segmental level at the same coordinates according to the atlas of Franklin and Paxinos [6], as well as gray matter landmarks. Positive c-Fos cells were identified using light field microscopy at (200×) and detected by the brown color of their nuclei. Positive nuclei were counted only when structures of the appropriate size and shape demonstrated clear increases in immunoreactivity compared to the background level. Questionable structures were examined at higher power (500×) and were not counted if identification remained uncertain [2,10]. The total number of positive cells in five similar sections through each area was counted in every animal. The number (mean ± S.E.) of positive cells in the area for one animal in each group ($n = 5$) was calculated.

### 2.6. Western blot analysis

Fifteen minutes after naloxone injection, the animals were decapitated and different areas of the brain were dissected and kept at −80 °C. The dissected brain tissue was homogenized by sonication in an ice-cold lysis buffer (50 mM Tris–HCl pH 7.5, 150 mM NaCl, 10 mM NaF, 10 mM EDTA, 0.5 mM dithiothreitol, 1 mM sodium orthovanadate, 10 mM sodium diphosphate decahydrate, 0.2 mM phenylmethylsulfonyl fluoride, 4 μg/ml aprotinin, 4 μg/ml leupeptin and 4 μg/ml pepstatin). The homogenate was centrifuged at 13,000 × $g$ for 20 min and the supernatant was used for the measurement of phosphorylation of CaMK II. Samples (20 μg of protein) were boiled in Laemmli sample buffer, separated on a 10% polyacrylamide gel and subsequently transferred to PVDF membranes (Millipore). The membranes were blocked with a Detector Block Kit (KPL) for 2 h at room temperature and probed with anti-phospho-CaMK IIα/β subunit antibody (polyclonal anti-rabbit peptide antibodies at 0.5 μg/ml, Upstate, VA, USA) overnight at 4 °C. Membranes were washed with TBST buffer (10 mM Tris–HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.4) and subsequently incubated with a goat anti-rabbit horseradish peroxydase-conjugated secondary antibody for 1 h at room temperature. The immune complexes were detected by chemiluminescence (ECL, Amersham) and exposed to X-ray film. The band intensities of the film were analyzed by densitometry. To confirm equal loading of each protein, membranes were stripped with stripping buffer (62.5 mM Tris–HCl, 100 mM 2-mercaptoethanol, and 2% SDS, pH 6.7) at 50 °C for 30 min, and then incubated with anti-CaMK IIα subunit antibody (monoclonal anti-rabbit peptide antibodies at 0.2 μg/ml, Sigma) and detected as described above.

### 2.7. Statistical analysis

The results are expressed as means ± S.E.M. for each group. Statistical analysis of the difference between groups was done with the one-way ANOVA and Student's $t$ test as a post hoc analysis. Differences were considered statistically significant at a level of $P < 0.05$.

## 3. Results

### 3.1. Effect of dizocilpine on morphine-induced analgesia

Acute morphine treatment (10 mg/kg s.c.) produced an antinociceptive effect in the hot plate test (Fig. 1A): the average analgesic latency in morphine-treated mice (25.5 ± 1.0 s) was significantly increased compared to that in saline-treated mice (11.0 ± 1.3 s). When dizocilpine (0.25 mg/kg i.p.) was administered 30 min before the morphine, it had no effect on the morphine-induced analgesia (23.8 ± 1.0 s) or the analgesic latency itself (7.4 ± 1.1 s).

### 3.2. Effect of dizocilpine on the development of morphine tolerance to analgesia

Tolerance to morphine was developed with the same administration schedule as dependence. The effect of dizocilpine co-administered with morphine on the development of morphine tolerance is shown in Fig. 1B and C shows the time course of development of morphine tolerance and the effect of co-administration of dizocilpine and morphine on it. Chronic administration of morphine alone induces tolerance to its antinociceptive effect. The analgesic latency in chronic morphine-treated mice on day 5 (11.8 ± 1.2 s) was not significantly different from the latency in chronic saline-treated mice (7.8 ± 0.9 s). The development of tolerance was significantly inhibited by the co-administration of dizocilpine and morphine. The analgesic latency (22.7 ± 1.8 s) in the chronic (dizocilpine + morphine)-treated group was significantly increased compared to the chronic (saline + morphine)-treated group or chronic saline-treated group. Chronic dizocilpine treatment alone had no effect on analgesic latency (5.7 ± 0.5 s) compared to chronic saline treatment.

### 3.3. Effects of dizocilpine on naloxone-precipitated withdrawal manifestations in morphine-dependent mice

Chronic morphine treatment produces physical dependence as assessed by a characteristic set of behavioral

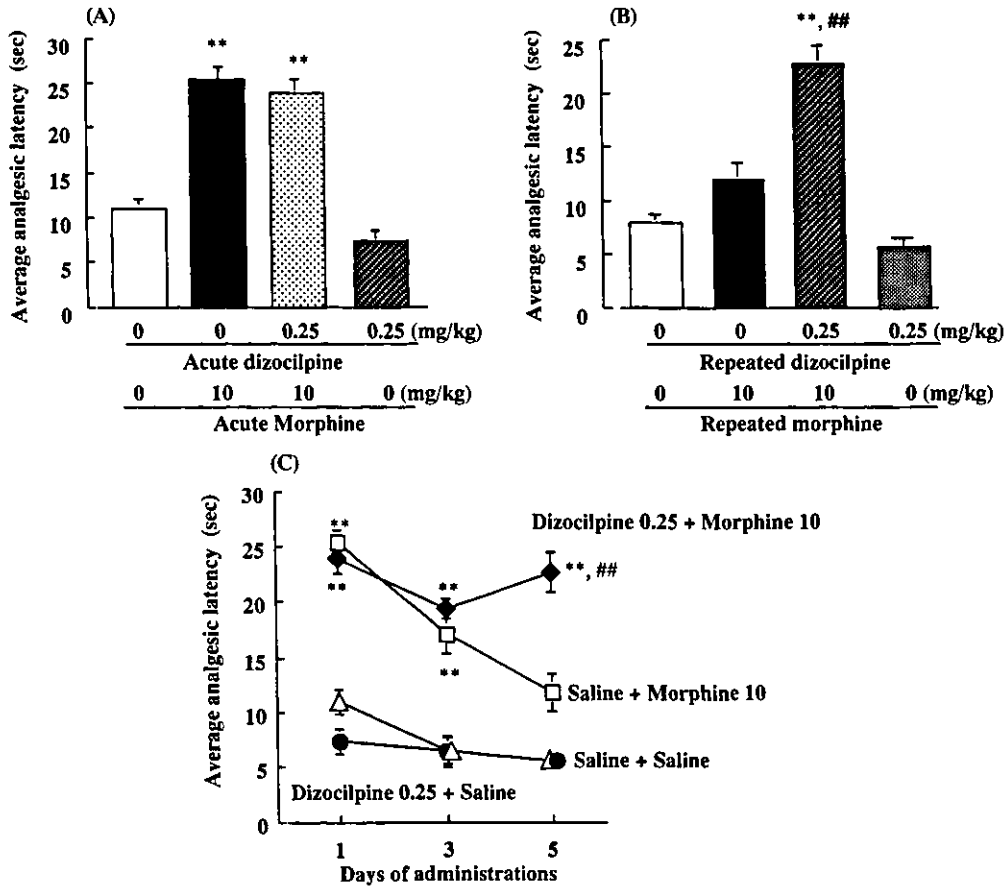


Fig. 1. Effects of dizocilpine on morphine-induced analgesia in mice. (A) Effect of acute dizocilpine treatment, (B) effect of repeated co-administration of dizocilpine and morphine, (C) the time course of tolerance to morphine-induced analgesia. The values are expressed as mean analgesic latency (hot plate) ± S.E.M. $^{**}P < 0.01$ vs. (saline + saline)-treated group. $^{\#\#}P < 0.01$ vs. (saline + morphine 10)-treated group ($n = 5$).

responses, including teeth chatter, erection, ptosis, chews, lacrimation, diarrhea, salivation, writhes, jumps, wet dog shakes and weight loss, following naltrexone challenge [26]. In agreement with previous reports, chronic morphine treatment produced a highly significant increase in withdrawal manifestations such as jumping, paw tremors, rearing, teeth chatter and diarrhea following the naloxone (5 mg/kg) challenge compared to the saline treatment (Fig. 2A–E). Co-administration of dizocilpine (0.25 mg/kg) and morphine (10 mg/kg) attenuated all withdrawal manifestations significantly (Fig. 2A–E). No significant difference could be detected between dependent mice and the dependent mice that received acute dizocilpine treatment (0.25 mg/kg) on the day of withdrawal. Further, chronic treatment with dizocilpine (0.25 mg/kg) alone also had no effect on withdrawal manifestations compared to the saline-treated group after the naloxone-challenge (Fig. 2A–E).

### 3.4. c-Fos protein expression

Naloxone-precipitated morphine withdrawal syndrome has been reported to associate with c-Fos protein expression in different areas of the brain [10,12]. The numbers of c-Fos-positive cells in the cingulate cortex (A) and cingulate thalamus (B) of morphine-dependent mice were significantly increased, compared to those in the saline-treated mice (Fig. 3). Chronic (dizocilpine + morphine)-treated mice showed a significant reduction of c-Fos protein expression in the above-mentioned areas compared to the chronic (saline + morphine)-treated mice or the dependent mice, which received acute dizocilpine treatment before the naloxone-challenge (Fig. 3). No significant difference was detected between the dependent mice and acute dizocilpine-treated dependent mice. Chronic dizocilpine treatment alone has no significant effect on the expression of c-Fos-positive cells (Fig. 3).

### 3.5. Changes in the phosphorylation of $Ca^{2+}$/calmodulin kinase II (p-CaMK II) levels

No difference in the total CaMK II level was detected between in the cingulate cortex and thalamus of each treatment following naloxone-challenge (data not shown). However, the mice with the naloxone-precipitated withdrawal syndrome showed a significant increase in p-CaMK II levels in the cingulate cortex, but not the thalamus. Although

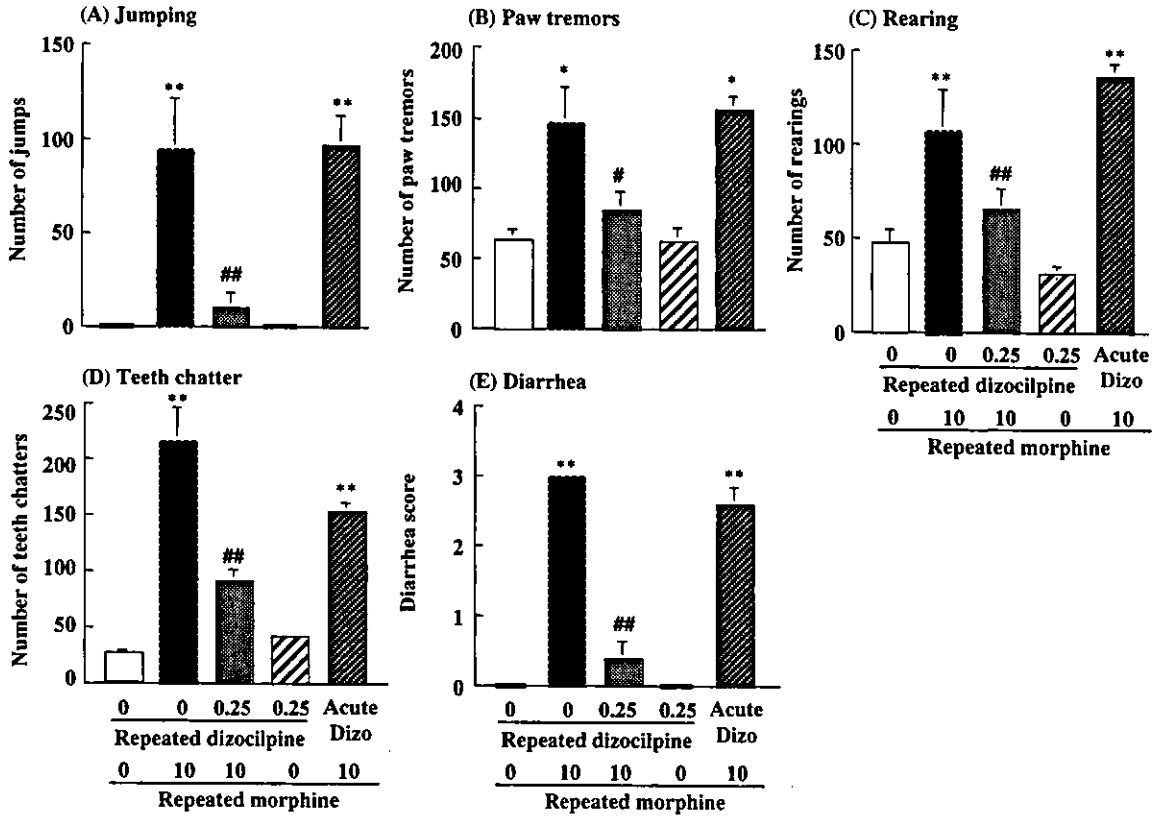


Fig. 2. Effects of co-administration of dizocilpine and morphine on the naloxone-induced withdrawal symptoms. (A) jumping, (B) paw tremor, (C) rearing, (D) teeth chatter, (E) diarrhea. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. chronic (saline + morphine 10)-treated group ($n = 5$). Dizo: dizocilpine.

dizocilpine itself did not significantly affect the level of p-CaMK II in the cingulate cortex, co-administration of dizocilpine significantly inhibited activation of CaMK II in the cingulate cortex (Fig. 4).

## 4. Discussion

We found that the chronic co-administration of dizocilpine and morphine inhibited the development of tolerance to morphine analgesia, as evidenced by a significant increase in the latency of analgesia in comparison with the chronic (saline + morphine)-treated group, but acute administration of dizocilpine had no effect on the morphine-induced tolerance. In addition, chronic dizocilpine treatment alone had no effect on the analgesic latency. Our results are consistent with previous reports that pretreatment with a non-competitive NMDA receptor antagonist, dizocilpine [8,34], can block the development of antinociceptive tolerance to morphine without affecting its antinociceptive actions [31,32]. An important characteristic of dizocilpine is that it attenuates the development of morphine tolerance without antagonizing the analgesic effect of morphine. In this respect it differs from the opioid receptor antagonists, naloxone [3] and β-funaltrexamine [4], which attenuate morphine tolerance only at doses that block the analgesia.

Chronic co-administration of dizocilpine (0.25 mg/kg) and morphine also inhibited the development of morphine dependence, evidenced by a significant reduction of withdrawal manifestations consistent with previous findings that selective antagonists for the NMDA receptor attenuate the manifestations of naloxone-induced withdrawal in morphine-dependent animals [14,26]. It is unlikely that the effect of dizocilpine is due to acute interaction between dizocilpine and morphine or naloxone on the day of withdrawal, since acute injection of dizocilpine before morphine or naloxone does not modify withdrawal behavioral manifestations in chronic morphine-treated mice. These results suggest that chronic co-administration of dizocilpine and morphine attenuates the development of morphine tolerance and dependence without affecting the acute effects of morphine.

Gene expression is thought to play an important role in many forms of neuronal plasticity. The severity of morphine dependence is related to the extent of morphine use, and withdrawal symptoms persist long after elimination [5,17]. Such long lasting behavioral modifications hint of plastic changes within the nervous system, some of which may be partially mediated by the regulation of gene expression

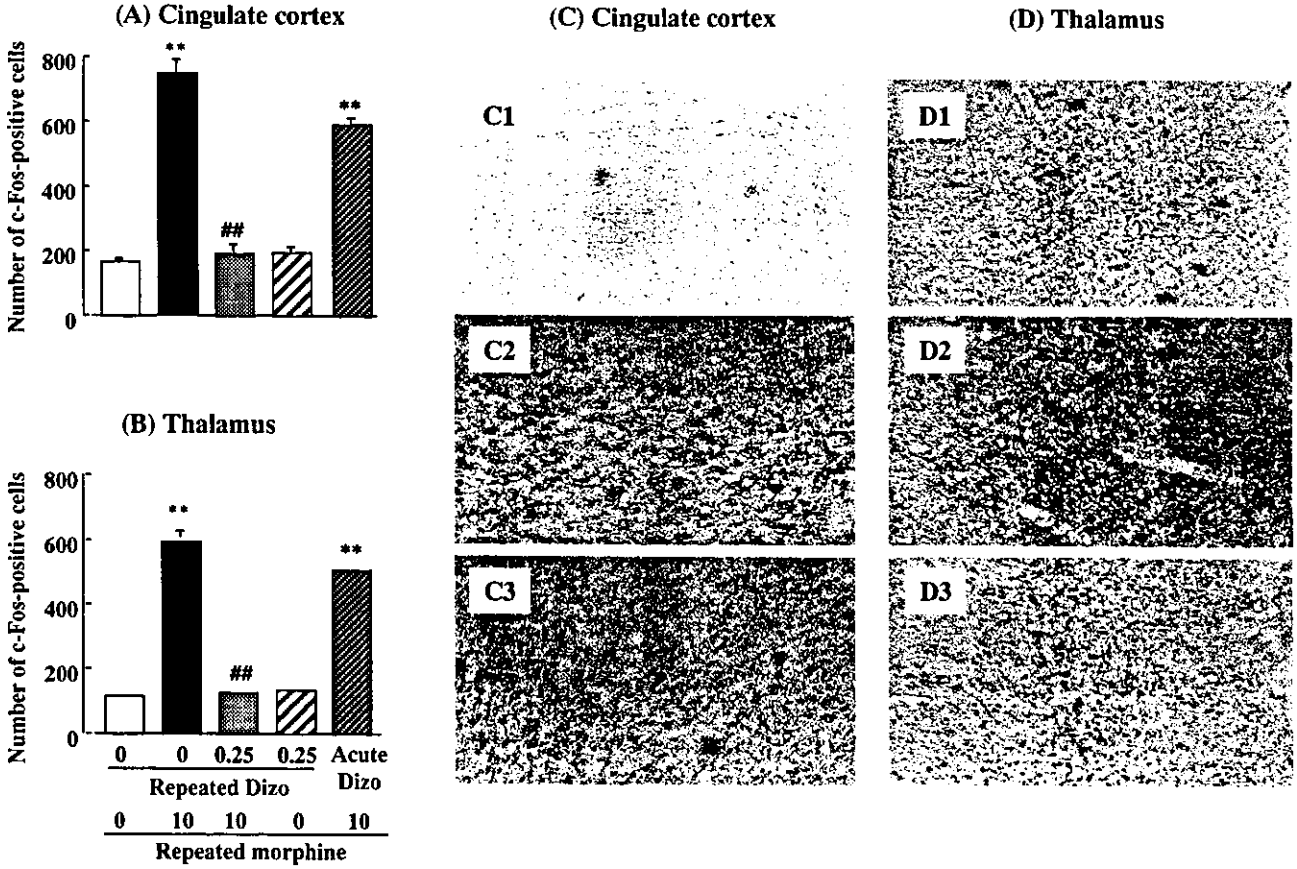


Fig. 3. Effects of repeated co-administration of dizocilpine and morphine on naloxone-induced c-Fos protein expression in the cingulate cortex (A) and cingulate thalamus (B). Representative photomicrographs of the cortex (C1–3) and thalamus (D1–3) for c-Fos protein histochemistry in different groups: (C1 and D1) = chronic (saline + saline)-treated group, (C2 and D2) = chronic (saline + morphine 10)-treated group, (C3 and D3) = chronic (dizocilpine 0.25 + morphine 10)-treated group. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#\#}P < 0.01$ vs. chronic (saline + morphine 10)-treated group ($n = 5$). Dizo: dizocilpine.

[9,24]. Acute and chronic morphine treatment decreases c-*fos* expression in the locus coeruleus [12]. Precipitated morphine withdrawal produced brain region-specific increases in the c-*fos* mRNA level. In the present study, the number of c-Fos protein-positive cells was significantly increased in the cingulate cortex and thalamus in morphine-dependent animals showing withdrawal manifestations, and the dependent mice injected with acute dizocilpine before the naloxone-challenge in comparison with chronic (saline + saline)-treated mice. In this study, interestingly, we observed a significant reduction in the number of c-Fos protein-positive cells in the group co-administered dizocilpine and morphine in the above-mentioned brain areas after naloxone-challenge. As regards c-Fos protein expression, our data are consistent with a previous report that levels of c-*fos* mRNA and protein are significantly increased in neuronal circuits mediating different functions during morphine withdrawal. Expression of c-Fos protein is significantly increased in several brain regions [10,30]. The frontal cortex and thamalus shows an increase of c-*fos* mRNA levels in morphine withdrawal [1,25]. Thus, this result supports our previous hypothesis that induction of c-Fos protein expression in different brain areas represents one feature of morphine withdrawal and may play a role in the expression of withdrawal manifestations [10].

The entry of $Ca^{2+}$ has the ability to affect numerous intracellular processes, including the activation of protein kinases [35]. Protein kinases have been reported to play an important role in various neuronal adaptive processes such as drug addiction [24]. Chronic opioid treatment causes adaptive increases in $Ca^{2+}$ accumulation and an increase in the expression of CaMK II [20]. The increase in $Ca^{2+}$ activates CaMK II [29]. CaMK II has an important role in the phosphorylation of CREB (cAMP response element binding protein) and the phosphorylation of CREB produces an increase in its activity, which leads to an increase in c-*fos* expression [29]. We investigated whether the $Ca^{2+}$/calmodulin-dependent signal pathway is involved in the dizocilpine-mediated attenuation of withdrawal syndrome and c-Fos protein expression. We did find an increase in the level of p-CaMK II only in the cingulate cortex. It has been suggested that the cortex is the terminal/intermedial area for noradrenergic neurons associated with drug addiction and plays a key role in the naloxone-precipitated morphine withdrawal syndrome [31].

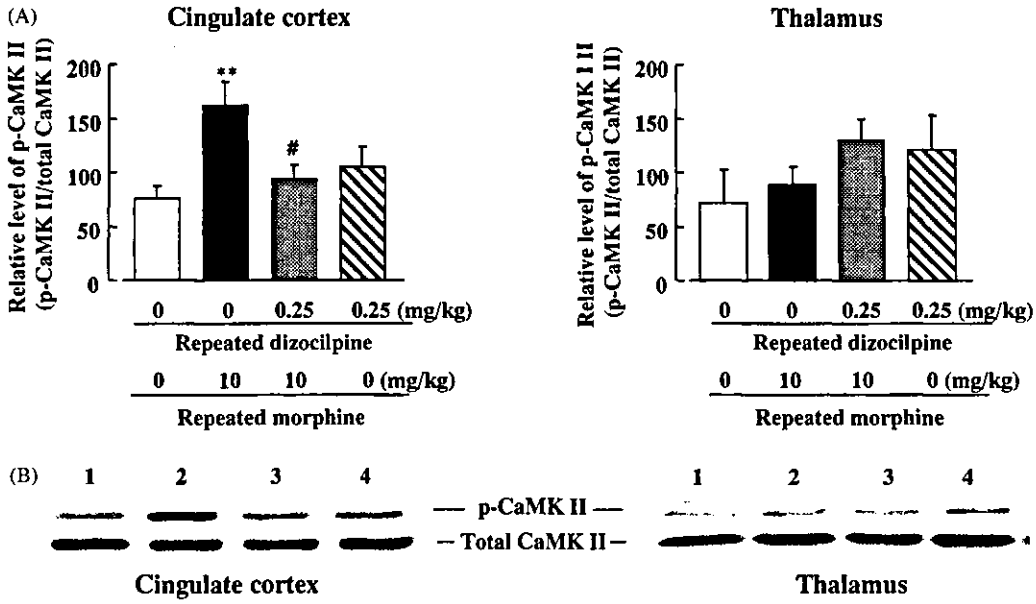


Fig. 4. The effects of dizocilpine on the p-CaMK II in the cingulated cortex and other brain areas of morphine-dependent mice following naloxone-precipitated withdrawal. (A) The ratio of p-CaMK II to total CaMK II in each brain region. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$ vs. chronic (saline + morphine 10)-treated group. (B) Lanes 1, 2, 3 and 4 represent chronic (saline + saline)-treated group, chronic (saline + morphine 10)-treated group, chronic (dizocilpine 0.25 + morphine 10) and chronic (dizocilpine 0.25 + saline)-treated group, respectively. $^{**}P < 0.01$ vs. chronic (saline + saline)-treated group, $^{\#}P < 0.05$ vs. chronic (saline + morphine 10)-treated group. Dizo: dizocilpine.

This activation of CaMK II after naloxone-precipitated withdrawal in the cingulate cortex was inhibited by repeated co-administration of dizocilpine and morphine. Activation of NMDA receptors leads to the opening of receptor-gated ion channels, which allow $Ca^{2+}$ to enter the neuron, where it participates in numerous processes, including the activation of protein kinases [35]. Dizocilpine is a non-competitive receptor antagonist that acts by blocking the ion channel [34]. According to these findings, we speculate that, the chronic co-administration of dizocilpine and morphine inhibits $Ca^{2+}$ participation in neurons. This inhibits the activation of CaMK II responsible for the phosphorylation of CREB. The lack of phosphorylation of CREB decreases its activity resulting in a reduced expression of c-Fos protein after the naloxone-challenge. These results support that chronic co-administration of dizocilpine and morphine, at least in part, through inhibition of CaMK II in the cingulate cortex, can attenuate the development of morphine tolerance, morphine dependence and c-Fos protein expression induced by naloxone-challenge. However, further study should be carried out to clarify other molecular mechanisms such as cyclic AMP and/or extracellular signal-regulated protein kinase signaling cascades, since there is a discrepancy between the changes of c-Fos protein and p-CaMK II in the thalamus.

In summary, we confirmed that dizocilpine attenuated the development of morphine dependence and tolerance, and found that naloxone-precipitated withdrawal-induced expression of c-Fos protein and activations of CaMK II in the cortex were prevented by repeated co-administration of dizocilpine and morphine. These results suggest that the preventive effect of dizocilpine results from the regulation of c-Fos protein expression, which is possibly involved in the activation of a $Ca^{2+}$/calmodulin-dependent signal cascade in the cortex.

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# Cyclic AMP/Protein Kinase A Signal Attenuates $Ca^{2+}$-Induced Fibroblast Growth Factor-1 Synthesis in Rat Cortical Neurons

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Fibroblast growth factor (FGF)-1 is increased in particular brain regions after birth, suggesting an involvement of some regulatory neuronal circuits. To address the neuronal activity responsible for FGF-1 synthesis, effects of various neurotransmitter receptor activation on cellular FGF-1 content were examined using cultured rat cortical neurons. Histamine, glutamate, carbachol, serotonin or γ-aminobutyric acid (GABA) caused an increase of FGF-1 content. Because this effect was mimicked by (1) *N*-methyl-D-aspartate, a glutamatergic agonist; (2) $Ca^{2+}$ ionophore; (3) depolarization with high concentration of KCl, but was abolished in $Ca^{2+}$-free medium, $Ca^{2+}$ influx was thought to trigger FGF-1 synthesis. Such $Ca^{2+}$-mediated enhancement of FGF-1 synthesis, however, did not occur in the presence of norepinephrine (NE), but was restored by KT-5720, an inhibitor of protein kinase A (PKA), suggesting an interplay between $Ca^{2+}$-activated and cAMP/PKA signals for neuronal FGF-1 synthesis. This mechanism was proved to function in vivo by stimulation of FGF-1 expression in neurons of the cerebral cortex after intracerebral administration of propranolol, an antagonist of adrenergic β receptors. This demonstrates that FGF-1 synthesis is essentially upregulated by $Ca^{2+}$ influx through excitatory neuronal activities, but such an effect is abolished by neurotransmission that evokes cAMP/PKA signals. FGF-1 produced is thought to act on establishment and maintenance of particular neuronal circuits in the brain, which may be one of the ways neurotransmitters regulate brain function.



**Key words:** fibroblast growth factor-1 (FGF-1); neurotrophic factor; synthesis; neurotransmitter; $Ca^{2+}$; cyclic AMP; protein kinase

Fibroblast growth factor (FGF)-1 is a prototype of the structurally related FGF family of proteins (Basilico and Moscatelli, 1992; Smallwood et al., 1996) that have 30–50% amino acid sequence homology and a similar exon/intron structure in the coding region of their gene (Wang et al., 1989; Goldfarb, 1990). FGF-1 promotes neuronal regeneration (Cordeiro et al., 1989) and survival (Unsicker et al., 1987) of cultured peripheral neurons, stimulates glial proliferation (Besnard et al., 1989; Davis and Stroobant, 1990), and exhibits neurotrophic effects on neuronal cultures and neuronal cell lines derived from the central nervous system (CNS) (Rydel and Greene, 1987; Unsicker et al., 1987; Lipton et al., 1988; Rifkin and Moscatelli, 1989; Mattson et al., 1989; Goldfarb, 1990). FGF-1 is expressed predominantly in neural tissues including brain, spinal cord, and retina, and exerts its effect through high-affinity receptors distributed throughout the nervous system (Heuer, 1990; Wanaka et al., 1990; Partanen et al., 1991). FGF-1 expression in brain is found exclusively in neuronal cells (Lipton et al., 1988; Heuer, 1990; Wanaka et al., 1990), but not in glial cells, and increases with brain development after birth (Ishikawa et al., 1991, Partanen et al., 1991). The role of FGF-1 in the CNS, however, is not yet well understood.

We have found that the FGF-1 level is increased within 3 weeks after birth, predominantly in the thalamus, pons/medulla, and cerebellum of rat brain, suggesting its involvement in the differentiation of particular neurons or neuronal circuits (Ishikawa et al., 1991). Such a development-dependent expression is observed in members of the neurotrophin family. Expression of nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) is enhanced postnatally in the brain regions in which FGF-1 expression is low, such as the hippocampus and cerebral cortex (Shelton and Reichardt, 1986; Das et al., 2001). NGF and BDNF have been shown expressed in an activity-dependent manner through excitatory and suppressive neuronal circuits (Zafra et al., 1990, 1991; Con-

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dorelli et al., 1994), demonstrating the involvement of neurotransmission in the neurotrophin synthesis (Thoenen et al., 1991). These findings suggest a possible activity- or transmission-dependent regulation of FGF-1 synthesis. Riva et al. (1996) have shown that cellular cAMP negatively regulates FGF-1 mRNA expression in cultured astrocytes; however, regulatory mechanisms of FGF-1 gene expression in neurons, the predominant source of FGF-1 in CNS, remain unknown.

We evaluated regulation of cellular FGF-1 in cultured rat cortical neurons using an enzyme immunoassay system (EIA), because of its high sensitivity, specificity for FGF-1, and superiority in quantification (Ishikawa et al., 1991). Our present results demonstrate that FGF-1 synthesis is enhanced by $Ca^{2+}$-activated signaling, but this enhancement is downregulated by cAMP/protein kinase A (PKA) signaling. Indeed, the blockade of cAMP/PKA signaling by propranolol, an antagonist of β-adrenergic receptors, administered into the adult rat cerebral cortex resulted in marked enhancement of FGF-1 mRNA and protein expressions. These in vitro and in vivo results suggest interplay between both signal transduction pathways to regulate FGF-1 synthesis in particular neurons of rat brain.

## MATERIALS AND METHODS

### Materials

FGF-1 and anti-FGF-1 antisera were prepared as described previously (Ishikawa et al., 1991). Anti-glial fibrillary acidic protein (anti-GFAP) antibody was purchased from DAKO Chemicals (Copenhagen, Denmark); and anti-microtubule-associated protein 2 (anti-MAP2) antibody, from Chemicon (Temecula, CA). γ-Aminobutyric acid (GABA), glutamate, carbachol, norepinephrine (NE), 5-hydroxytryptamine (5-HT; serotonin), histamine, *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), quisqualic acid, A23187, H89, and KT-5720 were obtained from Research Biochemicals (Natick, MA). α-Bungarotoxin, ranitidine, kainic acid and cycloheximide came from Sigma (St. Louis, MO).

### Cell Cultures

Neuronal cells were cultured from the cerebral cortices of 17-day-old rat embryos as described previously (Nakajima et al., 1993). Briefly, the cerebral cortices were incubated in phosphate-buffered saline (PBS) containing 0.25% trypsin, 10 mM glucose, and DNase (6 μg/ml; Sigma) for 20 min at 37°C, and triturated with a plastic pipette to dissociate the tissue into single cells. After centrifugation (900 × *g* for 3 min), the cell pellet was resuspended in medium composed with Dulbecco's modified Eagle medium (DMEM; Nissui, Tokyo, Japan) and nutrient mixture Ham's/F-12 (1:1; Gibco BRL), which contained 5% horse serum and 5% newborn calf serum. Suspended cells were plated in culture vessels ($10^5$ cells/$cm^2$) precoated with poly-DL-ornithine (0.5 μg/ ml; Sigma). After a 24-hr culture, the medium was changed to a serum-free one containing insulin (5 μg/ml; Sigma), transferrin (5 μg/ ml; Sigma), progesterone (2 pmol/ml; Sigma) and 5% bovine serum albumin (BSA). The cells were then cultured for 3 days, and provided for the experiments. Over 98% of the cells expressed MAP2 when stained with the specific antibody, demonstrating that most of cells in culture were neurons.

### Determination of FGF-1 Content

Cells were cultured in 35-cm dishes for 24 hr in the presence or absence of various reagents. The culture media were collected, and the cells were washed twice with PBS, gently detached with a rubber scraper from the culture dishes into 0.5 ml of 0.1 M Tris-HCl buffer (pH 7.6) containing 1 M NaCl, 2 mM EDTA, 2% BSA and 80 U aprotinin/ml, and disrupted by centrifugation at 100, 000 × *g* for 10 min. An aliquot of the culture media or the cell extracts were used for the EIA measurement. The cell number in five arbitrarily selected fields (total area, 2.4 $mm^2$) of each culture dish was counted under a phase-contrast microscope before scraping to calculate the total cell number. The values of FGF-1 content/$10^6$ cells were expressed as percent of the value obtained in the absence of the reagent.

The most critical event during extraction is the possible nonspecific binding of FGF-1 molecules to the surface of cell debris. To check this, we added exogenous FGF-1 to the extraction buffer before the second centrifugation. Recovery of exogenous FGF-1 (10–80 pg/ml) was about 95%, indicating that the loss of FGF-1 during extraction was negligible.

A two-site EIA for FGF-1 was carried out as described previously (Ishikawa et al., 1991). In short, immunoglobulin (Ig)G antibody (0.1 mg/ml) in 0.1 M Tris-HCl buffer (pH 7.6) was coated onto the well surface of 96-well U-bottom multiwell plates (20 μl/well) at room temperature for 1 hr. For evaluation of background signal, control wells were treated with normal rat IgG. The antibody IgG or control IgG solution was removed, and non-occupied space was blocked by incubation with 100 μl/well of 1% (wt/vol) skim milk for 1 hr. Each well was washed with 0.1 M Tris-HCl buffer (pH 7.6) containing 0.4 M NaCl, 0.1% BSA, 1 mM $MgCl_2$, and 0.02% $NaN_3$ (washing buffer) after every reaction. Each well then received 30 μl of test sample or serially diluted bovine FGF-1 as the standard. After incubation for 2 hr at room temperature, each well then received 30 μl of biotinylated affinity-purified antibody (0.2 μg/ml) in the washing buffer containing 1% normal rabbit serum. After overnight incubation at 4°C, 30 μl/well of β-D-galactosidase-conjugated streptavidin was added; 1 hr later, bound enzyme activity was measured by the addition of 30 μl of 30 μM 4-methylumbelliferyl-β-D-galactoside (Sigma). The amounts of 4-methylumbelliferone formed were analyzed fluorometrically (Model 850; Hitachi, Tokyo, Japan).

### Analysis of FGF-1 mRNA Expression

The reverse transcription polymerase chain reaction (PT-PCR) was used to evaluate the FGF-1 and β-actin mRNA levels, as described previously (Miwa et al., 1997; Nitta et al., 1999). Total RNA of cultured cells or brain tissues was prepared by using Isogen (Nippon Gene). First-strand cDNA was synthesized with PowerScript Reverse Transcriptase (Clontech Laboratories) according to the manufacturer's instruction. The synthesized cDNA was amplified under the following conditions: denaturation, 94°C, 30 sec; annealing, 68°C (FGF-1) or

55°C (β-actin), 30 sec; extension, 68°C, 1 min. The cycle was repeated 38 times for FGF-1, and 26 times for β-actin. The amounts of all PCR products were within range of linear increase at indicated thermal cycles. The following primers were used: FGF-1 forward-primer, 5′-TTCAACCTGCCTCTAGGA-AACT-3′, reverse-primer, 5′-CTTACAGCTCCCGTTCT-TCTTG-3′; β-actin forward-primer, 5′-GTGGGCCGCTCT-AGGCACCAA-3′, and reverse-primer, 5′-CTCTTTGATGTC-ACGCACGAT-3′. PCR products were electrophoresed in 2% agarose gels, and stained with ethidium bromide. DNA sequences of the PCR products were analyzed and their identity was confirmed.

### Intracellular cAMP Level

Cells cultured in 35-mm dishes were washed with PBS, scraped from the culture vessels, and suspended in 0.05 M acetate buffer, pH 5.8, containing 0.2 mg/ml BSA. The cells were then disrupted by sonication and centrifuged at 100,000 × *g* for 10 min. The supernatant fluids were used for determination of cAMP level, which was measured with an EIA kit (Amersham) according to the manufacturer's instructions.

### Monitoring of $Ca^{2+}$ Influx

Cortical neurons were cultured in 3.5-cm dishes precoated with poly-DL-ornithine ($10^5$ cells/$cm^2$). The dishes were then washed with 10 mM HEPES buffer (pH 7.4) containing 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 0.55 M glucose, after which 1 μM fluo-3 AM (Molecular Probes) was added and incubation was carried out for 30 min at room temperature. The fluorescence of individual cells was recorded at 488 nm with a fluorescence microscope (Axiovert S 100; Carl Zeiss, Germany) after the addition of 100 μM glutamate, 100 μM NE, or both together.

### Administration of Propranolol Into the Cerebral Cortex

All the experiments using animals were carried out according to the guidelines for animal experimentation of the NIH Guide for Care and Use of Laboratory Animals. Male Wistar rats (7 weeks old) purchased from Japan SLC (Shizuoka, Japan) were anesthetized with sodium pentobarbital (35 mg/kg, intraperitoneally), and fixed in a stereotaxic apparatus (Narishige, Tokyo). Five injections of 1 μl of propranolol dissolved in PBS (100 μg/μl) were made in the right side of the cerebral cortex. Stereotaxic coordinate of injection site a, b, c, d, or e (in mm anterior: −4.0, −3.0, −5.0, −5.0, or −3.0; lateral (lat.): 3.0, 2.0, 2.0, 4.0, or 4.0; height (ht.): 1.5, 1.3, 1.2, 1.4, or 1.8) was determined from the atlas of Paxinos and Watson (1998). PBS was then injected into the contralateral left side of the cerebral cortex. For RT-PCR analysis, rats were decapitated under the slight ether-anesthetized conditions at the indicated times after the injection. Brains were removed, and cut coronally into a 2-mm slice including all injection sites. The tissue containing all injection sites of propanolol or PBS was cut further into a $(2\ mm)^3$ cube, and used for RNA preparation. For immunohistochemical study, rats were anesthetized 24 hr after the injection, and cardio-perfused with 4% paraformaldehyde solution prepared with 0.1 M phosphate buffer, pH 7.3.

### Immunohistochemical Study of Brain Sections

The brains were dissected out, cut coronally into 5-mm slices, and post-fixed for 2 hr in cold 4% paraformaldehyde solution (the fixative). Slices were then soaked in PBS containing 30% (wt/vol) sucrose for 1 day, and frozen in embedding compound (Miles). Coronal sections of 30-μm thickness were cut with a cryostat (Model CM 1800; Leica), and then thawed on slide glasses bearing covalently linked amino groups (Matsunami Glass, Tokyo, Japan). Tissue sections were dried and soaked in the fixative for 15 min to cross-link covalently the tissue sections to slide glasses. Sections were rinsed in 0.1 M Tris-HCl buffer, pH 7.6, containing 0.3% (vol/vol) Triton X-100 for 15 min, boiled in Antigen unmasking solution (Vector) for 30 sec, and incubated for 1 day at 4°C with antibody against FGF-1 (Santa Cruz Biotechnology, Santa Cruz, CA), NeuN (Chemicon), or GFAP (Boehringer Mannheim Biochemicals) optimally diluted in PBS containing 10% FCS and 2% Block Ace (Dainippon Pharm., Osaka, Japan). Finally, the sections were reacted for 4 hr at 20°C with Alexa Fluor 488-labeled anti-rabbit IgG donkey antibody (Molecular Probes), or Alexa Fluor 546-labeled anti-mouse IgG goat antibody (Molecular Probes) diluted in PBS containing 10% FCS and 2% Block Ace, and observed for fluorescence with a confocal laser microscope (Model LSM510; Carl Zeiss).

## RESULTS

### Detection of FGF-1 Protein in Cultured Cells

For FGF-1 measurement, we used an EIA system having a sensitivity as low as 0.1 pg/ml of FGF-1 (Ishikawa et al., 1991). Even FGF-2, which has 55% amino acid sequence homology with FGF-1, did not give any significant signal. The intracellular FGF-1 content was $4.4 \pm 0.5$ pg/$10^6$ cells ($n = 6$) in cultured neurons, but the medium conditioned for 1 day by the cells was below the detection limit, confirming that FGF-1 lacks a signal peptide (Hicks et al., 1996). The intracellular FGF-1 content was maximally reduced to 20% of the original value by 24 hr after the addition of 100 μM cycloheximide, a protein synthesis inhibitor, demonstrating that most of the intracellular FGF-1 turned over within 24 hr. In light of this finding and the lack of secretion, the amount of intracellular FGF-1 was therefore considered to reflect the rate of FGF-1 synthesis.

### Regulation of Intracellular FGF-1 Content or mRNA Level in Cultured Neurons

Among the ligands for neurotransmitter receptor tested, histamine, glutamate, and carbachol caused over a fivefold increase in the FGF-1 content of cultured cortical neurons (Fig. 1A). The time-dependent change after the addition of glutamate is shown in Figure 1B. The increase was significant by 3 hr, maximal by 12 hr, and sustained until 24 hr, demonstrating that the response was rapid and long lasting. The involvement of $Ca^{2+}$-activated signaling was postulated, because histamine H2 receptor (Bossa et al., 1991; Koizumi and Ohkawara, 1999), glutamate receptor (Hartley et al., 1993), and muscarinic-acetylcholine receptor (Wang et al., 2002) were reported to mediate

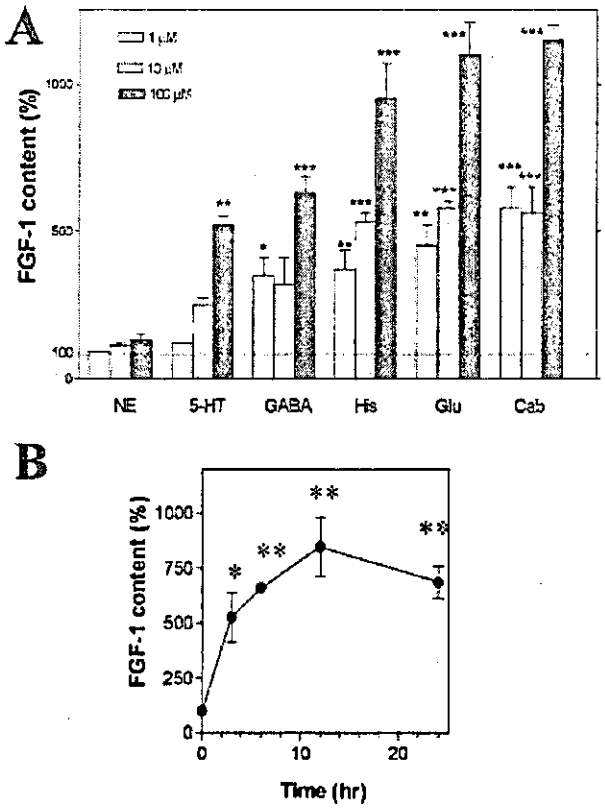


Fig. 1. Effects of various neurotransmitter receptor activation on FGF-1 content of cultured cortical neurons (**A**) and a time-dependent change in FGF-1 content after treatment with glutamate (**B**). Rat cortical neurons ($10^5$ cells/cm$^2$) were cultured in the serum-free defined medium containing insulin, transferrin, progesterone, and 5% BSA for 3 days. A: The cells were then treated for 24 hr with various ligands for neurotransmitter receptors prepared in the serum-free defined medium. The cell extracts were prepared and used for the EIA measurement of FGF-1. The FGF-1 content was 4.4 ± 0.5 pg/$10^6$ cells ($n$ = 6) in control cultures. The values are the mean ± SE of the percent increase of six culture dishes. Significance vs. no-treatment group, $*P < 0.05$; $**P < 0.01$, $***P < 0.005$, Bonferroni's $t$-test for multiple pairwise comparisons. B: Confluent neurons were cultured in the serum-free defined medium containing 100 μM glutamate for the indicated times. FGF-1 content in the cell extracts was measured by EIA. Values are the mean ± SE of the percent control of six culture dishes. The SE is not shown when it is less than the width of the symbol. Significance vs. control, $*P < 0.01$; $**P < 0.005$, Bonferroni's $t$-test for multiple pairwise comparisons. NE, norepinephrine; 5-HT, 5-hydroxytryptamine/serotonin; His, histamine; Glu, glutamate; Cab, carbachol.

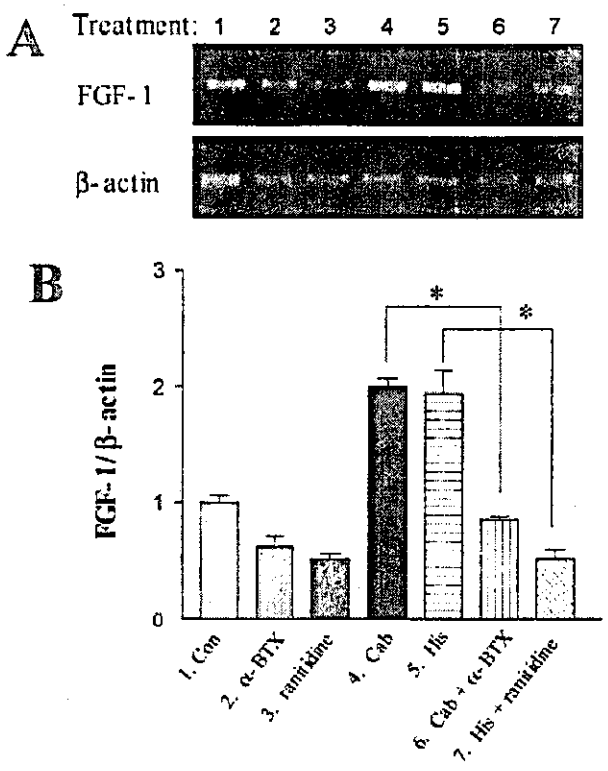


Fig. 2. Effects of antagonists against ACh or histamine receptors on FGF-1 mRNA expression of cultured cortical neurons. Rat cortical neurons were cultured for 3 days in the serum-free defined medium as described in the legend to Figure 1. **A:** 1. Control cells were maintained for 12 hr without any drugs. Cells were treated for 12 hr with: α-bungarotoxin (100 nM), an antagonist against nicotinic ACh receptors (2); ranitidine (50 μM), an antagonist against H2 receptors (3); carbachol (100 μM) alone (4); histamine (100 μM) alone (5); carbachol (100 μM) + α-bungarotoxin (100 nM) (6); or histamine (100 μM) + ranitidine (50 μM) (7). An aliquot of total RNA prepared from each culture was subjected to RT-PCR analysis using primers specific for FGF-1 or β-actin, and PCR products were electrophoresed in a 2% agarose gel. Gels were stained with ethidium bromide; photographs representing a typical result are shown. Structures of amplified DNA fragments were verified by sequencing analysis. The amounts of all PCR products were within range of linear increase at the indicated thermal cycles. **B:** The bands of PCR products corresponding to FGF-1 or β-actin mRNA-derived cDNA were densitometrically quantified, and the ratio of FGF-1/β-actin band intensity was calculated. Values are expressed as the means ± SE of the ratio of FGF-1/β-actin of five cultures. Significant differences were determined by Tukey's test. $*P < 0.005$. Con, control; α-BTX, α-bungarotoxin; Cab, carbachol; histamine, His.

$Ca^{2+}$ influx or intracellular $Ca^{2+}$ release. GABA moderately elevated the content at 1 μM and 100 μM, and 5-HT, at 100 μm. However, NE had no effect (Fig. 1).

The specificity of the ligands for neurotransmitter receptors such as carbachol and histamine was tested pharmacologically. First, the effect of carbachol on FGF-1 mRNA expression in cultured rat cortical neurons was examined in the presence or absence of α-bungarotoxin, an antagonist for nicotinic acetylcholine receptors (AChRs). FGF-1 mRNA expression was upregulated by the addition of carbachol in the absence of α-bungarotoxin, but unchanged in the presence of α-bungarotoxin (Fig. 2). Second, the effect of histamine on FGF-1 mRNA expression was evaluated in the pres-

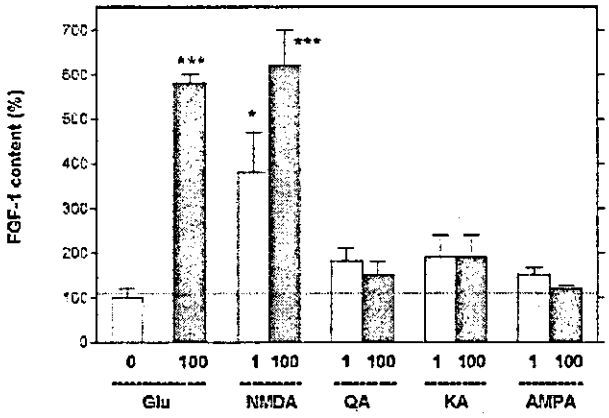


Fig. 3. Effects of the agonists for the glutamate receptors on FGF-1 content of cultured cortical neurons. Cortical neurons were treated with glutamate (Glu), or glutamate receptor agonists, NMDA, quisqualic acid (QA), kainic acid (KA) or AMPA for 24 hr at 1 or 100 μM. FGF-1 content in the cell extracts was measured by EIA. Intracellular FGF-1 content/$10^6$ cells is expressed as the percent of the value obtained in the absence of the reagent (control; Con). The values are the mean ± SE of six culture dishes. Significance vs. control, $*p < 0.05$; $***p < 0.001$, Bonferroni's $t$-test for multiple pairwise comparisons.

ence or absence of ranitidine, an antagonist for H2 receptors. FGF-1 mRNA expression was enhanced by the addition of histamine when ranitidine was absent, but unchanged when ranitidine was present (Fig. 2). These results demonstrated that FGF-1 mRNA expression by carbachol and histamine was specifically upregulated via α-bungarotoxin-sensitive AChRs and histamine H2 receptors, respectively. This implied that both drugs could enhance the synthesis of FGF-1 in a receptor-mediated specific manner.

To elucidate the mechanism of FGF-1 upregulation, we focused on the effects of glutamate. Glutamate receptors are activated selectively by agonists such as NMDA, quisqualic acid, kainic acid, or AMPA. Quisqualic acid is a full selective agonist for metabotropic glutamate receptors. The effect of glutamate was mimicked only by NMDA (Fig. 3), suggesting that activation of the NMDA glutamate receptor, resulting in $Ca^{2+}$ influx, is involved in the mechanism to increase the neuronal FGF-1 content. To test this possibility, we examined the effect of glutamate or carbachol on neurons under $Ca^{2+}$-free conditions. The absence of $Ca^{2+}$ completely abolished the effect (Fig. 4B), suggesting that FGF-1 synthesis is upregulated by the influx of $Ca^{2+}$ and its subsequent signaling. Agents that stimulate $Ca^{2+}$ influx via mechanisms different from the mechanism involving ligand binding-gated $Ca^{2+}$ channels were then examined. Cellular depolarization by a high concentration of KCl (30 mM), which induces $Ca^{2+}$ influx through voltage-dependent $Ca^{2+}$ channels (Tabuchi et al., 2000), and by the $Ca^{2+}$ ionophore A23187, which causes passive $Ca^{2+}$ incorporation through the membrane (Reed and Lardy, 1972), also increased the FGF-1 content to a level comparable to that obtained with glutamate (Fig. 3A). These results demonstrate that intracellular $Ca^{2+}$ influx is a key event to trigger FGF-1 gene expression in cultured cortical neurons.

### Interaction Between cAMP/PKA and $Ca^{2+}$-Activated Pathways in the Regulation of Neuronal FGF-1 Synthesis

The cAMP/PKA pathway may be involved in the mechanisms that regulate the FGF-1 content in neurons (Iyengar, 1996). We tested the effects of glutamate or carbachol on neuronal FGF-1 content in the presence of 100 μM NE, and found that the increase in FGF-1 content was suppressed completely (Fig. 5A). Furthermore, co-administration of NE with 10 μM KT-5720, an inhibitor of PKA, restored the stimulatory activity of the neurotransmitters (Fig. 5A), suggesting that the adrenergic β-receptor-mediated cAMP/PKA signal pathway was involved in inhibition of the $Ca^{2+}$-induced FGF-1 synthesis. The interaction between glutamate and NE was also evaluated by examining FGF-1 mRNA expression. The ratio of FGF-1 mRNA to that of β-actin was significantly higher in glutamate-treated cells than in cells treated with NE alone and with NE and glutamate in combination (Fig. 5B). This was in good agreement with the change in FGF-1 content, demonstrating that NE antagonized the $Ca^{2+}$-activated signal pathway leading to FGF-1 gene expression.

We next monitored cAMP content and $Ca^{2+}$ influx. Treatment with glutamate alone caused a slight but nonsignificant elevation of cAMP content during all experimental periods, whereas administration of NE alone or NE and glutamate in combination led to a significant increase in cAMP content (Table I). Combined treatment with NE and glutamate gave a much higher cAMP content than did NE alone at all times tested. These observations demonstrate that the high cAMP level was induced by the addition of NE irrespective of the coexistence of glutamate. The degree of $Ca^{2+}$ influx elicited by glutamate was essentially unchanged in the presence of NE (Fig. 6), suggesting that there is indeed interplay between cAMP/PKA and $Ca^{2+}$-activated signals in neuronal FGF-1 synthesis.

### Effects of Intracerebral Administration of Propranolol on Expression of FGF-1 Protein or mRNA in the Cerebral Cortex

To analyze the possible involvement of cAMP/PKA signal in the regulation of FGF-1 synthesis in the brain, we administered propranolol (100 μg/μl), a potent antagonist of adrenergic β-receptors, into the right side of the adult rat cerebral cortex, and compared the expression of FGF-1 mRNA with that of the left side, into which vehicle (PBS) had been injected (Fig. 7). FGF-1 mRNA was increased around the injection sites 6 and 12 hr after the injection, and returned to the original level by 24 hr (Fig. 8A). The increase of the expression occurred specifically and locally around the propranolol injection sites (Fig. 8B). The areas

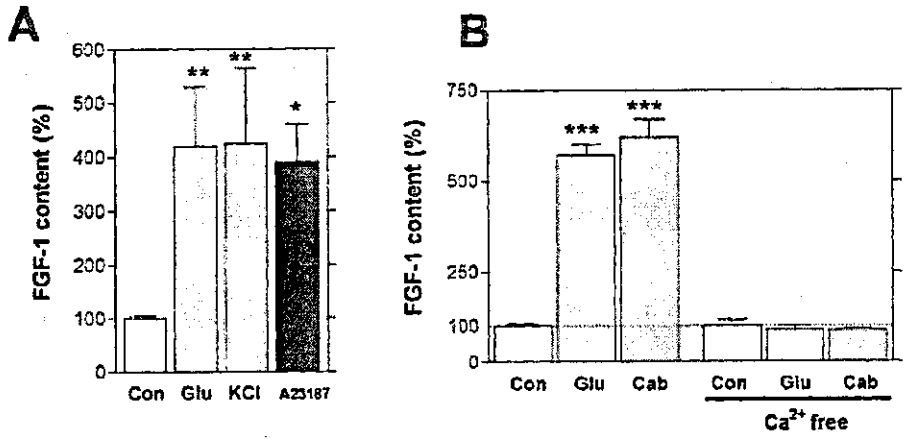


Fig. 4. Effect of agents that stimulate $Ca^{2+}$-influx (A) and the influence of $Ca^{2+}$-free medium (B) on FGF-1 content of cultured cortical neurons. A: Cortical neurons were treated with glutamate (100 μM, Glu), KCl (30 mM), or A23187 (0.6 μM) for 24 hr. B: Cortical neurons cultured in medium supplemented with $Ca^{2+}$ or not were stimulated with glutamate (100 μM, Glu) or carbachol (100 μM, Cab) for 24 hr. Intracellular FGF-1 content/$10^6$ cells is expressed as the percent of the value obtained in the absence of the reagent (control; Con). Values are the mean ± SE of six culture dishes. Significance vs. control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Bonferroni's $t$-test for multiple pairwise comparisons.

5 mm distant from the propranolol injection site (i.e., area 2 shown in Fig. 7) did not show significant elevation. These results demonstrated that FGF-1 mRNA expression in the cerebral cortex was facilitated by propanolol, suggesting negative regulation of FGF-1 synthesis via adrenergic β-receptors. Immunohistochemical study revealed that administered propranolol markedly upregulated FGF-1 immunoreactivity, and that the elevated FGF-1 immunoreactivity colocalized with NeuN antigen, a 46/48-kDa nuclear protein antigen used widely to identify postmitotic neurons (Weyer and Schilling, 2003), but not with GFAP, demonstrating that FGF-1 synthesis was facilitated in neurons, but not in astrocytes (Fig. 9).

## DISCUSSION

We showed that neuronal FGF-1 synthesis evoked through $Ca^{2+}$ channels such as the NMDA receptor was abolished by cAMP/PKA signal through adrenergic β-receptors in cultured rat cortical neurons. Furthermore, blockade of adrenergic β-receptors by propranolol in the adult rat cerebral cortex markedly facilitated expression of FGF-1 mRNA and protein. These results suggest a close interplay between neurotransmitter receptor activation that evoke $Ca^{2+}$-mediated signals and those that generate cAMP/PKA signals in the regulation of FGF-1 synthesis in rat brain.

In accordance with the lack of a signal peptide, we could detect FGF-1 only in the cells, but not in the culture medium, irrespective of the addition of reagents used in the present study. In addition to the concentration of FGF-1 within the cells, rapid intracellular turnover of FGF-1 protein enabled us to evaluate de novo synthesis by measuring cellular FGF-1 protein.

### $Ca^{2+}$-Activated Pathway to Upregulate FGF-1 Synthesis

Our present observations demonstrate that FGF-1 synthesis in cultured neurons is upregulated by $Ca^{2+}$ influx and its subsequent signal. $Ca^{2+}$ acts as a second messenger and triggers activity-dependent gene expression critical for adaptive changes in the nervous system (Finkbeiner and Greenberg, 1998), which includes gene expression of neurotrophic factors such as NGF, BDNF (Zafra et al., 1990; 1991; Rocamora et al., 1996; Shieh et al., 1998), neurotrophin-4/5 (NT-4/5) (Funakoshi et al., 1995), pleiotrophin (Amet et al., 2001), FGF-2 (Gomez-Pinilla et al., 1999), and neuregulin (Eilam et al., 1998). A leading paradigm of such regulation is activation of the nuclear transcription factor, $Ca^{2+}$/cAMP-responsive element binding protein (CREB) by $Ca^{2+}$/calmodulin (CaM) kinase IV (Sheng et al., 1990). Phosphorylated CREB drives the transcription of a many genes through interaction with its nuclear partner, CREB-binding protein (Chrivia et al., 1993). In the case of BDNF, one of the $Ca^{2+}$-responsive promoters is activated by CREB (Shieh et al., 1998). Instead, in the case of interleukin (IL)-6, another yet undefined factor may mediate the response to membrane depolarization (Sallmann et al., 2000). Amygdala stimulation of kindled animals greatly increased hippocampal and cortical BDNF mRNA levels; but the FGF-1 mRNA level was not altered, suggesting that BDNF and FGF-1 are recruited at different stages of kindling epileptogenesis (Simonato et al., 1998). These observations suggest that the expression of FGF-1 in neurons is regulated by a novel $Ca^{2+}$-responsive promoter element. The FGF-1 gene spans over 120 kb containing three protein-coding exons and at least four upstream untranslated exons (Liu et al.,

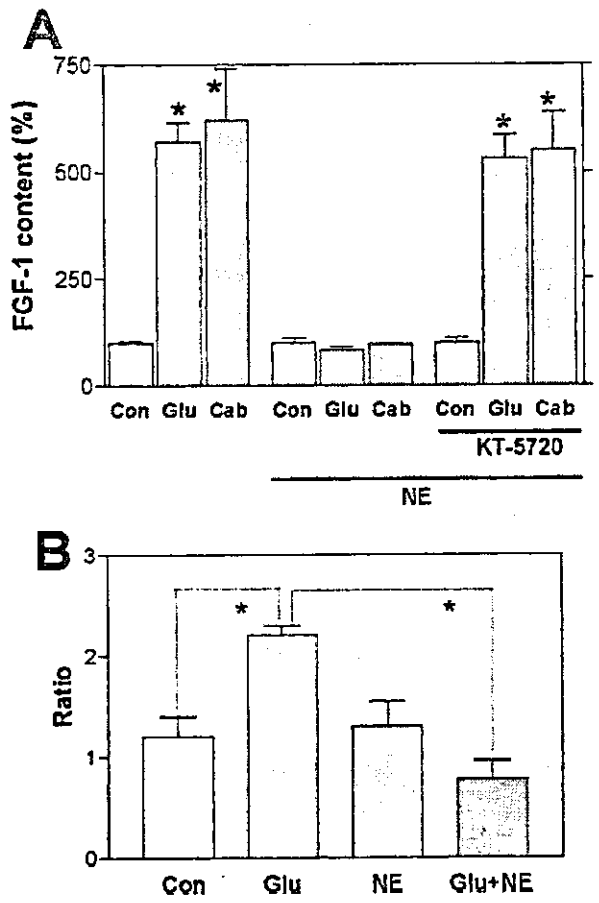


Fig. 5. Effect of coexistence of NE on glutamate- or carbachol-induced increase in FGF-1 content (**A**) or FGF-1 mRNA (**B**) of cultured cortical neurons. A: Cortical neurons were treated with glutamate (100 μM, Glu) or carbachol (100 μM, Cab) alone, or glutamate (100 μM) or carbachol (100 μM) with NE (100 μM) in combination for 24 hr. Co-administration of KT-5720 (10 μM) with NE was also carried out. Intracellular FGF-1 content/$10^6$ cells is expressed as the percent of the value obtained in the absence of the reagent (control; Con). The values are the mean ± SE of six culture dishes. Significance vs. control, $*P < 0.05$. Bonferroni's *t*-test for multiple pairwise comparisons. B: Cortical neurons were treated with glutamate (100 μM, Glu) or NE (100 μM) alone, or both in combination (Glu + NE) for 24 hr. Total RNAs were prepared, and an aliquot (500 ng) was reverse transcribed. Synthesized cDNAs of FGF-1 and β-actin were amplified by PCR, and the products were subjected to PAGE and visualized by ethidium bromide staining. The density of the corresponding bands was analyzed by image analysis software, and values are expressed as a ratio of the FGF-1:β-actin band density amplified from an identical RNA sample. Significance, $*P < 0.05$, Bonferroni's *t*-test for multiple pairwise comparisons.

**TABLE I. Intracellular cAMP Level After Treatment With Glutamate, Norepinephrine, or Both***

| Treatment | cAMP Level (fmol/dish) 10 min After treatment | 3 hr After treatment | 12 hr After treatment |
|---|---|---|---|
| No treatment | 14.2 ± 2.6 | 12.8 ± 0.7 | 16.5 ± 4.4 |
| Glutamate | 18.1 ± 3.3 | 16.3 ± 2.0 | 19.8 ± 4.0 |
| NE | 21.8 ± 5.4[a] | 21.4 ± 1.4[a] | 25.7 ± 4.0[a] |
| Glutamate + NE | 30.6 ± 2.4[c] | 24.9 ± 1.2[b] | 25.8 ± 3.5[a] |

*Cortical neurons ($10^5$ cells/$cm^2$) were cultured for the indicated times in serum-free medium supplemented with 100 μm, glutamate, 100 μm norepinephrine (NE), or both. Cells were washed with phosphate-buffered saline, disrupted by sonication, and centrifuged at 100,000 × *g* for 10 min. Supernatants were used for cAMP determination.
Significance vs. no-treatment group, [a]$P < 0.05$; [b]$P < 0.01$; [c]$P < 0.005$, Bonferroni's *t*-test for multiple pairwise comparisons.

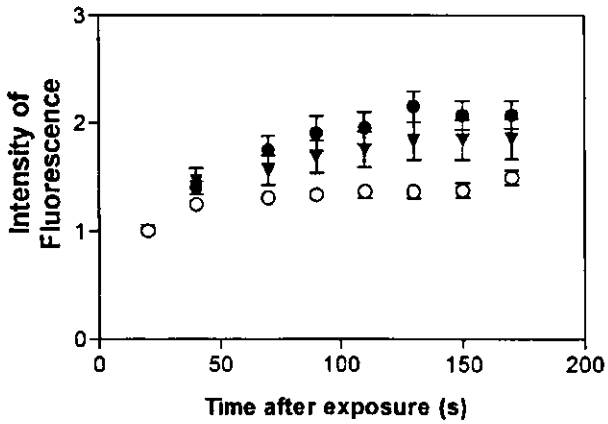


Fig. 6. Monitoring of $Ca^{2+}$ influx after exposure to glutamate or NE alone or combined. Cultured cortical neurons were incubated with 1 μM fluo-3 AM for 30 min at room temperature. The fluorescence of each neuron was recorded at 488 nm with a fluorescence microscope after the addition of vehicle (open circles), 100 μM glutamate (filled circles) or glutamate and NE (downward triangles). Values are mean ± SE of 30–40 cells. Significance vs. control, $*P < 0.05$; $**P < 0.01$; $***P < 0.001$, Bonferroni's *t*-test for multiple pairwise comparisons.

1998). Splicing of each of these untranslated exons to the first protein coding exon generates four different mRNA transcripts. FGF-1.B transcript is expressed selectively in neural tissues, and is localized predominantly in the brainstem, ventral spinal cord, and cerebellum (Alam et al., 1996). A 23-base pair (bp) *cis*-element identified in the FGF-1.B promoter is thought to be linked to the enhanced functional activity of the promoter (Myers et al., 1995). Furthermore, a splice variant of the E2-2 gene product represses the transcriptional activities of the FGF-1.B promoter (Liu et al., 1998). $Ca^{2+}$-triggered regulatory mechanisms for FGF-1 gene expression, however, remain unknown.

## Interplay Between $Ca^{2+}$-Activated and cAMP/PKA Signal Pathways

The cAMP/PKA pathway has been thought to regulate the activity of other signal transduction pathways (Iyengar, 1996). We found that the cAMP/PKA signal attenuated $Ca^{2+}$-induced FGF-1 synthesis. The degree of

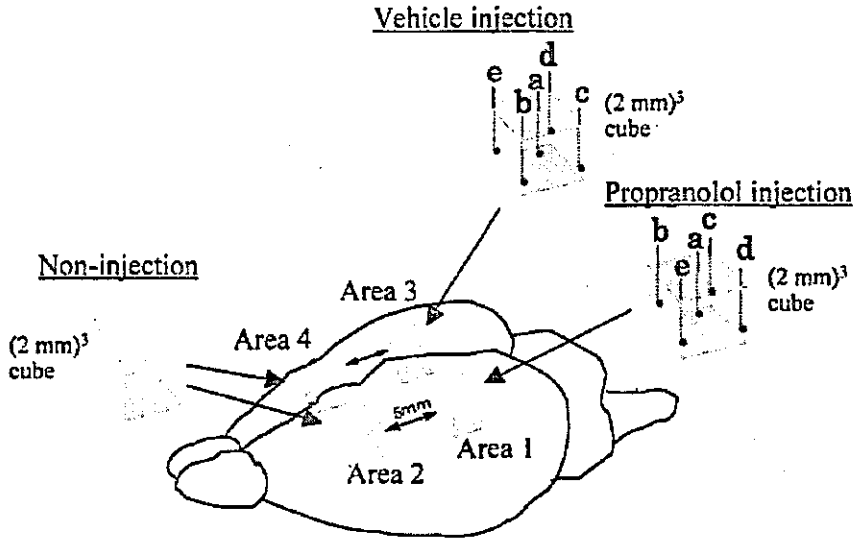


Fig. 7. Schematic illustration of the brain areas for vesicle or propranolol administration, and for RT-PCR or immunohistochemical analysis. One microliter each of vehicle (PBS) and propranolol (an antagonist of adrenergic β-receptors) in PBS (100 μg/μl) was injected into five sites from a–e within the brain areas 1 and 3, respectively. Area 1 or 3 was a (2 mm)$^3$ cube surrounded by bregma −3.0 to −5.0 mm, lat. 2.0 to 4.0 mm, ht. 0.5 to 2.5 mm of the cerebral cortex of male Wistar rats (7 weeks old). Stereotaxic coordinates of the injection sites in area 1 or 3 were as follows: **a**, bregma −4.0 mm, lat. 3.0 mm and ht. 1.5 mm; **b**, −3.0, 2.0, 1.3; **c**, −5.0, 2.0, 1.2; **d**, −5.0, 4.0, 1.4; **e**, −3.0, 4.0, 1.8. Noninjection area 2 (or area 4) includes a (2 mm)$^3$ cube area of bregma −1.0 to1.0 mm, lat. 2.0 to 4.0 mm, ht. 0.5 to 2.5 mm.

$Ca^{2+}$-influx by glutamate was essentially unchanged irrespective of the cellular cAMP content, suggesting that there is indeed interplay between cAMP/PKA and $Ca^{2+}$-activated signals in neurons. The regulatory mechanism(s) thus expected in vitro was confirmed in neurons of the adult rat cerebral cortex in vivo by analyzing effects of an adrenergic β-receptor antagonist: blockade of cAMP/PKA signal facilitated FGF-1 synthesis in cortical neurons. We could not observe such a change in astrocytes in vivo, however, which is probably because of a lack of FGF-1 synthesis in astrocytes of the mature rat brain (Wilcox and Unnerstall, 1991; Stock et al., 1992).

Elevation of intracellular cAMP activates PKA, which in turn phosphorylates some unknown protein "X" that might be involved in the major $Ca^{2+}$-dependent signaling pathway in FGF-1 gene expression. The phosphorylation inactivates X and blocks the FGF-1 gene expression, but dephosphorylation of X by phosphatase could reactivate the X. The resting levels of PKA and phosphatase activity in the cells are likely to reach a balance, leading to an equilibrium between active and inactive forms of X. As a candidate for X, $Ca^{2+}$/calmodulin (CaM) kinase kinase may be a plausible one. It is reported that CaM kinase cascades are blocked by cAMP/PKA-mediated inhibition of CaM kinase kinase by phosphorylation of Thr108, a mechanism to modulate the balance between cAMP- and $Ca^{2+}$-dependent signal transduction pathways (Matsushita and Nairn, 1999). Alternatively, in contrast to this consideration, there are reports showing alteration of $Ca^{2+}$ influx by cAMP/PKA signals (Yang et al., 1996) through phosphorylation of $Ca^{2+}$ channel proteins (Hell et al., 1995). It therefore cannot be excluded that cAMP/PKA signals alter the degree of $Ca^{2+}$ channel-gated influx and modify the subsequent $Ca^{2+}$-dependent signal cascade directing FGF-1 gene expression. Plural *cis*-elements of the promoter and *trans*-acting factors that respond to $Ca^{2+}$ may interplay in a complex fashion, depending on the $Ca^{2+}$ concentration, and $Ca^{2+}$/calmodulin kinase cascades may be transduced in different ways.

Enhanced expression of FGF-1 after administration of propranolol, a nonselective β-adrenergic antagonist, suggests that FGF-1 synthesis is negatively regulated via adrenergic β-receptors in cortical neurons in vivo. The adrenergic nerve fibers with NE as a neurotransmitter are projected densely from the locus coeruleus into the cerebral cortex. NE secreted in a neuronal activity-dependent manner may suppress FGF-1 synthesis in cortical neurons, as suggested from the present in vitro experiment. It is likely that FGF-1 synthesis is highly sustained by neurotransmitter receptor activation that causes $Ca^{2+}$ influx such as glutamate, acetylcholine, histamine, GABA, and serotonin in the absence of ligands of adrenergic β-receptors. These neurotransmitters can be substantially supplied from respective nerve terminals projected from areas other than the cerebral cortex.

FGF-1 synthesis regulated by neuronal activities in opposite ways is based on establishment and maintenance of particular neuronal circuits in the brain, which may be