Up-regulation of L-type high voltage-gated calcium channel subunits by sustained exposure to 1,4- and 1,5-benzodiazepines in cerebrocortical neurons

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Abstract

The aim of this study is to examine how sustained exposure to two 1,4-benzodiazepines (BZDs) with different action period, diazepam and brotizolam, and a 1,5-BZD, clobazam, affects L-type high voltage-gated calcium channel (HVCC) functions and its mechanisms using primary cultures of mouse cerebral cortical neurons. The sustained exposure to these three BZDs increased [$^{45}Ca^{2+}$] influx, which was due to the enhanced [$^{45}Ca^{2+}$] entry through L-type HVCCs but not through of Cav2.1 and Cav2.2. Increase in [^3H]diltiazem binding after the exposure to these three BZDs was due to the increase in the binding sites of [^3H]diltiazem. Western blot analysis showed increase of Cav1.2 and Cav1.3 in association with the increased expression of α2/δ1 subunit. Similar changes in [^3H]diltiazem binding and L-type HVCC subunit expression were found in the cerebral cortex from mouse with BZD physical dependence. These results indicate that BZDs examined here have the potential to increase L-type HVCC functions mediated via the enhanced expression of not only Cav1.2 and Cav1.3 but also α2/δ1 subunit after their sustained exposure, which may participate in the development of physical dependence by these BZDs.

Keywords: brotizolam, cerebrocortical neurons, clobazam, diazepam, drug dependence, L-type high voltage-gated calcium channels.

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1,4-Benzodiazepines (1,4-BZDs) are mainly used in various clinical fields as sedative-hypnotics, anxiolytics, muscle relaxants, and anticonvulsants according to their pharmacological differences in their pharmacokinetic properties and pharmacodynamic spectra instead of barbiturates, although pharmacological actions of 1,4-BZDs to enhance GABA$_A$ receptor functions are considered to be essentially similar among these BZDs. One of the reasons that the remarkable decline of the clinical use of barbiturates, which has been used as sedative-hypnotics before introducing clinical applications of 1,4-BZDs, is their relatively higher potential to develop physical dependence and abuse. In contrast, the potential of 1,4-BZDs to produce dependence is lower than that of barbiturates, although prolonged administration of 1,4-BZDs with shorter eliminating period tends to develop physical dependence and abuse when compared with those with longer ones (Schweizer and Rickels 1998; Chouinard 2004). In addition, the longer and the higher dose 1,4-BZDs are taken, the greater risk of developing physical dependence (Mackinnon and Paker 1982; Schweizer and Rickels 1998).

One of the notable neurochemical changes after long-term treatment with 1,4-BZDs is increase in the number of dihydropyridine-sensitive binding sites (Brennan and Littleton 1991). Moreover, withdrawal syndrome such as hyperexcitability found after chronic treatment with1,4-BZDs has

been reported to be attenuated by antagonists for L-type high voltage-gated calcium channels (HVCCs) (Hitchott *et al.* 1992; Watson and Little 2002). These results suggest that chronic treatment with 1,4-BZDs induces up-regulation of L-type HVCCs, which may in turn participate in producing pathophysiological conditions in the CNS observed in BZD-dependent animals, especially in animals with physical dependence.

Clobazam is one of 1,5-BZDs with anticonvulsant properties (Fielding and Hoffman 1979) and has been reported to be effective to therapy-resistant epilepsy (Koeppen *et al.* 1987; Remy 1994). Although prolonged administration of clobazam does not affect psychomotor performance (van der

Meyden *et al.* 1989), withdrawal symptoms such as hyper-excitation and reduced exploratory behaviors in the open field are reported in rats treated with clobazam, suggesting the possibility that clobazam has also a potential to produce physical dependence (Löscher and Rundfeldt 1990). However, there are few available data that clobazam causes physical dependence in human so far as we know and no studies investigating how L-type HVCC functions are changed after long-term treatment with this agent.

During the last two decades, various types of HVCCs including Cav2.1, Cav2.2, and L-type HVCCs have been found to consist of Cav, α2/δ, β, and γ subunits, among which Cav forms Ca^{2+} ionophores and the other subunits modify Cav functions (Hofmann *et al.* 1999; Stotz and Zamponi 2001). In the brains of animals with physical dependence induced by 1,4-BZDs as well as other drugs of abuse such as ethanol, morphine, and nicotine, up-regulation of L-type HVCC functions is caused by the increase in dihydropyridine binding sites of L-type HVCCs (Ramkumar and El-Fakahany 1988; Suzuki *et al.* 1995; Katsura *et al.* 2002, 2005 and Podhoma 2002). We have reported that increased L-type HVCC functions are because of the increased expression of Cav1.2 and Cav1.3 in the brains derived from physically dependent mice with ethanol and nicotine (Hayashida *et al.* 2005; Katsura *et al.* 2005) and in mouse cerebral cortical neurons in primary culture continuously exposed to nicotine (Katsura *et al.* 2002). In PC12 cells, sustained exposure to ethanol also up-regulates L-type HVCCs (Gerstin *et al.* 1998). These data suggest that up-regulation of L-type HVCC functions may be a neurochemical event accompanied with the development of physical dependence.

Based on these data, we therefore examined in this study whether long-term exposure to 1,4- and 1,5-BZDs produces increased L-type HVCCs and, if so, which subunits consisting of L-type HVCCs change using mouse cerebral cortical neurons having BZD receptors (Kuriyama *et al.* 1987).

Materials and methods

Primary culture of cerebral cortical neurons

Isolation and primary culture of cerebral cortical neurons were carried out according to the method described previously (Ohkuma *et al.* 1986) with a minor modification. In brief, the neopallium free of the meninges was removed from 15-day-old fetus of ddY strain mouse (Japan SLC, Inc., Hamamatsu, Japan) anesthetized with sodium pentobarbital, minced, dispersed by trypsin, and centrifuged at 1000 *g*. Thereafter, the isolated cells were placed on a poly-L-lysine-treated culture dish with Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and cultured at 37°C in humidified 95% air/5% CO_2 for 3 days. After the treatment of the cells with 10 μmol/L cytosine arabinoside in DMEM containing 10% horse serum for 24 h to suppress the proliferation of non-neuronal cells, the culture medium was exchanged to DMEM with 10% horse serum and the incubation was continued under the same conditions described above. The culture medium was exchanged to fresh DMEM with 10% horse serum every 4 days and the neurons were used for the experiments described blow on the 13th day of the culture. More than 95% of the primary cultured cells used here have been confirmed to be neurons by an immunohistochemical approach (Ohkuma *et al.* 1986; Mohri *et al.* 2003).

Sustained exposure of the neurons to BZDs and reagents

In the case of sustained exposure to 1,4- and 1,5-BZDs, and flumazenil, they were dissolved in 30% ethanol and added directly into the culture medium with 1/100 volume of the solutions containing these drugs. Nifedipine and protein kinase inhibitors, such as calphostin C, KT-5720, and KN-62 were dissolved in 100% dimethylsulfoxide (DMSO) and added directly into the culture medium with 1/5000 volume of the solutions containing these drugs. The control values for sustained exposure to these agents were determined with 0.3% ethanol and/or 0.05% DMSO. No difference in the leakage of lactate dehydrogenase activity and the activity of the neurons to extrude trypan blue dye were found in the presence and absence of 0.3% ethanol or 0.05% DMSO (data not shown).

We used the 13th day neurons in culture for [$^{45}Ca^{2+}$] influx and [^{3}H]diltiazem bindings and immunobloting, and BZDs were exposed to the neurons for 3 days prior to these experiments. Flumazenil and various types of HVCC antagonists were added into the incubation medium 15 s before the addition of BZDs. Protein kinase inhibitors were pre-loaded 30 min before the BZD exposure to the neurons.

Preparation of mice physically dependent on BZDs

Mice were intraperitoneally administered diazepam (16 mg/kg/day), brotizolam (16 mg/kg/day), and clobazam (16 mg/kg/day) for 5 days (Löscher *et al.* 1996; Tsuda *et al.* 1998) with a minor modification. Mice were then treated intraperitoneally with flumazenil (10 mg/kg) 2 h after the last dose of each BZD to precipitate withdrawal signs. Under such schedules to administer BZDs, all mice showed tremor, jumping, sniffing, and diarrhea (especially by brotizolam), indicating all BZDs produce physical dependence in mice.

Measurement of [$^{45}Ca^{2+}$] influx

Influx of [$^{45}Ca^{2+}$] into the neurons was measured according to the previous method (Ohkuma *et al.* 2001). In brief, after pre-incubation of the neurons in Ca^{2+}-free Krebs-Ringer bicarbonate buffer (pH 7.4; 137 mmol/L NaCl, 4.8 mmol/L KCl, 1.2 mmol/L KH_2PO_4, 1.2 mmol/L $MgSO_4·6H_2O$, 25 mmol/L $NaHCO_3$, and 10 mmol/L glucose) containing 20 mmol/L HEPES (KRB-H) at 37°C for 10 min, the neurons were incubated in fresh and warm (37°C) Ca^{2+}-free KRB-H. The reaction was initiated by simultaneous addition of 30 mmol/L KCl and 2.7 mmol/L $CaCl_2·H_2O$ (1.0 μCi of [$^{45}Ca^{2+}$]Cl_2/dish). After the incubation of the neurons at 37°C for 2 min, the radiolabeled Ca^{2+}-containing incubation buffer was discarded and the neurons were washed five times with ice-cold KRB-H containing 2.7 mmol/L $CaCl_2·H_2O$ (total volume; 7.5 mL). Thereafter, the neurons were scraped off from a culture dish with 0.5 mol/L NaOH. An aliquot of the alkaline digested neurons was neutralized with equimolar acetic acid and then used to measure

radioactivity accumulated in the neurons by liquid scintillation spectrometry. The effect of flumazenil, a central BZD receptor antagonist, on [^{45}Ca^{2+}] influx induced by sustained exposure to 1,4- and 1,5-BZDs was examined by the addition of flumazenil 15 s before the sustained treatment with the neurons with these BZDs for 3 days.

The basal values of [^{45}Ca^{2+}] influx were determined in the absence of 30 mmol/L KCl. Sustained exposure to DMSO and ethanol used for dissolved BZDs did not affect the basal value of [^{45}Ca^{2+}] influx (data not shown). In addition, the KCl-induced [^{45}Ca^{2+}] influx in the neurons cultured with 0.3% ethanol and 0.05% DMSO for 3 days was no significantly different from that without these vehicles (data not shown). The data obtained after the exposures to these vehicles were therefore used as the control.

The present study also measured the effect of nifedipine (0.1 μmol/L), an antagonist selective to L-type HVCCs, on the increase of 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx induced by sustained exposure to BZDs by the addition of nifedipine at 12 and 24 h after the initiation of sustained exposure to BZDs.

We also examined which type of HVCCs is up-regulated by sustained exposure to BZDs in the presence or absence of HVCC inhibitors, such as ω-conotoxin GIVA (ω-CTX), ω-agatoxin VIA (ω-ATX), and nifedipine. These inhibitors were added into the culture medium 15 s before the addition of BZDs.

In order to examine how protein kinases such as protein kinase C (PKC), protein kinase A (PKA), and Ca^{2+}/calmodulin-dependent protein kinase II (CaM kinase II) participate in the up-regulation of HVCC functions induced by sustained exposure to BZDs, we measured 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx immediately after the sustained exposure of the neurons with each BZD (diazepam 0.3 μmol/L; brotizolam 1 μmol/L; and clobazam, 1 μmol/L) plus each inhibitor of the kinases for 3 days. The used inhibitors for PKC, PKA, and CaM kinase II were calphostin C (1 μmol/L), KT-5720 (30 μmol/L), and KN-62 (1 μmol/L), respectively. Each inhibitor was added into the culture medium 15 s before the addition of BZDs. The sustained exposure to these drugs for 3 days did not change the leakage of lactate dehydrogenase activity from the neurons and the neuronal activity to exclude trypan blue dye, when compared with those of non-treated neurons (control) (data not shown).

Measurement of [^{3}H]diltiazem binding

[^{3}H]Diltiazem binding was carried out according to the previous method with a minor modification (Garcia *et al.* 1989; Schaeffer *et al.* 1991; Katsura *et al.* 2002). The particulate fractions prepared from the neurons were suspended in 50 mmol/L Tris–HCl buffer (pH 7.4) containing 1 mg/mL of bovine serum albumin (200 μg protein/500 μL of assay volume) and incubated with [^{3}H]diltiazem (0.125–32 nmol/L) at 25°C for 120 min. The reaction was terminated by filtration of the reaction mixture through Whatman GF/B filters pre-coated with 0.3% polyethyleneimine at 4°C for 5 h, under vacuum followed by three times washes of the filters with ice-cold 50 mmol/L Tris–HCl buffer. The filter was then used to measure the radioactivity retained on the filter by liquid scintillation spectrometry. Specific binding was calculated by subtraction of non-specific binding obtained in the presence of 10 μmol/L non-labeled diltiazem from total binding determined without non-labeled diltiazem.

As the bindings of [^{3}H]diltiazem to the particulate fractions from the neurons exposed to the vehicle (ethanol) to dissolve BZDs for 3 days showed no significant difference from those without the vehicles (data not shown), we determined that the data from the neurons cultivated with the vehicles were used as the control.

The particulate fractions from the neurons used for [^{3}H]diltiazem binding were prepared as described below. The neurons were washed three times with ice-cold phosphate-buffered saline (PBS; 137 mmol/L NaCl, 2.7 mmol/L KCl, 10 mmol/L Na$_2$HPO$_4$, and 1.8 mmol/L KH$_2$PO$_4$, pH 7.4), scraped off from the dishes with ice-cold 50 mmol/L Tris–HCl buffer (pH 7.4), homogenized with a Polytron homogenizer, and centrifuged (48 000 g, 4°C, 20 min). The washing procedure by centrifugation after homogenization was carried out four times before stocking at –80°C for at least 24 h. Before the binding experiment, the frozen pellet was thawed, suspended with the same buffer used for preparing the particulate fractions, and washed three times as described above.

In the case of the binding to the particulate fractions of the cerebral cortex from animals physically dependent on BZDs, the mice administered BZDs as described above were decapitated 2 h after the last dose of BZDs. The cerebral cortex was then removed and homogenized in ice-cold 50 mmol/L Tris–HCl buffer (pH 7.4) with a Polytron homogenizer, and centrifuged (48 000 g, 4°C, 20 min). The washing procedure by centrifugation was carried out four times before stocking at –80°C for at least 24 h. Before the binding experiment, the frozen pellet was thawed, suspended with the ice-cold 50 mmol/L Tris–HCl buffer, and washed three times as described above.

Immunoblots for subunits of HVCCs

Extraction of proteins from the neurons for electrophoresis was carried out as follows (Katsura *et al.* 2002). The neurons were washed five times with ice-cold 0.15 mol/L NaCl including calpain inhibitor set and protease/phosphatase inhibitor cocktail, fixed with 6% trichloroacetic acid in 0.15 mol/L NaCl at 4°C, scraped off from the dishes, and centrifuged (10 000 g, 5 min, 4°C). The pellet was washed with ice-cold Tris–HCl buffer (pH 7.4), mixed with the sample buffer [4% sodium lauryl sulfate, 12% β-mercaptoethanol, 20% glycerol, calpain inhibitor set, and protease/phosphatase inhibitor cocktail in 100 mmol/L Tris–HCl (pH 6.8)], sonicated (1 min), boiled (3 min), and finally centrifuged (10 000 g, 60 min, 4°C). The resultant supernatant was stored at –80°C until use.

To extract protein from the cerebral cortex, the mouse was anesthetized with sodium thiopental 2 h after the last dose of BZDs. A Teflon cannula was inserted into the left ventricle and the abdominal aorta was cut off. The brain was perfused with ice-cold 0.15 mol/L NaCl with the flow rate of 20 mL/min for 1 min, and then perfused with 6% trichloroacetic acid in 0.15 mol/L NaCl for 3 min with same flow rate. The cerebral cortex was dissected, sonicated with the sample buffer [4% sodium lauryl sulfate, 12% β-mercaptoethanol, 20% glycerol, calpain inhibitor set, and protease/phosphatase inhibitor cocktail in 100 mmol/L Tris–HCl (pH 6.8)] for 1 min, boiled for 3 min, and finally centrifuged (10 000 g, 60 min, 4°C). The resultant supernatant was stored at –80°C until the experimental use.

After electrophoresis (applied proteins, 20 μg/lane) with size of 10 × 10 cm and thickness of 0.5 mm (20 mA, 90 min), proteins separated on the gel were transferred to a nitrocellulose filter with a semidry type transblotter (160 mA, 120 min). The nitrocellulose filters were incubated with antibodies against each subunit of the HVCCs (Cav1.2, Cav1.3, Cav2.1, Cav2.2, α2/δ1, and β4) (diluted 1 : 200 to 1 : 1000 in PBS containing 0.1% normal serum) after washes of the filters with PBS and subsequent blocking with PBS containing 5% skim milk at 22–25°C for 60 min. After the filters were washed four times with Tris-buffered saline (0.15 mol/L NaCl in 20 mmol/L Tris–HCl, pH 7.4) containing 0.05% Tween 20, the antigenic proteins were stained using anti-rabbit IgG antibody conjugated with alkaline phosphatase (diluted 1 : 2500). The bands of the proteins in the gels were stained with Coomassie Brilliant Blue (Katsura *et al.* 2002).

The relative intensity of immunoreactive bands was quantified using IMAGEMASTER 1D Elite software (Amersham Biotech, Buckinghamshire, UK), and the data were estimated as percentage of each control.

The single bands for respective HVCC subunits were detected under these conditions, and the detected size of each unit (kDa) was Cav1.2 (210), Cav1.3 (260), Cav2.1 (220), Cav2.2 (250), α2/δ1 (150) and β4 (60), respectively.

There were no significant differences in the expression of the subunits of HVCCs measured in this study in the neurons exposed to 0.03% ethanol and those without the vehicles (data not shown). Similarly, no significant differences between the neurons treated with or without 0.05% DMSO. Therefore, the control values were obtained from the neurons cultured with 0.3% ethanol or 0.05% DMSO for 3 days.

Measurement of protein
The protein content in the neurons was determined by the previous method (Lowry *et al.* 1951) using bovine serum albumin as standard.

Statistical analysis
Each value of the data was expressed as the mean ± SEM. The statistical significance was assessed by the methods as described in each legend of figures following the application of one-way ANOVA.

Chemicals
[^{3}H]Diltiazem (3.1 TBq/mmol) and [^{45}Ca]Cl$_2$ (0.3511 GBq/mg) were purchased from New England Nuclear (Boston, MA, USA). Antibodies against Cav2.1 (ACC-001), Cav2.2 (ACC-002), Cav1.2 (ACC-003), Cav1.3 (ACC-005), and α2/δ1 (ACC-015) subunits were products of Alamone Labs Ltd. (Jerusalem, Israel). An antibody against β4 (C-5863), flumazenil and protein kinase inhibitors such as calphostin C, KT-5720, and KN-62 were the products of Sigma-Aldrich Co. (St Louis, MO, USA). Nifedipine, diazepam and brotizolam were obtained from Wako Pure Chemical Industries (Osaka, Japan). ω-CTX and ω-ATX were purchased from the Peptide Institute, Inc. (Tokyo, Japan). Phosphatase/protease inhibitor Cocktail and calpain inhibitor set were the products of Nacalai Tesque (Kyoto, Japan) and Calbiochem (Darmstadt, Germany), respectively. Clobazam is a kind gift of Dainippon Sumitomo Pharma Co., Ltd. (Osaka, Japan). Other reagents used were locally available and of analytical grade.

Results

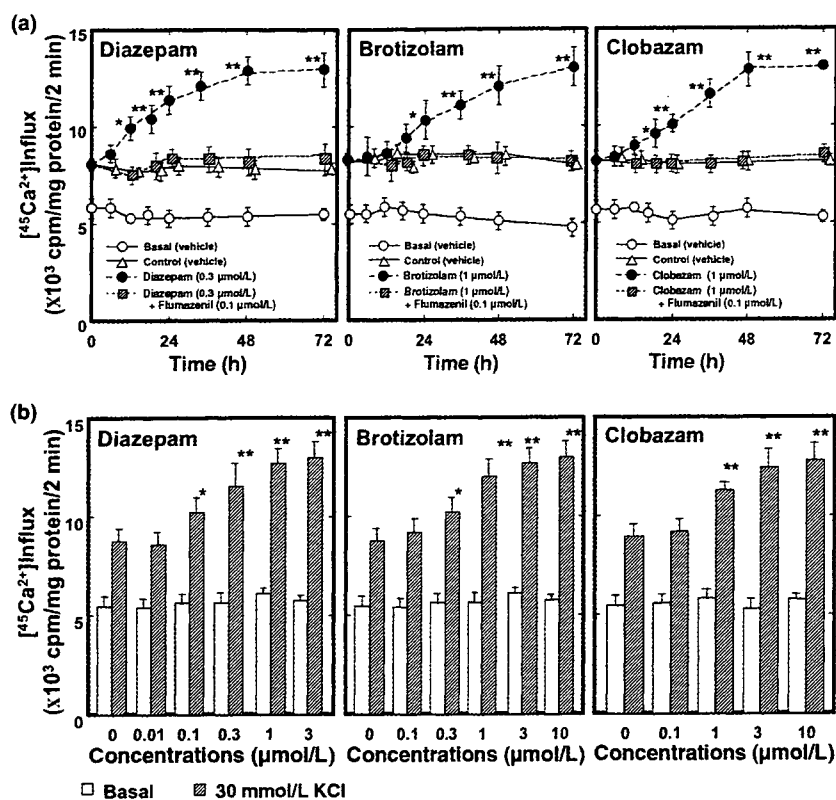### [^{45}Ca^{2+}] influx after sustained exposure to 1,4- and 1,5-BZDs
As shown in Fig. 1a, the sustained exposure to 1,4-BZDs (0.1 μmol/L diazepam and 1 μmol/L brotizolam) and a 1,5-BZD, clobazam (1 μmol/L), gradually increased the high potassium (30 mmol/L KCl)-induced [^{45}Ca^{2+}] influx into the neurons in a time-dependent manner (at 6, 12, 18, 24, 36, and 46 h) and the maximal [^{45}Ca^{2+}] influxes were observed after the sustained exposure to each BZD for 48 h. Thereafter, the maximal influx was maintained until 3 days after the initiation of their sustained exposures. On the other hand, the 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx in the neurons not exposed to BZDs remained at the level similar to that found before the exposure. In addition, the basal [^{45}Ca^{2+}] influx, which means the [^{45}Ca^{2+}] influx without the stimulation by 30 mmol/L KCl, was not significantly changed during the experiments (Fig. 1a).

After the cortical neurons were exposed to the multiple doses of BZDs for 72 h, each BZD enhanced the KCl-induced [^{45}Ca^{2+}] influx in a dose-dependent manner (Fig. 1b). Among these BZDs the potential of diazepam to induce the increase of the KCl-evoked [^{45}Ca^{2+}] influx is the strongest when evaluated on the base of EC$_{50}$ values and clobazam has the lowest potential (Table 1).

The concomitant exposure of the neurons to flumazenil, an antagonist of central type of BZD receptors, with BZDs completely abolished the increase in the KCl-evoked [^{45}Ca^{2+}] influx induced by sustained exposure to BZDs (Fig. 1a), indicating that the enhancement of the KCl-induced [^{45}Ca^{2+}] influx after the sustained exposure to each BZD occurs through the activation of central BZD receptors by these 1,4- and 1,5-BZDs. On the other hand, flumazenil alone showed no effects on the KCl-evoked [^{45}Ca^{2+}] influx (data not shown).

Effects of various inhibitors for P/Q- , N-, and L-type HVCCs
In order to examine through which types of HVCCs mediate the increase in the KCl-evoked [^{45}Ca^{2+}] influx induced by sustained exposure to BZDs for 3 days, the KCl-evoked [^{45}Ca^{2+}] influx in the neurons after the exposure to BZDs for 3 days was measured in the presence of various inhibitors for each HVCC (ω-CTX, ω-ATX, and nifedipine for P/Q- , N-, and L-type HVCCs, respectively). Figure 2 shows that only nifedipine has an inhibitory effect on the increased KCl-evoked [^{45}Ca^{2+}] influx induced after sustained exposure to these three BZDs, and no suppression of the KCl-induced [^{45}Ca^{2+}] influx are observed in the concomitant presence of the inhibitors for Cav2.1 and Cav2.2, indicating that the increased [^{45}Ca^{2+}] influx produced after sustained exposure to BZDs is mediated via L-type, but not Cav2.1 and Cav2.2.

Fig. 1 Changes in 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx after sustained exposure to BZDs in primary cultures of mouse cerebral cortical neurons. The procedure to measure [^{45}Ca^{2+}] influx into the neurons was described in the text in detail. 1,4- and 1,5-BZDs were dissolved in 30% ethanol and then added into the culture medium. The basal and KCl-induced [^{45}Ca^{2+}] influxes in the neurons exposed to ethanol (final concentration, 0.3%) for 3 days were not significantly different from those without the vehicles (data not shown). The control values were determined in the neurons exposed to ethanol. Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. (a) Time course of changes in 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx after sustained exposure to BZDs. *$p < 0.05$ and **$p < 0.01$ versus the control (Dunnett's test). Diazepam, 0.3 μmol/L, brotizolam, 1 μmol/L, clobazam, 1 μmol/L. (b) Effects on 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx after sustained exposure to various concentrations of BZDs for 72 h. *$p < 0.05$ and **$p < 0.01$ versus the control (Dunnett's test).

Table 1 EC$_{50}$ values of 1,4- and 1,5-BZDs for 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx after sustained exposure to BZDs in primary cultures of mouse cerebral cortical neurons

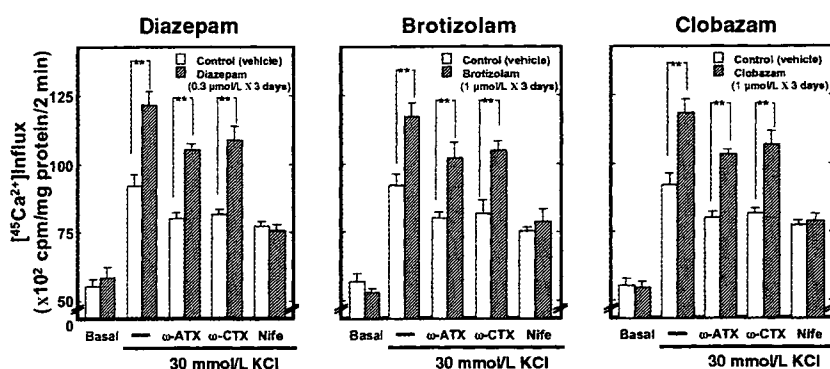|	EC$_{50}$ value (nmol/L)
Diazepam	220.7 ± 13.2
Brotizolam	892.2 ± 44.7[a]
Clobazam	2100.3 ± 189.6[a]

The procedure to measure [^{45}Ca^{2+}] influx into the neurons was described in the text in detail. 1,4- and 1,5-BZDs were dissolved in 30% ethanol and then added into the culture medium. The KCl-induced [^{45}Ca^{2+}] influxes in the neurons exposed to ethanol at its final concentration (0.3%) for 3 days were not significantly different from those without the vehicles. Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. [a]$p < 0.05$, compared with diazepam (Bonferroni's test).

Effects of nifedipine added during sustained exposure to BZDs on [^{45}Ca^{2+}] influx

After the exposure to BZDs for 12 and 24 h, nifedipine (0.1 μmol/L) was added into the culture medium containing BZDs and the neurons were further incubated with BZDs plus nifedipine. It is noteworthy that nifedipine added 12 h after the initiation of BZD exposure significantly and time-dependently suppressed the BZD-induced increase of KCl-evoked [^{45}Ca^{2+}] influx during following exposure to BZDs plus nifedipine, whereas nifedipine added 24 h after the initiation of BZD exposure showed any affects on the increased [^{45}Ca^{2+}] influx (Fig. 3).

Changes in the parameters of [^{3}H]diltiazem binding after sustained exposure to BZDs

From the results that the sustained exposure to BZDs facilitated [^{45}Ca^{2+}] entry into the neurons through L-type

Fig. 2 Effects of inhibitors of HVCCs on 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx after sustained exposure to BZDs. The procedure to measure [^{45}Ca^{2+}] influx into the neurons was described in the text in detail. 1,4- and 1,5-BZDs were dissolved in 30% ethanol and then added into the culture medium. The basal and KCl-induced [^{45}Ca^{2+}] influxes in the neurons exposed to ethanol at its final concentration (0.3%) for 3 days were not significantly different from those without the vehicles (data not shown). The control values were determined in the neurons exposed to ethanol. Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. **$p < 0.01$ (Bonferroni's test). ω-ATX (0.1 μmol/L); ω-CTX (0.1 μmol/L); Nife (nifedipine, 0.1 μmol/L).
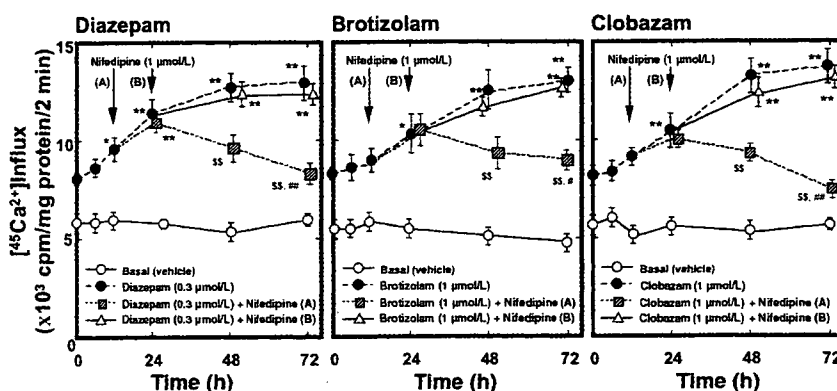
Fig. 3 Effect of nifedipine added during sustained BZD exposure on 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx. The procedure to measure [^{45}Ca^{2+}] influx into the neurons was described in the text in detail. Nifedipine (0.1 μmol/L) was added 12 h (a) or 24 h (b) after the initiation of sustained BZD exposure and then the neurons were further incubated with BZDs plus nifedipine. KCl-induced [^{45}Ca^{2+}] influx was measured 72 h after the initiation of sustained exposure to BZDs. Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. *$p < 0.05$ and **$p < 0.01$ versus the value measured before the initiation of sustained BZD exposure (Bonferroni's test), ##$p < 0.01$ versus the value measured after sustained exposure to BZDs alone for 24 h (Bonferroni's test), $^{\$\$}p < 0.01$ versus each value measured after sustained exposure to BZD alone at the time indicated in the figure.

HVCCs, we examined how the properties of [^{3}H]diltiazem binding to neuronal membrane change using the particulate fractions from the primary cultures of neurons. As shown in Fig. 4, all BZDs examined in this study increased [^{3}H]diltiazem binding and this change is due to the increase in B_{max} values with no changes in K_d values.

## Changes in L-type subunit expression after sustained exposure to BZDs

The increase in B_{max} values of [^{3}H]diltiazem binding indicates that [^{3}H]diltiazem binding sites, that is, Cav1.2 and Cav1.3 (Hofmann et al. 1999; Muth et al. 2001), on neuronal membrane are up-regulated by the sustained exposure to BZDs. Therefore, the expression of subunits

consisting of L-type HVCCs was measured using western blot analysis.

All of BZDs examined significantly enhanced the expression of Cav1.2 and Cav1.3 as well as α2/δ1 subunit, whereas the expression of Cav2.1, Cav2.2, and β4 subunit showed no significant changes (Fig. 5a and b).

Effects of calphostin C (1 μmol/L), KT-5720 (30 μmol/L), and KN-62 on [^{45}Ca^{2+}] Influx

We measured the effects of inhibitors of PKC in addition to CaM kinase II and protein kinase on the 30 mmol/L KCl-induced [^{45}Ca^{2+}] influx to clarify the mechanisms of the increased functions of L-type HVCCs induced by sustained exposure to BZDs. As shown in Fig. 6, all of examined
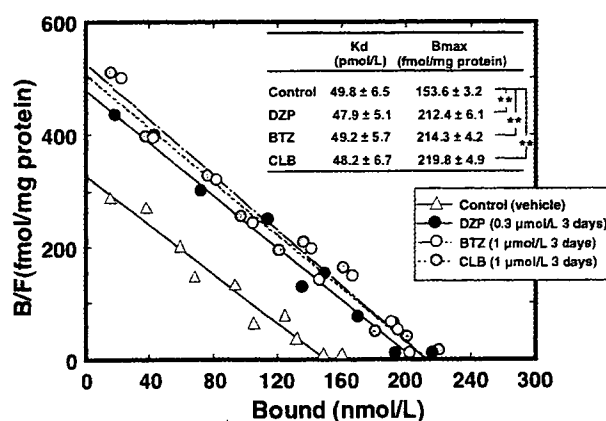
Fig. 4 Changes in the parameters of [³H]diltiazem binding to the particulate fractions from the neurons exposed continuously to BZDs. The procedure to measure [³H]diltiazem binding to the particulate fractions prepared from the neurons continuously exposed to BZDs for 3 days was described in the text in detail. 1,4- and 1,5-BZDs were dissolved in 30% ethanol and then added into the culture medium. The [³H]diltiazem bindings in the neurons exposed to ethanol at its final concentration (0.3%) for 3 days were not significantly different from those without the vehicles (data not shown). The control values were determined in the neurons exposed to ethanol. $**p < 0.01$ (Bonferroni's test). Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. DZP (diazepam, 0.3 µmol/L); BTZ (brotizolam, 1 µmol/L); CLB (clobazam, 1 µmol/L).

inhibitors for protein kinases did not show any effects on the [⁴⁵Ca²⁺] influx. These results therefore suggest that the phosphorylation by these protein kinases was not at least involved in the up-regulation of α1 subunits of L-type HVCCs induced after sustained exposure to BZDs.

Changes in [³H]diltiazem binding and L-type subunit expression in the cerebral cortex from mice physically dependent on BZDs

The continuous treatment with BZDs significantly increased [³H]diltiazem binding to the particulate fractions of the cerebral cortex (Fig. 7a). Moreover, the increases in Cav1.2, Cav 1.3, and α2/δ1 subunits, but not β4 subunit, were found in the cerebral cortex of mice physically dependent on BZDs (Figs. 7b and c).

Discussion

In the present study, we examined whether sustained exposure to 1,4-BZDs such as a long-acting BZD, diazepam, and a short-acting brotizolam modified L-type HVCC functions because chronic treatment of PC12 cells with chlordiazepoxide increased dihydropyridine-sensitive binding sites (Brennan and Littleton 1991). Moreover, whether a 1,5-BZD, clobazam, with a low potential to induce psychomotor behaviors (van der Meyden *et al.* 1989), alters L-type

HVCC functions was also investigated as there are little available data on these points until now.

Previous reports suggest that up-regulation of L-type HVCC functions is an important factor to participate in the development of physical dependence by drugs of abuse such as ethanol, morphine, and nicotine (Little 1991; Walter and Messing 1999). In physical dependence by 1,4-BZDs, up-regulation of L-type HVCCs is also considered to be involved in the development of their physical dependence (Brennan and Littleton 1991; Hitchott *et al.* 1992). We have also demonstrated that increased L-type HVCC functions occur in the brain of mouse showing physical dependence by administration of nicotine and ethanol (Hayashida *et al.* 2005; Katsura *et al.* 2005) and in the cerebral cortical neurons exposed continuously to these agents (Katsura *et al.* 2002). The changes in L-type HVCC functions after sustained exposure to BZDs are in good agreement with those by ethanol and nicotine and are considered to reflect to neurochemical changes in the brain during the development of BZD physical dependence. Moreover, as shown in this study, the complete inhibition of increase in [⁴⁵Ca²⁺] influx by sustained exposure to BZDs by flumazenil, a non-selective antagonist for BZD receptors, indicate that the up-regulation of L-type HVCCs is due to the direct effect of BZD to interact with BZD receptors.

The Scatchard analysis revealed that the binding sites of [³H]diltiazem are up-regulated on the neuronal membrane by the sustained exposure to BZDs. In addition, diltiazem, one of benzothiazepines, binds to the transmembrane region of repeat IV of the Cav1.2 and Cav1.3 (Kuniyasu *et al.* 1988; Catterall and Striessnig 1992; Watanabe *et al.* 1993). Taken together with these data, it is likely that sustained exposure to a 1,5-BZD, clobazam, as well as 1,4-BZDs (diazepam and brotizolam) increases Cav1.2 and Cav1.3 expression.

In order to check the possibility of the increase in Cav expression, we examine expression patterns of various subunits consisting of L-type HVCCs after sustained exposures of the mouse cerebrocortical neurons to both 1,4- and 1,5-BZDs by western blot analysis. As a result, the expression of Cav2.1 and Cav2.2 (Stotz and Zamponi 2001), respectively, showed no changes and these data are considered to be in good agreement with the data showing that the increase in the KCl-evoked [⁴⁵Ca²⁺] influx is not affected by the inhibitors for these two types of HVCCs. On the other hand, western blot analysis demonstrates the enhanced expression of both Cav1.2 and Cav1.3 forming the Ca²⁺ ionophores of L-type HVCCs. These results and the data on the KCl-evoked [⁴⁵Ca²⁺] influx and [³H]diltiazem binding certainly indicate that sustained exposure to both 1,4- and 1,5-BZDs up-regulates L-type HVCC functions through increased expression of Cav1.2 and Cav1.3.

The previous investigations have revealed that these accessory subunits modify Cav functions. α2/δ subunit is reported to increase the current amplitude (Felix *et al.* 1997;
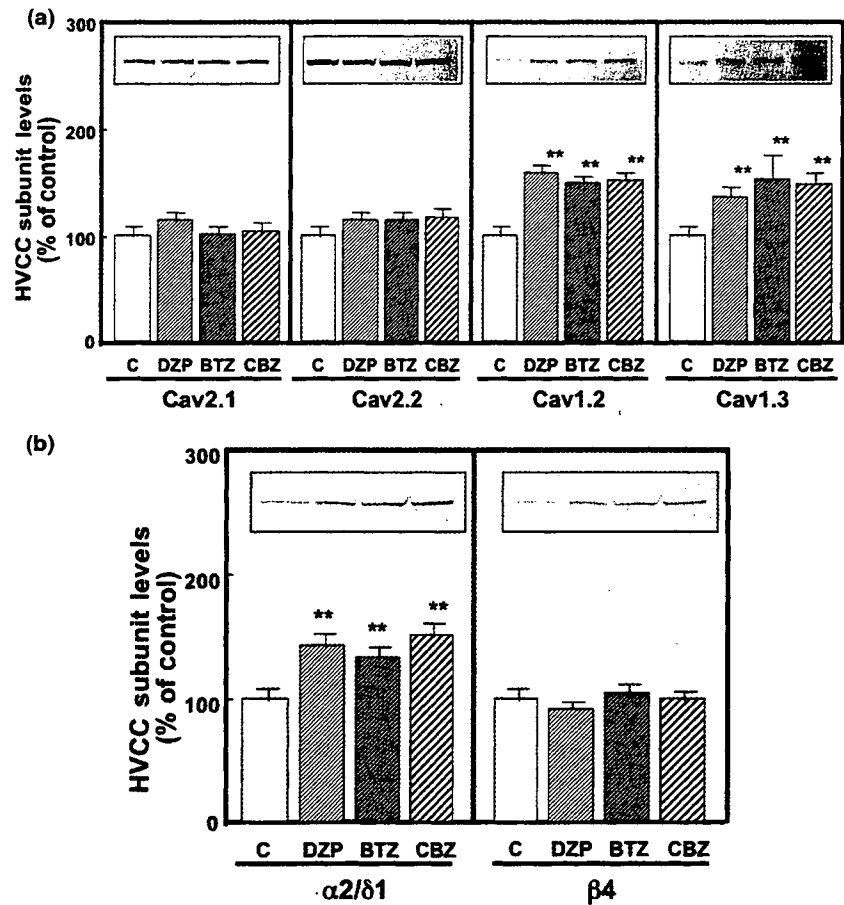
Fig. 5 Changes in the expression of various subunits of HVCCs after sustained exposure of the mouse cerebral cortical neurons to BZDs for 3 days. 1,4- and 1,5-BZDs were dissolved in 30% ethanol and then added into the culture medium. The expression of each subunit in the neurons exposed to ethanol at its final concentration (0.3%) for 3 days were not significantly different from those without the vehicles (data not shown). The control values were determined in the neurons exposed to ethanol. (a) Changes in the Cav expressions. (b) Changes in the expression of $\alpha2/\delta1$ and $\beta4$ subunits. $^{**}p < 0.01$ (Bonferroni's test). Each value was expressed as the mean ± SEM, which was obtained five separate experiments. C, control; DZP (diazepam, 0.3 μmol/L); BTZ (brotizolam, 1 μmol/L); CBZ (clobazam, 1 μmol/L).
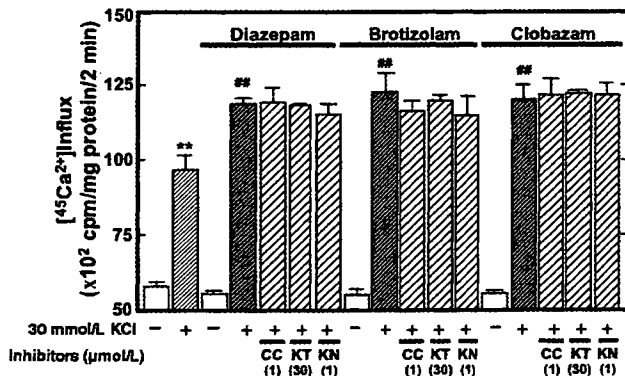

Fig. 6 Effects of inhibitors for protein kinase C, protein kinase A, and Ca^{2+}/calmodulin-dependent protein kinase II on increased 30 mmol/L KCl-induced [$^{45}Ca^{2+}$] influx induced by sustained exposure to BZDs. The procedure to measure [$^{45}Ca^{2+}$] influx into the neurons was described in the text in detail. Each inhibitor added the culture medium 15 s before the addition of BZDs. After the exposures to BZD and each inhibitor for 3 days, the 30 mmol/L KCl-induced [$^{45}Ca^{2+}$] influx was measured. Each value was expressed as the mean ± SEM, which was obtained five separate experiments, each of which was carried out in triplicate. $^{**}p < 0.01$ versus the basal influx (Bonferroni's test), $^{##}p < 0.01$ versus 30 mmol/L KCl alone (Bonferroni's test). CC, calphostin C; KT, KT-5720; KN, KN-62.

Klugbauer et al. 1999) and to enhance open probability of the channels (Shistik et al. 1995). Therefore, the increased expression of $\alpha2/\delta$ subunit may further facilitate Ca^{2+} entry through Cav into the neurons. Although the role of another subunit, $\beta4$ subunit, is to increase Ca^{2+} currents through the channels formed by Cav via modulation of inactivation kinetics and interaction with second messenger regulation (Lacerda et al. 1991; Varadi et al. 1991; Isom et al. 1994; Qin et al. 1998), pathophysiological significance of the reduced expression of $\beta4$ subunit on Ca^{2+} entry through Cav is not clear at present.

The present study demonstrates that the difference of the stage to inhibit L-type HVCC functions by nifedipine during sustained exposure to BZDs shows the different effects on the increase in KCl-evoked [$^{45}Ca^{2+}$] influx induced by sustained exposure to BZDs. That is, the increased KCl-evoked [$^{45}Ca^{2+}$] influx is significantly inhibited by nifedipine during 12 h after the initiation of sustained BZD exposure and the addition of nifedipine 24 h after the initiation of sustained BZD exposure no longer shows any affects on the increased KCl-evoked [$^{45}Ca^{2+}$] influx. These results suggest that during 24 h after the initiation of sustained exposure to BZDs some biochemical machinery triggers and/or facilitates neurochemical processes to stimulate the increased L-type
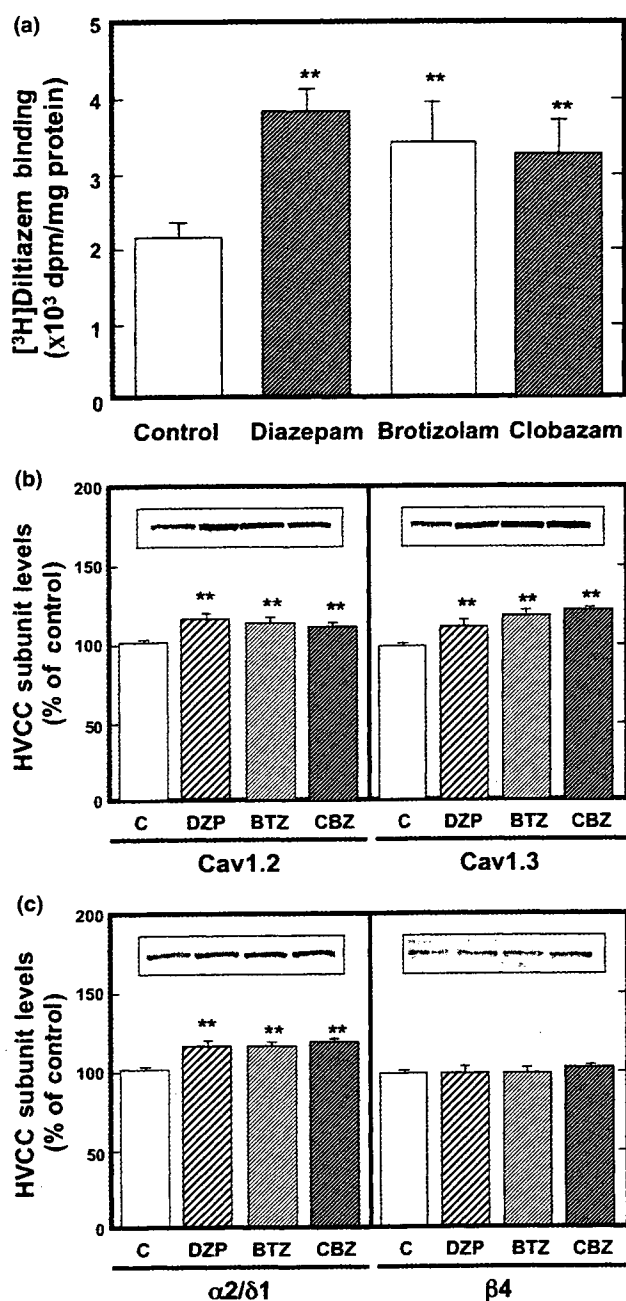
Fig. 7 Changes of [^{3}H]diltiazem binding to the particulate fractions and the expression of various subunits of HVCCs in the cerebral cortex from mouse physically dependent on BZDs. The procedures to measure [^{3}H]diltiazem binding and expression of HVCC subunits and to prepare mouse physically dependent on BZDs were described in the text in detail. Each value was expressed as the mean ± SEM, which was obtained 6 mice. (a) [^{3}H]Diltiazem binding. **$p < 0.01$ versus the control (Bonferroni's test). (b and c) Expression of HVCC subunits. **$p < 0.01$ versus control (Bonferroni's test).

HVCC functions. However, the exact mechanisms to initiate such neurochemical events remain to be elucidated at present.

There have been few reported data on the Cav up-regulation by chronic treatment with BZDs, although a previous study suggests the possibility that chronic ethanol treatment up-regulates Cav through PKCδ in PC12 cells (Gerstin *et al.* 1998; Walter *et al.* 2000). Chronic administration of antipsychotic drugs and drugs of abuse increase the density of HVCC in the CNS (Gerstin *et al.* 1998) and increase in intracellular Ca^{2+} concentration serves as an important factor to produce neuropsychotic signs and symptoms found in patients suffering from drug dependence (Antkiewicz-Michaluk 1995). Increase in intracellular Ca^{2+} concentration turns on the process of protein synthesis such as activation of transcription factors and accelerated activation of protein kinases participating phosphorylation of various functional proteins (Burgoyne 2007), which may in turn facilitate the up-regulation of L-type HVCCs found after sustained exposure to drugs of abuse. So this study examined the effects of inhibitors of PKC in addition to CaM kinase II and PKA on the facilitation of the 30 mmol/L KCl-induced [$^{45}Ca^{2+}$] influx induced by sustained exposure to BZDs, because these experiments may define the possibility that these protein phosphorylation systems participate in the increased functions of L-type HVCCs under the conditions with sustained BZD exposure. As a result, all of examined inhibitors for the protein kinases did not show any effects on the 30 mmol/L KCl-induced [$^{45}Ca^{2+}$] influx. These results therefore suggest that the phosphorylation by these protein kinases does not at least participate in the Cav up-regulation induced after sustained exposure to BZDs. Such differences concerning the involvement of protein phosphorylation systems between the previous (Gerstin *et al.* 1998) and present studies might be because of the differences in cell types used in experiments as well as the difference in the used drugs to induce physical dependence. On the other hand, based on the report that chronic amphetamine treatment-induced changes in L-type HVCCs involves activation of mitogen-activated protein kinase phosphatase pathway (Rajadhyaksha *et al.* 2004), mitogen-activated protein kinase phosphatase system is considered to be a possible candidate to regulate L-type HVCCs during chronic exposure to BZDs. However, the exact mechanisms up-regulating Cav under the sustained exposure to BZDs remain to be elucidated in future.

The significant increases in both [^{3}H]diltiazem binding and levels of Cav1.2, Cav1.3, and α2/δ1 subunit indicate that the responses of L-type HVCC subunits in the cerebral cortex of animals chronically treated with BZDs parallel to those in the primary cultures of cerebral cortical neurons continuously exposed to BZDs, that is, the behaviors of L-type HVCC subunit expression after sustained exposure to BZDs well reflects the changes in L-type HVCC subunit expression in the cerebral cortex from mice physically dependent on BZDs. Similar parallel responses of L-type HVCC subunit expression after long-term treatment with

drugs of abuse such as ethanol and nicotine in the cerebral cortex of mice and primary cultures of cerebral cortical neurons were reported (Katsura *et al.* 2002, 2005, 2006; Hayashida *et al.* 2005).

1,4-Benzodiazepines with shorter half-life ($t_{1/2}$) in the blood have reported to tend to develop physical dependence and addiction in clinical use than those with longer $t_{1/2}$ (Chouinard 2004). Although diazepam has long $t_{1/2}$ (20–80 h) (Busto *et al.* 1986) and $t_{1/2}$ of brotizolam is relatively shorter (3.6–7.9 h) (Bechtel 1983, 1990) than diazepam, both 1,4-BZDs produce the increase in L-type HVCC functions with similar order of potentials when evaluating the EC_{50} values for increase in [$^{45}Ca^{2+}$] influx. Therefore, it is considered that the difference in $t_{1/2}$ between these two BZDs may not be necessarily a factor to provide the difference in the potency to up-regulate L-type HVCC subunit expression occurring in the development of physical dependence.

Clobazam is an effective antiepileptic drug in most varieties of seizures and epilepsies (Remy 1994) and its $t_{1/2}$ of elimination is about 60 min in rodents (Caccia *et al.* 1980). The EC_{50} value of clobazam to induce L-type HVCC up-regulation is about 2 $\mu mol/L$, which is higher than those of diazepam and brotizolam. As far to the potential of clobazam to increase L-type HVCC functions, clobazam is less effective than 1,4-BZDs, which may be partially related to the lower incidence of physical dependence. However, as the potential to increase L-type HVCC subunit expression is not necessarily close to the potential to produce physical dependence as mentioned above, it is supposed that clobazam with longer duration of its administration may cause the development of physical dependence in human. Indeed, clobazam was reported to have a possible activity to develop physical dependence in experimental animals (Löscher and Rundfeldt 1990).

The binding sites of BZDs are well known to be localized in $GABA_A$ receptor/BZD receptor/Cl^- ionophore complex ($GABA_A$ receptors) and BZDs positively facilitate $GABA_A$ receptor functions. $GABA_A$ receptor functions were negatively modified by antagonists of L-type HVCCs (Das *et al.* 2004). Although it is therefore supposed that up-regulation of L-type HVCCs produced by chronic exposure to BZDs may enhance $GABA_A$ receptor functions, pathophysiological significance of increased L-type HVCC functions in BZD dependence in association with possible changes of $GABA_A$ receptor functions are not clear at present.

In summary, we examined the effects of continuous exposure to two 1,4-BZDs with different action period, diazepam and brotizolam, and a 1,5-BZD, clobazam, on the expression of L-type HVCC subunits. The sustained exposure to these BZDs increased Ca^{2+} entry through L-type HVCCs and enhanced B_{max} values of [^3H]diltiazem binding, which was due to the up-regulate expression of Cav1.2 and Cav1.3 in association with the increased expression in $\alpha2/\delta1$ unit.

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Increase in Diazepam Binding Inhibitor Expression by Sustained Morphine Exposure Is Mediated Via μ-Opioid Receptors in Primary Cultures of Mouse Cerebral Cortical Neurons

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Our previous in vivo experiment demonstrates that chronic morphine treatment up-regulates diazepam binding inhibitor (DBI) transcripts in mouse cerebral cortex, although detailed mechanisms were unclear (Katsura et al. [1998] J. Neurochem. 71:2638–2641). This study sought to elucidate the precise mechanisms of DBI mRNA up-regulation by long-term treatment with morphine using primary cultures of mouse cerebral cortical neurons. A significant increase in DBI mRNA was observed after sustained exposure to 0.3 μM morphine for 2 days, and the maximal expression occurred after 2 days of exposure, whereas transient exposure to 0.3 μM morphine for 15 min, 1 hr, and 3 hr produced no changes in the expression. Continuous exposure to DAMGO also significantly increased DBI mRNA expression, which was completely abolished by a selective antagonist of μ-opioid receptors, β-funaltrexamine (β-FNA). The morphine-induced increase in DBI mRNA expression and its content were completely inhibited by naloxone and β-FNA, and the inhibitory potential of naloxonazine was about half that of β-FNA. On the other hand, κ- and δ-opioid receptor antagonists showed no effects on the morphine-induced increase in DBI mRNA. In addition, both a calmodulin antagonist and a CaM II kinase inhibitor significantly suppressed the morphine-induced increase in DBI mRNA. These results indicate that the increase in DBI expression is induced by continuous activation of μ-opioid receptors but not of κ- and δ-opioid receptors and is regulated by the calcium/calmodulin-related phosphorylation system. © 2007 Wiley-Liss, Inc.

Key words: diazepam binding inhibitor; morphine; opioid receptors; cerebral cortical neurons; mouse brain

Chronic administration of morphine produces physical dependence, which is exhibited as various spe-cific behavioral and vegetative signs such as jumping, convulsion, tremors, diarrhea, and anxiety after with-drawal of morphine or administration of opioid receptor antagonists (Wei et al., 1973; Suzuki, 1990; Amgo et al., 1995). Clinical findings and experimental investigations using animal models for dependence on several drugs of abuse have indicated that long-term treatment with these drugs induces the interaction of neurotransmitter recep-tors, which involves alterations of neuronal functions to produce neurological signs and withdrawal symptoms (Harris and Aston-Jones, 1994; Matthes et al., 1996).

Diazepam binding inhibitor (DBI) is an endoge-nous anxiogenic neuropeptide present in γ-aminobutyric acid (GABA)-ergic neurons that has pharmacological properties as an inverse agonist for benzodiazepine receptors (Guidotti et al., 1986; Shoyab et al., 1986) and produces anxiety and fear (Corda et al., 1984). Increase in cerebral DBI induces behavioral changes in experi-mental animals (Guidotti et al., 1983; Corda et al., 1984; Alho et al., 1985), and several behaviors observed in ani-mals after treatment with DBI seem to resemble those observed in morphine-dependent and -withdrawn ani-mals (Suzuki, 1990; Bhargava, 1994). In addition, we have previously demonstrated that the expressions of

DBI and its mRNA elevate in the brains of chronically nicotine-treated and -withdrawn mice and in primary cultures of cerebral cortical neurons exposed to these agents for the long term, and such an increase of DBI mRNA is completely antagonized by simultaneous treatment with antagonists for neural nicotinic acetylcholine (nACh) receptors (Katsura et al., 1995a,b, 1998b, 2000). Similar results were obtained in the brains of morphine-dependent and -withdrawn mice (Katsura et al., 1998a). With these data taken into account, drugs of abuse are thought to modify the DBI level in brain via activation of neural nACh and opioid receptors.

The opioid receptor-like (ORL1) receptor is a G-protein-coupled receptor (GPCR) that shares high sequence identity with the three classically recognized opioid receptor types, μ, κ, and δ receptors (van Lee et al., 1999), and these opioid receptors are considered to be involved in the development of morphine dependence, especially morphine's psychological dependence (Suzuki et al., 1993; Matthes et al., 1996; van Lee et al., 1999). In the present study, we have examined which subtypes of opioid receptors are involved in the increase in DBI mRNA expression produced by long-term exposure of the mouse cerebral cortical neurons to morphine.

MATERIALS AND METHODS

Isolation and Primary Culture of Mouse Cerebral Cortical Neurons

Isolation and primary culture of cerebral cortical neurons were carried out according to the method described previously (Ohkuma et al., 1986). In brief, the neopallium free of the meninges was removed from a 15-day-old fetus of ddY strain mouse (Japan SLC, Inc., Hamamatsu, Japan) that had been anesthetized with sodium pentobarbital, minced, dispersed by trypsin, and centrifuged. Thereafter, the isolated cells were inoculated on a poly-L-lysine-treated culture dish with Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% fetal calf serum and cultured at 37°C in humidified 95% air/5% CO_2 for 3 days. After treatment of the cells with 10 μM cytosine arabinoside in DMEM containing 10% horse serum for 24 hr to suppress the proliferation of nonneuronal cells, the culture medium was exchanged to DMEM with 10% horse serum and the incubation was continued under the same conditions as described above. The culture medium was changed to fresh DMEM with 10% horse serum every 4 days. More than 98% of the primary cultured cells used here have been confirmed to be neurons by an immunohistochemical approach (Ohkuma et al., 1986).

Short- and Long-Term Treatments of Opioid Receptor Agonists and Antagonists

For studying the effects of long-term exposure to morphine and a μ-opioid receptor agonist (DAMGO: D-Ala[2], NMe-Phe[4], Gly-ol[5] · enkephalin) on DBI expression, the drugs were diluted with Hank's solution and directly added into the culture medium according to the previous method, with minor modifications (Katsura et al., 2000). Morphine- and DAMGO-containing culture medium was exchanged

every 24 hr during the experimental period indicated in the figures. Cells cultivated under conditions in the absence of morphine were used as the control. To examine the effects of short-term exposure to morphine on DBI mRNA expression, the neurons were exposed to 0.3 μM morphine for 0.25, 1, and 3 hr, and then the culture medium was exchanged to fresh culture medium without morphine for further culture up to 72 hr after the initiation of the exposure to morphine.

During long-term exposure to morphine, the culture medium with and without morphine was exchanged every 24 hr. The opioid receptor antagonists used in the present study were diluted with Hank's solution and added into the culture medium 5 min before the stimulation with morphine. In the experiments to check effects of exposure to opioid receptor antagonists on morphine-induced DBI mRNA expression, the medium containing morphine and/or antagonists except naloxonazine was exchanged every 24 hr. The medium containing naloxonazine was exchanged every 6 hr to avoid the decrease in drug concentration by its degradation. In addition, to examine the possibility of activation of heterodimers forming with μ- plus δ-opioid receptors by naloxonazine (Law and Loh, 1999), the morphine-induced DBI mRNA levels after exposure to 1 and 10 μM naloxonazine for 3 and 6 hr were examined.

Long-Term Treatment With Morphine Plus a Calmodulin Antagonist (W-7) or a CaM Kinase II Inhibitor (KN-62)

To examine the effects of the calcium/calmodulin-related phosphorylation system, the neurons were incubated with 0.3 μM morphine plus 1 μM W-7 or 10 μM KN-62 for 3 days, and then DBI mRNA level was measured.

Amplification of DBI cDNA

DBI cDNA was amplified from the brains by polymerase chain reaction (PCR) using a set of oligonucleotides (CTTGATTGCTCCTGCTTCTG, CTCGTCTACCTTTT CCACAT) specific for the mouse DBI cDNA sequence (Kato, 1991). After the amplification, the cDNA was ligated with EcoRI adapter and inserted into the EcoRI site of pUC18.

Preparation of mRNA and RNA Blot Hybridization

mRNA was obtained from neurons cultured for 14 days. After the neurons were washed with ice-cold phosphate-buffered saline (PBS; pH 7.4), they were scraped off with a rubber policeman. Poly(A)$^+$ RNAs were isolated using FASTTrack, and RNA blot hybridization was performed as previously described (Katsura et al., 1995a,b). Denatured poly (A)$^+$ RNA (1 μg) was electrophoresed on 1.1% agarose gel containing formaldehyde. Gels were stained with ethidium bromide and photographed under UV illumination to assess the integrity of the RNA by visual inspection. RNA was transferred to a nitrocellulose filter (NitroPure; Osminics Inc.) using 20× standard saline citrate (SSC) buffer by the capillary blotting method, and the completeness of transfer was confirmed by examining gels for ethidium fluorescence. The DBI cDNA fragment (302 bp) from mouse brain was labeled by

the Klenow fragment with random primer and [α-32P]dCTP. Antisense oligomer (5'-AGGTCTCAAACATGATCTGGG TCA-3') for β-actin (Ponte et al., 1984) synthesized by an Applied Biosystems DNA synthesizer was end-labeled by terminal deoxynucleotidyl transferase with [α-^{32}P]dCTP. These denatured DNAs were used as probes in the hybridization. The baked filter was hybridized with 50 mM sodium phosphate buffer (pH 7.0), 5× SSC, 50 mg/ml salmon sperm DNA, 1× Denhardt's solution, 30% formamide, 10% dextran sulfate, and ^{32}P-labeled probe at 42°C for 24 hr. The filters were washed with 0.2× SSC and 0.1% sodium lauryl sulfate at 50°C and autoradiographed. The radioactive intensities of the bands were represented as arbitrary units determined by Fujix FLP 3000G System (Fuji-Film Co., Ltd., Tokyo, Japan). β-Actin mRNA was determined using the method previously reported (Ponte et al., 1984; Katsura et al., 1995a,b).

The contents of poly(A)$^+$ RNAs isolated from the mouse cerebral cortical neurons after the treatments with morphine and/or several opioid receptor antagonists were similar to those from the neurons not treated with these agents (data not shown). In Northern blot analysis, a single band of approximately 650 bp, which was in agreement with the reported size of mouse DBI mRNA (Mocchetti and Santi, 1991), was detected with the probe for DBI in the mouse cerebral cortical neurons.

In the present study, the levels of DBI mRNA were normalized according to the message of β-actin signal, and the levels of β-actin mRNA in the neurons treated with morphine, DAMGO, and opioid receptor antagonists used here were almost same as those in the nontreated neurons (data not shown).

Preparations of Authentic DBI and Antisera to DBI

Authentic DBI protein and an antigen to produce antisera against DBI peptide were synthesized as described previously (Katsura et al., 1998b). In brief, mouse brain cDNA was amplified by PCR with Tag primer and mouse DBI primer. The amplified DNA fragment was digested with AflIII and PmlI, cloned into the NcoI/SmaI site of pTrc99A prokaryote expression vector, and introduced into Escherichia coli. After the culture reached early log phase, the cells were collected, disrupted, and sonicated. The lysate was centrifuged, and the soluble protein included DBI protein was precipitated and purified.

The antisera against DBI peptide were obtained from rabbits injected intradermally with 0.2 mg of the DBI protein in 2 ml of complete Freund adjuvant according the method previously reported (Katsura et al., 1998b). The antisera specific for DBI were purified by affinity chromatography using Protein G sepharose 4FF (Amersham Biosciences Corp., Piscataway, NJ), by which DBI-IgG antibody was isolated (titer: 1:40,000 dilution compared with the standard antisera, IWAKI 0005; Asahi Techno Glass, Tokyo, Japan).

Measurement of DBI Protein

The measurement of DBI protein in the neurons was carried out according to the method previously reported (Katsura et al., 1998b). In brief, The neurons treated with mor-

phine were sonicated with 1 mM acetic acid, heated at 95°C for 15 min, and finally centrifuged. After application of the supernatant onto an Ultrafree C3-LCC column (molecular cutoff size <5,000; Millipore, Bedford, MA), the eluate was lyophilized and stored at −80°C until use.

The DBI content in the neurons was measured by a technique of ELISA. After the sample was diluted with ice-cold PBS (10 μg/35 μl in assay volume) and incubated in a 96-well plate precoated with the first antibody (a DBI-IgG antibody) at 37°C for 60 min, the reaction mixture was further incubated at 37°C for 60 min with the second antibody [a horseradish peroxides (HRP)-labeled DBI-IgG antibody]. Finally, the reaction mixture was removed by aspiration, 10 mM o-phenylendiamine/5 mM H_2O_2 reagent was added, and the optical density was measured at 492 nm. Cerebral DBI in the concentration range of 0.5–20 ng/ml assay volume was detectable by this assay method, and the cross-reactivity with another neuropeptide produced by the decomposition of DBI, octadecaneuropeptide, was less than 10% of the total optical density (Katsura et al., 1998b).

[³H]Naloxone Binding

To confirm the presence of opioid receptor subtypes such as μ, κ, and δ opioid receptors in the neurons used in the present study, [³H]naloxone binding was measured in the presence of antagonists of each opioid receptor subtype, because [³H]naloxone at nanomolar concentrations is reported to be able to recognize these three subtypes of opioid receptors (Chang et al., 1982).

The binding assay was carried out according to the method previously reported (Mogil et al., 1994; Cox et al., 1998), with a minor modification. The particulate fractions for using the binding assay were prepared as described below. The neurons were rinsed three times with ice-cold PBS, pH 7.4, 137 mM NaCl, 2.6 mM KCl, Na₂HPO₄ · 12 H₂O, 1.8 mM KH₂PO₄ and then scraped off from the dish with 50 mM Tris-HCl buffer (pH 7.4) followed by the homogenization with a Polytron homogenizer (setting 6, 30 sec, 4°C). The homogenate was centrifuged (48,000g, 4°C, 20 min), and the resultant pellet was resuspended with 50 mM Tris-HCl buffer. These washing procedures were carried out again, and the pellet (the particulate fractions) thus obtained was stored at −80°C for at least 24 hr.

The binding of [³H]naloxone to the particulate fractions was carried out according to the method previously reported (Mogil et al., 1994; Cox et al., 1998), with a minor modification. The particulate fractions suspended with 50 mM Tris-HCl buffer (400 μg protein/ml) were incubated at 25°C for 30 min with 5 nM [³H]naloxone. The reaction was terminated by filtration of the reaction mixture through Whatman GF/B filters (presoaked with 0.3% polyethyleneimine at 4°C for 5 hr) under vacuum followed by three times washes of the filters with ice-cold 50 mM Tris-HCl buffer. The filter was then used to measure the radioactivity retained on the filter by liquid scintillation spectrometry. Specific binding was calculated by subtraction of nonspecific binding obtained in the presence of 10 μM nonlabeled naloxone from total binding determined without nonlabeled naloxone.

Measurement of Neural Viability

For examining the leakage of lactic dehydrogenase (LDH) activity from the neurons, LDH activity released from the neurons was measured according to the previous method, with a minor modification (Ohkuma et al., 1994; Katsura et al., 2002). After the exposure of the neurons to opioid receptor ligands for 3 days, the neurons were washed three times with ice-cold KRB-HEPES and then incubated in KRB-HEPES at 37°C for 1 hr without any drug. The incubation buffer was pooled at 4°C, and the neurons were dissolved with 2% Triton X-100. The activities of LDH in the pooled incubation buffer and the dissolved neurons were measured using a LDH cytotoxicity detection kit (TaKaRa Biomedicals, Tokyo, Japan). The leakage of LDH activity into the incubation buffer was represented as a percentage of total LDH activity (activity determined in the buffer plus that detected in the dissolved neurons).

Trypan blue dye exclusion test was carried out as described below. After the neurons were incubated with 0.5% trypan blue dye in PBS for 10 min, the neuronal toxicity was evaluated as the percentage of positively stained neurons in the total cells (positively plus negatively stained neurons). As described above, the effects of W-7 and KN-62 on the neuronal viability were also measured.

Measurement of Protein

The protein content in the neurons was determined by the method of Lowry et al. (1951), with bovine serum albumin as standard.

Statistical Analysis

Each value in the data was expressed as the mean ± SEM. Statistical significance was assessed by Dunnett's and Bonferroni's tests as described in the figure legends following application of one-way ANOVA.

Materials

Morphine was obtained from Shionogi & Co., Ltd. (Osaka, Japan). New England Nuclear (Boston, MA) and PerkinElmer Inc., Wellesley, MA) were the sources of $[\alpha-^{32}P]dCTP$ and $[^{3}H]$naloxone (2.0 TBq/mmol), respectively. FASTTrack was a product of Invitrogen (San Diego, CA). Random primed DNA labeling kit was supplied by Boehringer-Mannheim (Mannheim, Germany). Terminal deoxynucleotidyl transferase and LE agarose were obtained from Nacalai Tesque (Kyoto, Japan). DAMGO, β-funaltrexamine (β-FNA), naloxonazine, nor-binaltorphimine (nor-BNI), and naltrindole (NTI) were purchased from Tocris Cookson Inc., (Ellisville, MO). Naloxone, W-7, and KN-62 were obtained from Sigma-Aldrich Co. (St. Louis, MO). Other reagents used were commercially available and of analytical grade.

RESULTS

Effect of Short-Term Exposure to Morphine on DBI mRNA Expression

The exposure of the neurons to morphine (0.3 μM) for 0.25, 1, and 3 hr induced no alterations in DBI
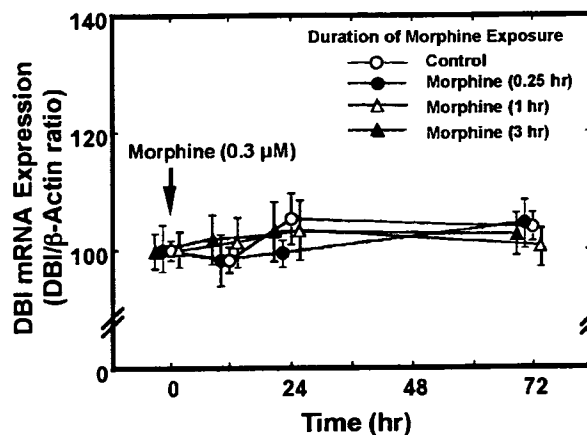

Fig. 1. Time course of changes in DBI mRNA expression after short-term exposures of mouse cerebral cortical neurons to morphine. Each value represents the mean ± SEM obtained from four separate experiments.

mRNA expression up to 3 days after the morphine exposure (Fig. 1).

Effect of Long-Term Exposure to Morphine on DBI mRNA Expression

Continuous morphine exposure for 2 days significantly increased DBI mRNA expression. The maximal expression was also observed after the sustained exposure for 2 days, and this increased level was maintained when continuously exposing the neurons to morphine for 3 or 5 days (Fig. 2A). Figure 2B shows the effects of various concentrations of morphine exposed for 3 days on DBI mRNA expression. Morphine in the range of concentrations from 0.1 to 3 μM dose dependently increased the DBI mRNA expression, and the maximal expression occurred at 0.3 μM (Fig. 2B).

Effect of Naloxone on Morphine-Induced Increase in DBI Expression

β-Actin mRNA level showed no changes after morphine treatment (Fig. 3A). In addition, naloxone (1 μM) alone did not show any effects on the expression of DBI and β-actin mRNA (Fig. 3A). Naloxone inhibited the morphine-induced increase in DBI mRNA expression in a dose-dependent manner (Fig. 3B). At the concentration of 0.01 μM, naloxone significantly suppressed the increased DBI mRNA expression induced by morphine, and 1 μM naloxone completely abolished the stimulatory effect of morphine on DBI mRNA expression (Fig. 3B). Similarly, increases in DBI content were observed after long-term treatment with morphine, and this increase was completely abolished by concomitant exposure to morphine and naloxone (Fig. 3C).
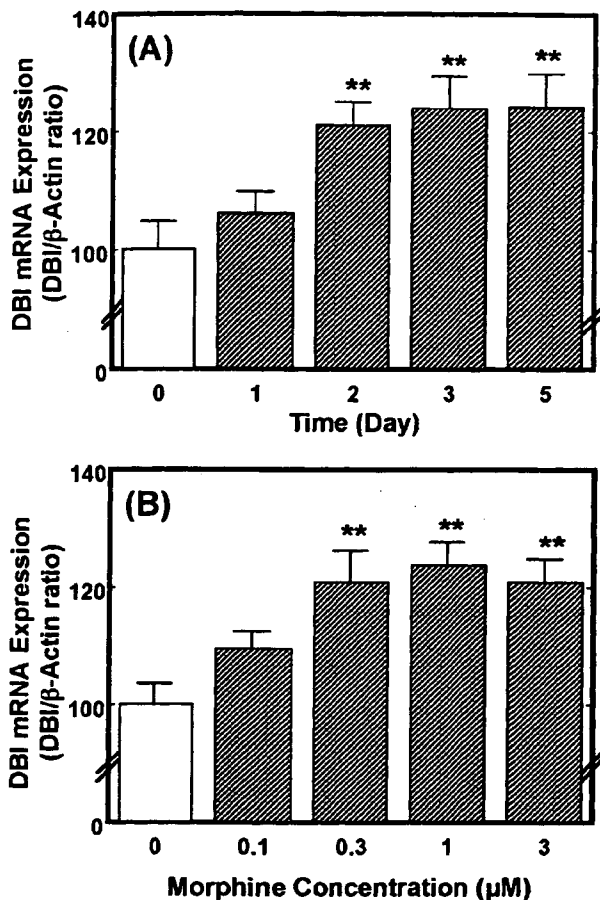
Fig. 2. Effect of morphine exposure on DBI mRNA expression in mouse cerebral cortical neurons. **A:** Time course of DBI mRNA expression exposed to morphine (0.3 μM). **B:** Effect of exposure to various concentrations of morphine (3 days) on DBI mRNA expression. Each value represents the mean ± SEM obtained from five separate experiments. $**P < 0.01$, compared with the value determined in the absence of morphine (Dunnett's test).

Presence of Opioid Receptor Subtypes in the Neurons Using [³H]Naloxone Binding

As shown in Figure 4A, the binding of [³H]naloxone to the particulate fractions was dose dependently inhibited by each inhibitor selective to μ (β-FNA), κ (nor-BNI), and δ (NTI) opioid receptors. The maximal inhibitions by these inhibitors were found at the concentrations of 0.01 μM of each inhibitor, indicating that the neurons used in the present study have all these opioid receptor subtypes.

The simultaneous incubation of β-FNA, nor-BNI, and NTI (concentration of each inhibitor 0.01 μM) completely inhibited the [³H]naloxone binding to the particulate fractions (Fig. 4A). On the other hand, a μ1-opioid receptor antagonist, naloxonazine, also inhibited the binding, and the maximal inhibition was obtained at 0.01 μM (Fig. 4B).

Effect of Opioid Receptor Antagonists on Morphine-Induced Increase in DBI Expression

The morphine-induced increase in DBI mRNA was reduced by simultaneous exposure to β-FNA by about 100% as in the case of naloxone (Fig. 5A). Naloxonazine, which has been considered as an antagonist selective to μ1-opioid receptors, reduced the increase to about 50% (Fig. 5A). It is noted that the medium change containing naloxonazine was carried out every 6 hr, and, even when changing every 3 hr, its partial inhibitory effect was similar to that in the case of changing medium every 6 hr (data not shown). Although naloxonazine significantly inhibited the morphine-stimulated DBI mRNA, its maximal effect was observed at the concentration of 0. 01 μM, and no further inhibition occurred even at higher concentrations (Fig. 5A).

On the other hand, nor-BNI and NTI had no inhibitory effects on the increase in DBI mRNA expression by morphine (Fig. 5A). The single application of the opioid receptor antagonists also showed no effects on basal DBI mRNA expressions (data not shown). Responses of DBI protein levels to the long-term exposure to morphine with or without opioid receptor antagonists were similar to those of DBI mRNA (Fig. 5B).

Effect of Long-Term Exposure to DAMGO on DBI mRNA Expression

A significant increase in DBI mRNA expression was observed after the exposure of DAMGO for 3 days. The maximal expression was observed at 1 μM DAMGO, and this DAMGO-induced increase was antagonized by β-FNA (Fig. 6).

Effects of W-7 and KN-62 on Morphine-Induced Increase in DBI mRNA Expression

As shown in Figure 7, both a calmodulin antagonist (W-7) and a CaM kinase II inhibitor (KN-62) significantly suppressed the morphine-induced increase in DBI mRNA level.

Changes in Neuronal Viability With Sustained Exposure to Opioid Receptor Ligands and Inhibitors for Calcium/Calmodulin-Related Phosphorylation System

The leakage of LDH activity from the neurons exposed to 0.3 μM morphine for 3 days was not different from that from the nontreated neurons (nontreated neurons 2.2% ± 0.2% of total LDH, morphine-treated neurons 2.1% ± 0.3%, N = 5). Similarly, the exposures to DAMGO and other opioid receptor antagonists used in this study showed no changes in LDH leakage from the neurons or the activity of the neurons excluding trypan blue dye during their exposure period of 3 days (data not shown). The continuous exposure to W-7 and KN-62 also did not affect the leakage of LDH activity from the neurons and neuronal activity of excluding dye (data not shown).

DISCUSSION

Abrupt withdrawal from morphine in human and experimental animals physically dependent on morphine triggers the manifestation of depression, fear, and anxiety as withdrawal syndrome, and a series of our studies sug-

(A)

(B)

Naloxone (µM)

Morphine (0.3 µM, 3 days)

(C)

gests that the DBI may cause anxiety appearing as a behavior of withdrawal syndrome in animals physically dependent on drugs of abuse such as ethanol and nicotine (Katsura et al., 1995a, 1998b, 2000). In the case of chronic treatment of mice with morphine, abrupt withdrawal of morphine or administration of naloxone after daily increasing doses of morphine to mice induced remarkable behavioral changes, such as jumping, ptosis, tremor, and diarrhea, and cerebral DBI mRNA significantly increased in morphine-treated mice (Katsura et al., 1998a). Moreover, we have already reported that the increase in DBI expression is found after continuous exposure to ethanol in the cerebral cortical neurons (Katsura et al., 1995a, 1998b) and mice physically dependent on ethanol (Katsura et al., 1995a), which implies that the changes in DBI expression in the neurons might be a neurochemical event occurring in the brains of animals dependent on drugs of abuse, as previously reported (Williams et al., 2001).

In this study, we have investigated whether morphine after short- and long-term exposure changes DBI mRNA expression and its content even in primary cultures of mouse cerebral cortical neurons and which opioid receptor subtypes are involved in morphine-induced DBI mRNA expression, because the chronic treatment with morphine produces the increase in DBI mRNA expression in mouse cerebral cortex (Katsura et al., 1998a). The expression of DBI mRNA showed no significant changes after short-term exposure of the neurons to morphine as presented here. Similarly, a single dose of morphine induced no alterations in DBI mRNA expression in the mouse cerebral cortex (Katsura et al., 1998a). On the other hand, the long-term exposure to morphine significantly increased DBI mRNA expression and its content in the mouse cerebral cortical neurons, whereas no changes in β-actin mRNA expression were found. These results indicate that sustained exposure of the neurons to morphine is crucial to increase cerebral DBI mRNA.

To examine whether the enhanced expression of DBI mRNA by the continuous exposure to morphine is mediated via the stimulation of opioid receptors, DBI mRNA levels in the neurons were measured in the concomitant presence of morphine and naloxone, a compet-
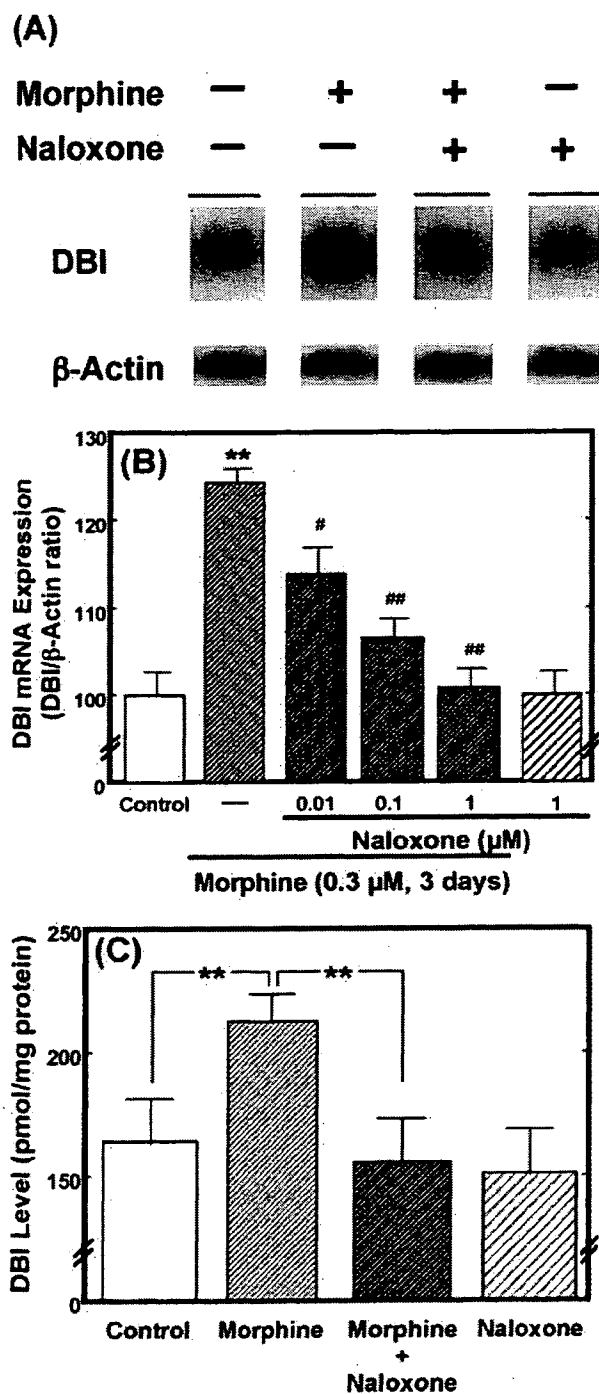
Fig. 3. Effect of naloxone on DBI mRNA and DBI peptide expressions in cerebral cortical neurons exposed to morphine. **A:** Typical Northern blots for DBI and β-actin mRNA in morphine-treated neurons in the presence of naloxone. **B:** Effects of exposure to various concentrations of naloxone on morphine-induced increase in DBI mRNA expression. Each value represents the mean ± SEM obtained from five separate experiments. $**P < 0.01$, compared with the control value (Bonferroni's test). $^{#}P < 0.05$ and $^{##}P < 0.01$, compared with the value determined in the presence of morphine alone (Dunnett's test). **C:** Effect of sustained exposure to morphine and naloxone on DBI content in mouse cerebral cortical neurons. Each value represents the mean ± SEM obtained from five separate experiments. $**P < 0.01$ (Bonferroni's test).

(A)

(B)

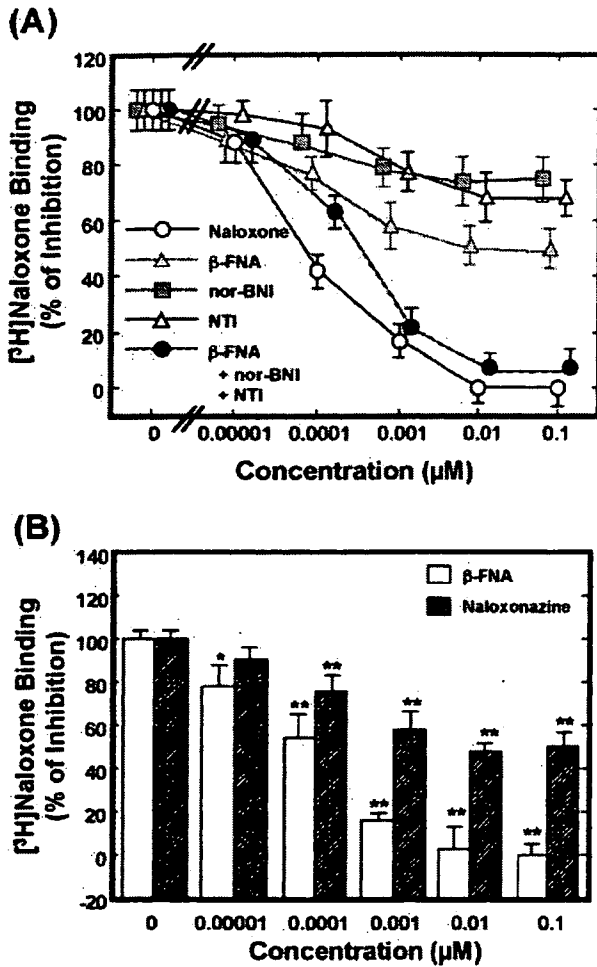
Fig. 4. Displacement of [³H]naloxone binding to particulate fractions from cerebral cortical neurons by naloxone, β-FNA, nor-BNI, and NTI (A) and by β-FNA and naloxonazine (B). Each value represents the mean ± SEM obtained from four separate experiments run in triplicates. *P < 0.05 and **P < 0.01, compared with each control (Dunnett's test). β-FNA, β-funaltrexamine; nor-BNI, nor-binaltorphimine; NTI, naltrindole.

(A)

Morphine (0.3 µM, 3 Days)

(B)

Morphine (0.3 µM, 3 Days)

Fig. 5. Effects of β-FNA, naloxonazine, nor-BNI, and NTI on DBI mRNA (A) and DBI protein (B) in cerebral cortical neurons exposed to morphine. One microgram of poly(A)⁺ RNA was applied to each lane. Each value represents the mean ± SEM obtained from four separate experiments. A: **P < 0.01 compared with the basal value (Bonferroni's test). *P < 0.05 and ##P < 0.01 compared with the value determined in the presence of morphine alone (Bonferroni's test). B: *P < 0.05 and *P < 0.01 (Bonferonni's test). N, naloxone; Nal, naloxonazine; BNI, nor-BNI.

itive antagonist specific for opioid receptors. Naloxone inhibited the morphine-induced increase in DBI mRNA in a dose-dependent manner, although β-actin mRNA level showed no changes. Moreover, naloxone alone did not show any effects on DBI mRNA expression. The increases in DBI content were also observed by long-term treatment with morphine, and this increase was completely abolished with concomitant exposure of the neurons to morphine and naloxone. The similar suppression of increased DBI mRNA by naloxone administration was reported in the morphine-dependent mouse brain (Katsura et al., 1998a). Accordingly, it is concluded that the morphine-induced increase of DBI expression is mediated via the activation of opioid receptors.
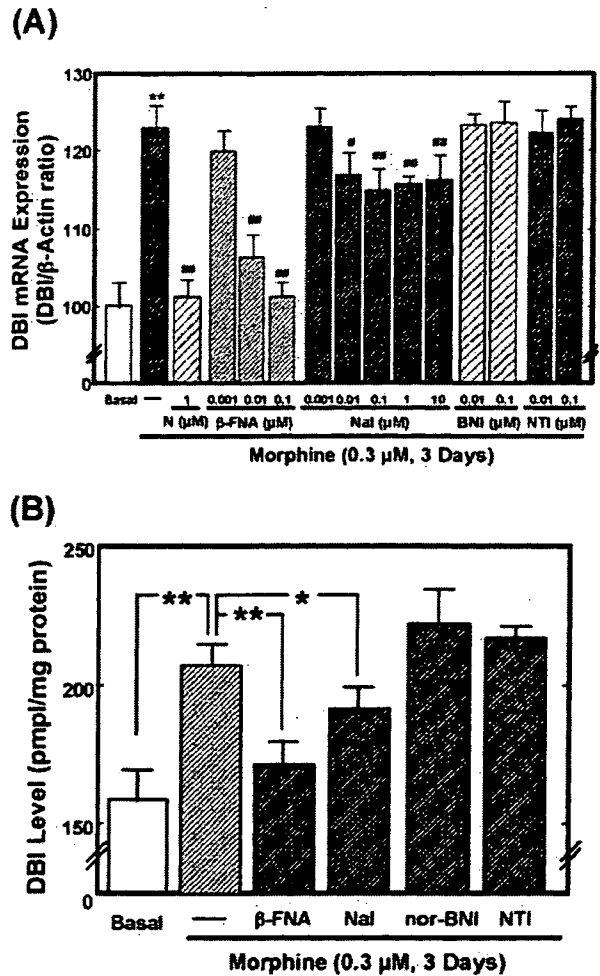
A binding study using [³H]naloxone establishes that the primary cultures of mouse cerebral cortical neurons used in this study have three opioid receptor subtypes, so we examined which subtypes of opioid receptors participate in the morphine-induced increase in DBI mRNA expression. The abolishment of the morphine-induced increases in DBI mRNA by β-FNA, a selective µ-opioid receptor antagonist, and the increase by DAMGO in DBI mRNA expression clearly indicate that µ-opioid receptors, but not κ- and δ-opioid receptors, are those that induce the increase of DBI mRNA expression.
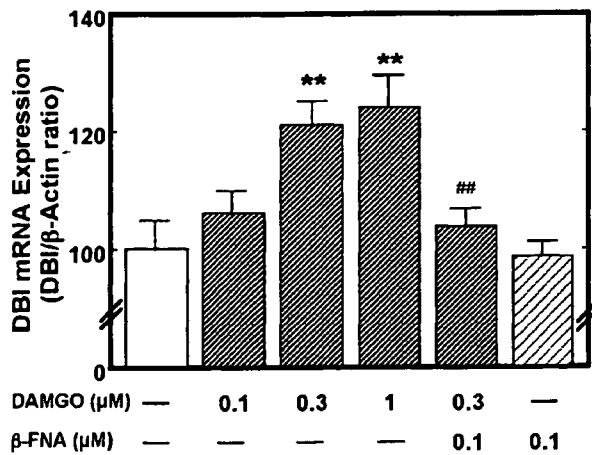
Fig. 6. Effects of DAMGO and β-FNA on DBI mRNA expression in mouse cerebral cortical neurons. Each value represents the mean ± SEM obtained from four separate experiments. $**P < 0.01$, compared with the basal value determined in the absence of all drugs (Bonferroni's test). $##P < 0.01$, compared with the value determined in the presence of morphine alone (Bonferroni's test). DAMGO, D-Ala²,NMe-Phe⁴,Gly-ol⁵ · enkephalin A; β-FNA, β-funaltrexamine.
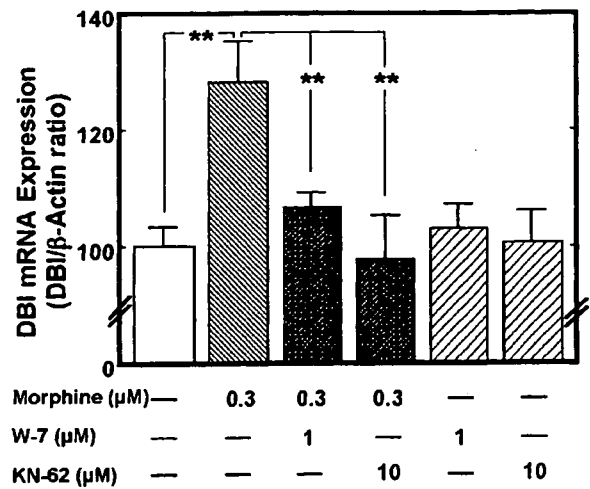

Fig. 7. Effects of W-7 and KN-62 on morphine-stimulated increase in DBI mRNA expression in mouse cerebral cortical neurons. Each value represents the mean ± SEM obtained from four separate experiments. $**P < 0.01$ (Bonferroni's test).

Naloxonazine, which has been considered to be a μ1-opioid receptor antagonist, showed partial inhibition of the morphine-induced increase of DBI mRNA expression. A previous study using μ1-opioid receptor gene-deficient mice (CHBK mice) and opioid receptor knockout mice revealed that both μ1- and μ2-opioid receptors participate in the establishment of psychological and physical morphine dependence (Suzuki et al., 1992, 1993; Matthes et al., 1996). Therefore, it is feasible that posttranslational modifications, alternative mRNA splicing, tissue distribution of more than one receptor gene and/or scaffolding with additional proteins, or homo- and/or hetero-dimerization of the existing μ-opioid receptor proteins could result in these various additional pharmacological phenotypes (Waldhoer et al., 2004). However, the concept of μ-opioid receptor subtypes is controversial, because recent investigations demonstrate that the μ-opioid receptor gene (OPRM1) has single cDNA coding regions (Cadet, 2004), and living cells have many splice variants of OPRM1 gene as μ-opioid receptors (Pan et al., 1999, 2001, 2005; Bolan et al., 2004; Waldhoer et al., 2004; Choi et al., 2005). Moreover, selectivity of opioid actions is supposed to be achieved by varying efficacies and affinities of opioid compounds at different μ-opioid receptor variants (Chavkin et al., 2001; Pasternak et al., 2004; Ikeda et al., 2005; Choi et al., 2006). Therefore, the lower effects of naloxinazine vs. naloxone may be due to such different efficacies of this compound at splice variants of μ-opioid receptors.

In addition, a second possibility for the lower effect of naloxonazine is considered. As shown in Figure 5A, 1 and 10 μM naloxonazine after a 6-hr exposure induced

50% inhibition of the expression, and this inhibitory rate was similar to that with 0.01 μM naloxonazine. Based on such data, the possibility that naloxonazine inhibits not only μ-opioid receptors themselves but also hetrodimers formed with μ- plus δ-opioid receptors cannot be ruled out, because heterodimers of opioid receptors have different pharmacological responses to their ligands from their respective opioid receptor monomers consisting of heterodimers (Cvejie and Devi, 1997; Jordan and Devi, 1999; Law and Loh, 1999; George et al., 2000) and the partial inhibitory potential of high concentrations of naloxonazine on the morphine-induced increase in DBI mRNA presented in this study may be due to the action of naloxonazine on the heterodimers composed of μ- and δ-opioid receptors. However, the exact reasons for the partial antagonistic effects of naloxonazine remain to be elucidated.

The instability of naloxonazine may induce its partial inhibitory effects. That is, during the incubation period with the neurons at 37°C, it degrades and loses its inhibitory potential. However, such event is unlikely to occur, because the effects of 3-hr exposure were similar to those of 6-hr exposure.

Although the detailed mechanisms of the increased expression of DBI mRNA after long-term exposure to morphine are not clear at present, a transient exposure to morphine does not produce the increase in DBI mRNA, because short-term morphine exposure does not increase the expression as shown in this study. In the case of nicotine-induced increase in DBI mRNA expression, inhibitors of CaM kinase II and calmodulin completely suppress the increased DBI mRNA induced by sustained exposure to nicotine (Katsura et al. 2000).

Chronic administration of morphine has also been reported to increase CaM kinase II activity (Brüggemann et al., 2000; Hamdy et al., 2004). From these data, we examine the involvement of calmodulin and CaM kinase II in the morphine-induced DBI mRNA increase. As shown in this study, the inhibitors for calmodulin and CaM kinase II completely suppress the morphine-induced DBI mRNA increase. Taken together with these data, the increase in DBI mRNA with sustained exposure to morphine may be mediated through mechanisms in which the calcium/calmodulin-related phosphorylation system participates.

Morphine exerts various pharmacological actions on central nervous functions, such as learning and memory facilitation, increase in spontaneous activity, convulsion, and modification of psychomotor activity (Bhargava, 1994). Among these alterations in the central nervous function, anxiety and aggression are thought to be at least in part induced by DBI, because DBI serves as an endogenous substance inducing anxiety in animals and human (Guidotti et al., 1983; Payeur et al., 1992; Ferrarese et al., 1993). The time-dependent behaviors and pharmacological properties of the response of DBI mRNA and its protein in the cerebral cortex from alcohol-dependent mouse are very similar to those in primary cultures of cerebral cortical neurons continuously exposed to ethanol (Katsura et al., 1995a,b, 1998b). Moreover, the pharmacological behaviors of DBI mRNA in the brain prepared from morphine-dependent mice (Katsura et al., 1995a) are considered to be very similar to those in this study. Therefore, we consider that neurons continuous exposed to morphine are a suitable tool for clarifyinbg the mechanisms of increasing DBI, and such data are also useful in resolving the functional significance of the cerebral increase of DBI with its relationship to animal behaviors.

In summary, DBI and its mRNA expressions increased in the mouse cerebral cortical neurons with sustained exposure to morphine. Simultaneous exposure of the neurons with morphine and μ-opioid receptor antagonists completely abolished these elevations of DBI mRNA expression and DBI contents. These results indicate that continuous functional interaction between morphine and opioid receptors in cerebral cortical neurons has a critical role in the increased cerebral DBI mRNA expression.

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