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Deltamethrin, a type II pyrethroid insecticide, has neurotrophic effects on neurons with continuous activation of the *Bdnf* promoter

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ABSTRACT

Pyrethroids, widely used insecticides with low acute toxicity in mammals, affect sodium channels in neurons. In a primary culture of rat cortical neurons, deltamethrin (DM), a type II pyrethroid, markedly enhanced the expression of brain-derived neurotrophic factor (BDNF) exon IV-IX (*Bdnf eIV-IX*) mRNA. In this study, we found that DM has a neurotrophic effect on cultured neurons and investigated the mechanisms responsible for it. One μM DM increased cell survival, neurite complexity and length. Neurite complexity and length were reduced not only by a blockade of cellular excitation with GABA or Ca^{2+} influx via L-type voltage-dependent calcium channels with nicardipine, but also by a blockade of TrkB, a specific receptor for BDNF, with TrkB/Fc. These data indicate DM has neurotrophic actions. DM-induced *Bdnf eIV-IX* mRNA expression through the calcineurin and ERK/MAPK pathways, the increase of which was reduced by $GABA_A$ receptor activation. Using a promoter assay, we found that Ca^{2+}-responsive elements including a CRE are involved in the DM-induced activation of the *Bdnf* promoter IV (*Bdnf-pIV*). The intracellular concentration of Ca^{2+} and activation of *Bdnf-pIV* remained elevated for, at least, 1 and 24 h, respectively. Moreover, $GABA_A$ receptor activation or a blockade of Ca^{2+} influx even after starting the incubation with DM reduced the elevated activity of *Bdnf-pIV*. These data demonstrated that the prolonged activation of *Bdnf-pIV* occurred because of this continuous increase in the intracellular Ca^{2+} concentration. Thus, DM has neurotrophic effects on neurons, likely due to prolonged activation of *Bdnf* promoter in neurons.

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1. Introduction

Brain-derived neurotrophic factor (BDNF) is a neurotrophin with important roles in controlling neuronal development, survival, and plasticity. Much evidence has recently emerged that BDNF affects dendritic structures (Gorski et al., 2003), which are thought to be essential to synaptic plasticity. In addition, the level of BDNF expression in the brain changes in patients with neurodegenerative disorders including Alzheimer's, Parkinson's and Huntington's disease or psychiatric disorders including depression and schizophrenia (Angelucci et al., 2005; Zuccato and Cattaneo, 2007; Mattson, 2008). Thus, it seems highly possible that the expression of BDNF in the brain plays a fundamental role in expressing a variety of neuronal functions and its impairment induces neuronal diseases.

The BDNF gene (*Bdnf*) consists of eight 5′ exons (exons I ∼ VIII) and a common 3′ exon (exon IX), encoding a preproBDNF protein (Aid et al., 2007). Nine promoters, *Bdnf* promoter I ∼ IX (*Bdnf-pI ∼ pIX*), were mapped upstream of the exons, each of which is controlled differently in the brain (Timmusk et al., 1993; Aid et al., 2007; Pruunsild et al., 2011). The *Bdnf* transcript containing exon IV (*Bdnf eIV-IX*) is the most abundant and widely detected in the rat brain (Metsis et al., 1993; Aid et al., 2007). A series of analyses have already demonstrated that the promoters of *Bdnf-pI* and *pIV* are activated by the influx of Ca^{2+} into neurons through L-type voltage-dependent Ca^{2+} channel (L-VDCC), which can be caused by membrane depolarization with high concentrations of KCl in culture (Tao et al., 1998, 2002; Tabuchi et al., 2000, 2002; Chen et al., 2003). The Ca^{2+} signals evoked via L-VDCC consequently activate *Bdnf-pI* and *-pIV* through Ca^{2+} signal-responsive *cis*-elements including a cAMP-response element (CRE) which binds the CRE-binding protein (CREB).

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Pyrethroids are highly active synthetic derivatives of natural pyrethrins which are toxins found in the flowers of *Chrysanthemum cinerariaefolium*. Since pyrethroids are rapidly metabolized in mammals and have low toxicity, they have been widely used to control insect pests in agriculture and in the home. Pyrethroid insecticides are classified into two major groups on the basis of chemical structure (Miyamoto et al., 1995): type II pyrethroids with a cyano moiety at the α-position (i.e., cypermethrin and deltamethrin), and type I pyrethroids, like permethrin, without it. Pyrethroids prolong the opening of the voltage-sensitive sodium channel (VSSC) and raise a prolonged sodium tail current in mammalian as well as invertebrate neurons (Vijverberg et al., 1982; Narahashi, 1985).

Previously, we found that the administration of deltamethrin (DM), a type II pyrethroid, to cultured rat cortical neurons markedly increased *Bdnf eIV-IX* mRNA expression, to more than 100 times the control level through the Ca^{2+} signals evoked via L-VDCC (Imamura et al., 2006). In this study, we investigated whether or not the neurotrophin-like effects on cell survival and morphological changes are induced by DM and, if so, by what mechanisms these neurotrophic effects are induced.

2. Materials and methods

2.1. Chemicals

Technical-grade deltamethrin (DM), (*S*)-α-cyano-3-phenoxybenzyl-(1*R*,3*R*)-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropanecarboxylate, was purchased from Wako Pure Chemicals (Tokyo, Japan). A stock solution of pyrethroids was prepared with dimethyl sulfoxide (DMSO), purchased from Sigma-Aldrich (St. Louis, MO). APV, nicardipine, Bay K8644, FK506, GABA, muscimol and baclofen were obtained from Sigma-Aldrich. TrkB/Fc was from R&D Systems (Minneapolis, MN). BAPTA-AM and U0126 were purchased from Merck (Darmstadt, Germany). These chemicals were used to clarify the signaling pathways induced by DM. The concentrations of the drugs used in this study were determined by results obtained previously (Tabuchi et al., 2000; Imamura et al., 2006; Yasuda et al., 2007).

2.2. Primary culture of rat cortical neurons

A primary culture of cortical cells was prepared from the cerebral cortexes of 17-day-old Sprague–Dawley rat embryos (Japan SLC, Hamamatsu, Japan) as described previously (Imamura et al., 2006). The cells were suspended in DMEM (Invitrogen, Carlsbad, CA) containing 10% fetal calf serum, 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the mRNA analysis, reporter assays, and MTT assays, the dissociated cells were seeded at 2.5×10^6 cells in 35 mm culture dishes (Iwaki, Tokyo, Japan) that had been coated with 0.1% polyethylenimine (Sigma–Aldrich). Under such conditions, Kato-Negishi et al. (2004) have demonstrated that spontaneous Ca^{2+} oscillations can be synchronously induced among neurons, indicating that active synapses are formed in this culture. For immunostaining, cells were plated at 7.0×10^5 cells/well onto 18 mm coverslips. The coverslips were placed in 12-well plates (Iwaki) and coated with poly-D-lysine (Sigma–Aldrich). The cells were grown for 3 days, and then the medium was replaced with serum-free DMEM containing 1 mM sodium pyruvate, 100 U/mL penicillin, and 100 μg/mL streptomycin. For the intracellular Ca^{2+} imaging, the cells were seeded at a density of 1.2×10^6 cells in 35 mm glass-based dishes (Iwaki) coated with poly-L-lysine (Sigma–Aldrich), and thereafter incubated in neurobasal medium containing 2% B-27 supplement (Invitrogen), 2 mM L-glutamine, and 2 mg/mL gentamycin for 3 days, after which half of the medium was changed.

2.3. MTT assay

The MTT assay, in which the conversion of MTT to colored formazan is measured, was performed according to the method of Hansen et al. (1989). After stimulation for the period indicated, 300 μL of a 5 mg/mL MTT solution was added to 2 mL of the culture medium and the cells were incubated in a CO_2 incubator for 1 h. Then, 1 mL of the culture medium was discarded, and 500 μL of extraction buffer (20% SDS and 50% dimethylformamide) was added. Cells were incubated overnight at 37 °C before optical density (OD) was measured at 570 nm.

2.4. Immunostaining and morphological analysis

The procedure was performed as described previously (Ishikawa et al., 2010). To monitor the effect of DM on the morphology of cortical neurons, a green fluorescent protein (GFP) expression vector (1 μg/well) was transfected at 4 days in culture using the calcium phosphate precipitation method. At 6 days in culture, after treatment with the drugs for 6 h, cells were fixed by placing them in PBS containing 4% formaldehyde and 4% sucrose for 15 min and then permeabilized by placing them in PBS containing 0.3% Triton X-100, 3% bovine serum albumin, and 3% normal goat serum for 1 h. The cells were incubated with anti-GFP and microtubule-associated protein 2 (MAP2) primary antibodies in the presence of blocking solution (PBS with 3% BSA and 3% normal goat serum) at 4 °C overnight. After being washed in PBS, the cells were incubated for 1 h at room temperature with Alexa488- and Alexa594-conjugated secondary antibodies against rabbit or mouse IgG diluted in blocking solution. After another wash, nuclei were counter-stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) (Invitrogen). Finally, coverslips were mounted with Fluoromount (Diagnostic BioSystems, Pleasanton, CA). The rabbit polyclonal antibodies used were anti-GFP (1:500; Invitrogen) and Alexa 488-conjugated anti-rabbit IgG (1:1000, Invitrogen). The mouse monoclonal antibodies used were anti-MAP2 (1:1000, Sigma) and Alexa594-conjugated anti-mouse IgG (1:1000, Invitrogen).

After immunostaining, the cells were processed for microscopy (BX50-34-FLA-1, Olympus, Tokyo, Japan), as described previously (Sholl analysis, Ishikawa et al., 2010). A series of concentric circles at 20-μm intervals were centered on the cell body and the number of intersections with GFP-positive processes was recorded as an index of neurite complexity (Sholl, 1953). The length of neurites was measured by tracing all of the neurites starting at the cell body and extending within a circle of 400-μm diameter using Image J software. More than 120 neurons were totally evaluated from three independent experiments ($n = 3$). We confirmed that the GFP-positive cells used for the morphological analysis were MAP2-positive cells (Fig. S1). Namely, we observed all kinds of neurons without any selection of cells for measurements.

2.5. RNA isolation and quantitative RT-PCR analysis

Total cellular RNA was extracted by the acid guanidine phenol–chloroform method. The isolation of RNA from cultured cells or rat tissues was described in detail previously (Fukuchi et al., 2009). In brief, total cellular RNA was extracted using TRIsure (BIOLINE, London, UK) and treated with RNase-free DNase I (TaKaRa, Shiga, Japan) to prevent contamination by genomic DNA. After the removal of DNase I, total RNA was quantified with a NanoDrop 1000 (Thermo Fisher Scientific, Wilmington, DE). One microgram of RNA was used for reverse transcription with SuperScript II (Invitrogen). Then quantitative PCR was performed using the Stratagene Mx3000p Real-Time PCR system (Stratagene, La Jolla, CA) in 20 μL of a reaction mixture containing 1 × SYBR Green QPCR master mix (Stratagene), 2 μL of cDNA solution, and 0.2 μM of primer pairs. The thermal profile for real-time PCR was as follows: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 45 s, annealing at 57 °C (dependent on Tm °C of primers) for 45 s, and extension at 72 °C for 1 min. Standard curves were generated for each gene using a plasmid DNA dilution series containing the target sequences. For the amplification of rat *Bdnf eIV-IX* cDNA, *Bdnf eIV* sense (5′-TCGGCCACCAAAGACTCG-3′) and *eIX* antisense (5′-GCCCATTCACGCTCTCCA-3′) primers were used. For the internal control, *Gapdh* cDNA was amplified using rat *Gapdh* sense (5′-ATCGTGGAAGGGCTCATGAC-3′) and antisense (5′-TAGCCCAGGATGCCCTTTAGT-3′) primers. The mRNA levels were computed from the threshold cycle (C_t) value and normalized to the concentration of *Gapdh* mRNA.

2.6. DNA transfection and luciferase assay

DNA transfection was carried out over four days in culture. A plasmid DNA containing *Bdnf-pIV* was prepared to generate pGL4.12-*Bdnf-pIV* by inserting the amplified DNA fragments covering the region (−629 to +281) of *Bdnf-pIV* into the *Hind*III site of the pGL4.12-Basic firefly luciferase reporter vector (Promega, Madison, WI). As an internal control vector, phRL-TK(−) was purchased from Promega. Plasmid DNA was transfected using the calcium phosphate/DNA precipitation method as described previously (Imamura et al., 2006). In brief, the precipitates were prepared by mixing 1 volume (67 μL) of plasmid DNA (5.3 μg; pGL4.12-*Bdnf-pIV*:phRL-TK(−) = 10:1) in a 250 mM $CaCl_2$ solution with an equal volume of 2 × HEPES-buffered saline, and added to a 35-mm dish. The dish was washed two times with PBS and replenished with fresh serum-free DMEM. After 2 days, the transfected cells were stimulated for 6 h, and cell lysates were extracted with passive lysis buffer (Promega). Dual (firefly and *Renilla*) luciferase activity was measured using a Dual-Luciferase Reporter assay system (Promega) with a TD-20/20 luminometer (Promega).

2.7. Intracellular Ca^{2+} imaging

The cells were incubated with 10 μM fura-2 acetoxymethylester (fura-2 AM) in neurobasal medium for 30 min at 37 °C. They were then washed with balanced salt solution (150 mM NaCl, 5 mM KCl, 1.2 mM $CaCl_2$, 1.2 mM $MgCl_2$, 10 mM D-glucose and 25 mM HEPES; pH 7.4) and mounted on an inverted fluorescence microscope (U7795-13, Hamamatsu Photonics). Digital images of fura-2 fluorescence, recorded by a high-sensitivity CCD camera (C9100-13, Hamamatsu Photonics) for 4 min at 0.5 s intervals were analyzed by a digital image processor (AQUACOSMOS,

Hamamatsu Photonics). The ratio of 510 nm emission fluorescence at 340 nm excitation to that at 380 nm excitation, termed "Ratio (F340/380)" was used as an indicator of the intracellular concentration of Ca^{2+} ($[Ca^{2+}]_i$) in single cells.

2.8. Statistics

All data were expressed as the mean ± S.E.M. for a number of separate experiments performed, as indicated in the corresponding figures. Statistical analyses were performed using a one-way analysis of variance (ANOVA) with the Tukey–Kramer or Sheffe's F test, or repeated measure ANOVA with the Bonferroni/Dunn test (See Figure legends).

3. Results

3.1. Effect of DM on the survival of cortical neurons in culture

Pyrethroid insecticides prolong the opening of the voltage-sensitive sodium channel (VSSC), giving rise to a sodium tail current in both insects and mammals (Soderlund et al., 2002). Previously, we demonstrated that *Bdnf eIV-IX* mRNA expression increased 3 h after incubation with 10 nM to 10 μM DM. Expression peaked with 1 μM DM with the level being about 150 times higher than that of the control (Imamura et al., 2006). To detect the neurotrophic action of DM on neurons, we first examined the effect of DM on cell survival in culture. We incubated the cortical neurons with 0.1, 1 or 10 μM DM for 12 or 24 h and measured cell survival using a MTT assay. As shown in Fig. 1, the viability of the neurons was increased by the incubation with 0.1 or 1 μM but not 10 μM at 12 and 24 h compared to the control. Using DAPI staining, we also checked for changes in nuclear morphology characteristic of apoptosis at 1 μM DM but did not detect any evidence of condensed nuclei, an indicator for apoptosis (Fig. S1).

3.2. Morphological changes of neurons induced by DM

To further detect the neurotrophic action of DM, we next examined the effect of DM on the morphology of neurons. For this purpose, we cultured the cells with 1 μM DM for 6 h after introducing the GFP expression vector at 4 DIV and incubating the cells for another 2 days, and then processed the cultures for the fluorescence microscopic observation of GFP-positive cells. Using immunostaining with anti-MAP2 antibody, we focused on GFP-positive and MAP2-positive cells, including all types of neurons in culture (Fig. S1). The administration of DM increased changes in neurite structures (Fig. 2). To quantify the structural changes, we performed a Sholl analysis (Sholl, 1953; Ishikawa et al., 2010), enabling us to detect changes in neurite complexity by measuring the number of crossings between neurites and circles with different diameters. As shown in Fig. 2A, B, and C, addition of DM increased the number of crossings in cultures harvested 6 h after the incubation, indicating that DM promotes neurite complexity. We also measured total length of neurites using Image J software and found that DM increased the length of neurites (Fig. 2D).

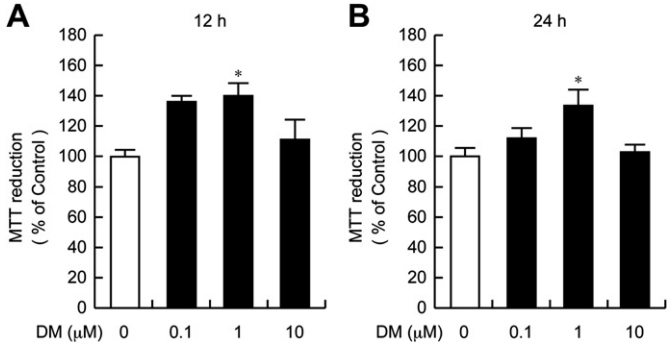

Fig. 1. The effect of DM on MTT-reducing activity. To quantify cell viability, cultured cortical neurons were treated with DM for 12 h (A) or 24 h (B), and then the MTT assay was performed. The OD value of the unstimulated sample was regarded as 100% survival. Values represent the mean ± S.E.M ($n = 3–4$). *$p < 0.05$ compared with the control using a one-way ANOVA with the Tukey–Kramer test.

Since DM increases neuronal excitability through its effect on VSSC that depolarizes the membrane and subsequently openings of L-VDCC that cause the influx of Ca^{2+} into neurons, we examined the effect of blockade of Ca^{2+} influx via L-VDCC or activation of GABA receptors on the DM-induced increase in neurite complexity and length. As shown in Fig. 2A, B, and D, the DM-induced stimulation of neurite complexity and length was effectively reduced in the presence of 5 μM nicardipine, a blocker of L-VDCC. Upon treatment of cells with 100 μM GABA, the DM-induced increase in neurite complexity (at 20, 60, 80, 100, 120, and 140 μm from cell body) was reduced, and the length of neurites not changed compared to the control (Fig. 2A, C and D). Furthermore, addition of 100 ng/mL TrkB/Fc, a blocker for the interaction of BDNF with TrkB receptors, effectively reduced the DM-induced change in neurite complexity and length (Fig. 2E, F, and G). Addition of GABA tended to increase the neurite complexity and length (Fig. 2A, C and D).

3.3. Involvement of calcineurin pathway induced by Ca^{2+} influx through L-VDCCs in the DM-induced increase in Bdnf eIV-IX mRNA expression

To clarify the mechanisms responsible for the DM-induced neurotrophic activity, we investigated the effects of DM on *Bdnf eIV-IX* mRNA expression. We first confirmed that the pathway including VSSCs, L-VDCCs, and ERK/MAPK is responsible for the marked induction of *Bdnf eIV-IX* mRNA expression (data not shown), consistent with our previous report (Imamura et al., 2006). In this study, we further found that administration of 10 μM BAPTA-AM, an intracellular Ca^{2+} chelator, almost completely repressed the induction, whereas that of 1 μM Bay K8644, an agonist for L-VDCCs, enhanced the mRNA expression induced by DM (Fig. 3A and B). Although Bay K8644 increased the expression, the effect was quite weak compared to that of DM (Fig. 3B). In addition, the DM-induced *Bdnf eIV-IX* mRNA expression was markedly inhibited by 5 μM FK506, a potent inhibitor for protein phosphatase 2B, calcineurin, and partially by 20 μM U0126, an inhibitor for MEK1/2 (Fig. 3C). The simultaneous addition of 5 μM FK506 and 20 μM U0126 strengthened the inhibition compared to that achieved by either inhibitor alone (Fig. 3C).

3.4. DM-induced Bdnf eIV-IX mRNA expression was inhibited by the activation of $GABA_A$ receptors

Since addition of GABA reduced the DM-induced increase in neurite complexity and length (Fig. 2), we examined if the $GABA_A$ or $GABA_B$ receptor is effective at reducing the excitability evoked by DM. As shown in Fig. 4A, the addition of 100 μM GABA prior to the stimulation of cortical neurons with DM markedly reduced *Bdnf eIV-IX* mRNA expression. This reduction was also detected on the addition of 10 μM muscimol, an agonist for the $GABA_A$ receptor (Fig. 4B). On the other hand, addition of 25 μM baclofen, an agonist for the $GABA_B$ receptor, did not reduce the DM-induced expression (Fig. 4C).

3.5. The DM-induced activation of Bdnf-pIV via Ca^{2+}-responsive elements

Next, we examined whether and if so how *Bdnf-pIV* is activated by stimulation with DM. Three Ca^{2+}-responsive *cis*-elements (CaREs), called CaRE1, CaRE2 (or UBE), and CaRE3 (or CRE) have already been identified within *Bdnf-pIV* (Tao et al., 1998, 2002; Chen

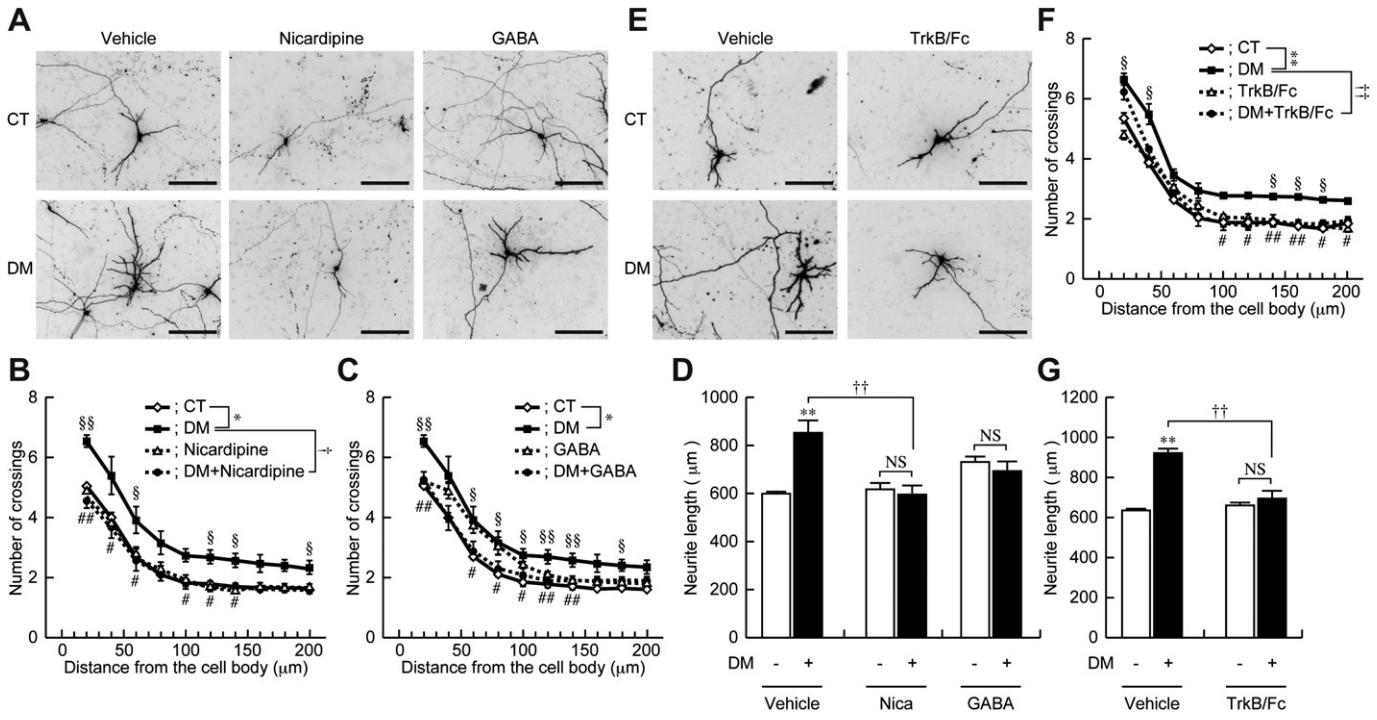

Fig. 2. The effect of DM on neuronal morphology. At 4 days in culture, rat cortical neurons were transfected with a GFP plasmid for immunostaining. Two days after transfection, the neurons were pretreated with 5 μM nicardipine, 100 μM GABA (A, B, C and D) and 100 ng/mL TrkB/Fc (E, F and G) for 10 min before being 1 μM DM was administered. The neurons were fixed after 6 h DM incubation. Representative images of untreated or pretreated neurons are shown (A and E). A measure of the number of neurites was obtained for each cell scored by placing a circle 20–200 μm in radius (20 μm interval) over the center of the cell and counting the number of times the circle intersects emanating from the cell body (B, C and F). Values represent the mean ± S.E.M ($n = 3$). §$p < 0.05$ and §§$p < 0.01$ versus the control, #$p < 0.05$ and ##$p < 0.01$ compared with the DM-treated neurons, using a one-way ANOVA with the Scheffe's F test at each length from 20 to 200 μm in radius. *$p < 0.05$ versus the control, †$p < 0.05$ compared with the DM-treated neurons, using a repeated measure ANOVA with the Bonferroni/Dunn test. Scale bar, 100 μm. The length of neurites was also measured by tracing all of the neurites starting at the cell body and extending within a 400 μm diameter (D and G). Values represent the mean ± S.E.M ($n = 3$). **$p < 0.01$ versus the control, ††$p < 0.01$ compared with the DM-treated neurons, using a one-way ANOVA with the Tukey–Kramer test. NS, not significant.

et al., 2003). These *cis*-elements are responsible for the Ca^{2+} signal-responsive activation of *Bdnf-pIV* induced by membrane depolarization. As shown in Fig. 5A and B, *Bdnf-pIV* was activated by DM, the actions of which were abolished by the prior addition of 5 μM nicardipine but not with 200 μM APV, an antagonist for NMDA receptors. In addition, the DM-induced activation of *Bdnf-pIV* was inhibited by 10 μM BAPTA-AM but further enhanced by 1 μM Bay K8644 (Fig. S2). To examine the effect of CaREs on the DM-induced *Bdnf-pIV* activation, we used two kinds of *Bdnf-pIV* constructs. In the first construct, CaRE1,2,3m, CaRE1, 2, and 3 are all mutated. In the second construct, CaRE3m, only CaRE3 is mutated (Fig. 5C). As shown in Fig. 5D and E, the DM-induced activation of *Bdnf-pIV* was reduced by the mutation of CaRE3 (or CRE), the inhibitory level of which was lower than that of CaRE1, 2 and 3.

3.6. Maintenance of the elevated intracellular concentration of Ca^{2+}

Using a calcium-imaging analysis with fura-2, we next examined the changes in $[Ca^{2+}]_i$ after addition of DM. As shown in

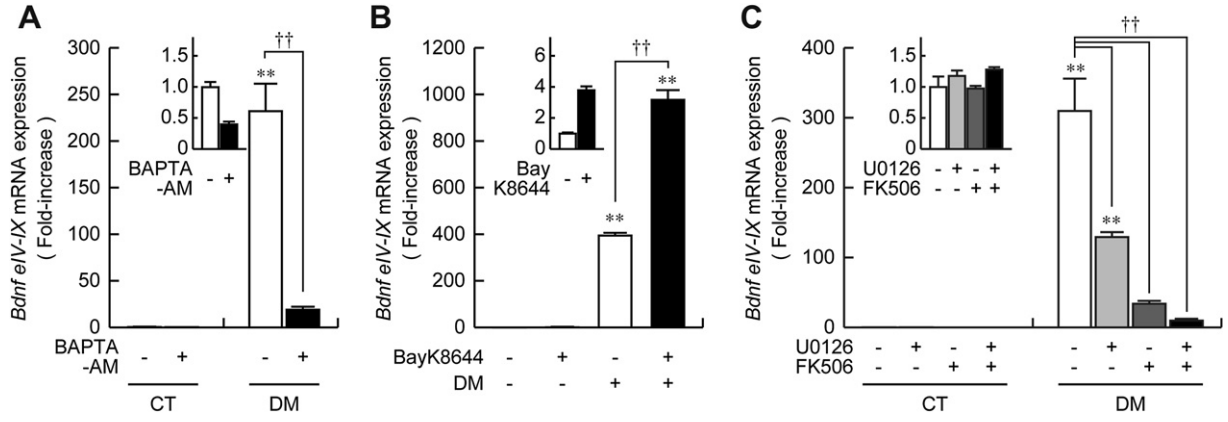

Fig. 3. The effect of blocking the intracellular Ca^{2+} signaling cascade on DM-induced *Bdnf eIV-IX* mRNA expression. Rat cortical neurons were pretreated with 10 μM BAPTA-AM (A), 1 μM Bay K8644 (B), or 20 μM U0126 plus 5 μM FK506 (C) for 10 min before the addition of 1 μM DM. Total RNA was prepared for measurement 3 h later. The ratio of mRNA expression relative to the control is shown. Values represent the mean ± S.E.M ($n = 3$–5). **$p < 0.01$ compared with the same sample without DM (A and C) or the control (B, DM(−)/Bay K8644(−)), ††$p < 0.01$ versus DM alone using a one-way ANOVA with the Scheffe's F test.

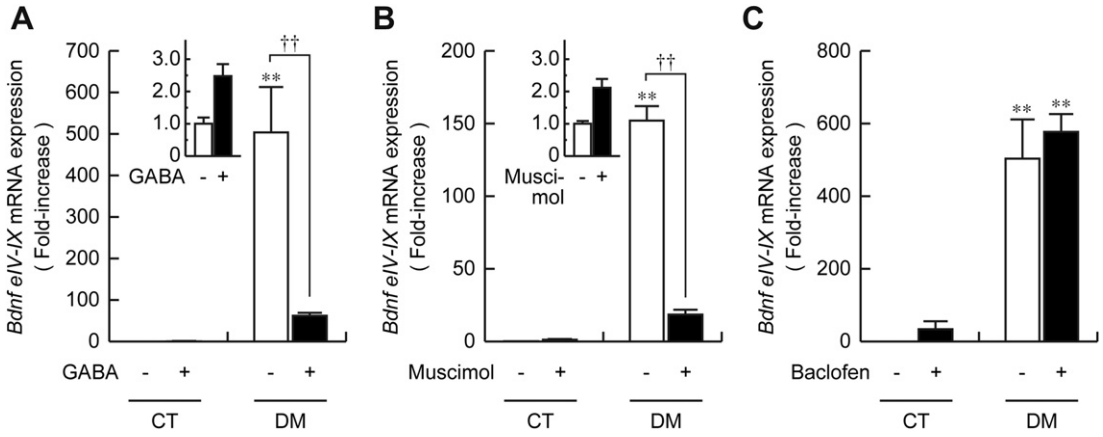

Fig. 4. The effect of GABAergic drugs on DM-induced *Bdnf eIV-IX* mRNA expression in rat cortical neurons. Ten minutes before the addition of 1 μM DM, the cells were treated with 100 μM GABA (A), 10 μM muscimol (B), or 25 μM baclofen (C), and incubated for another 3 h. The ratio of mRNA expression relative to the control is shown. Values represent the mean ± S.E.M ($n = 3$–4). $^{**}p < 0.01$ compared with the control, $\dagger\dagger p < 0.01$ versus DM with no pretreatments using a one-way ANOVA with the Scheffe's F test.

Fig. 6A(a), the $[Ca^{2+}]_i$ gradually increased on average till at least 4 min after the addition of DM, though the pattern of increase varied among individual neurons (data not shown). Upon depolarization, in contrast, the $[Ca^{2+}]_i$ transiently increased just after the treatment with 25 mM KCl but rapidly decreased at 2 and 4 min (Fig. 6A(b)). The $[Ca^{2+}]_i$ elevated by DM was evident even 10 min or 1 h after the incubation, while the $[Ca^{2+}]_i$ elevated by depolarization decreased to almost the same level as the control (Fig. 6B). We could not examine how long the $[Ca^{2+}]_i$ was elevated by DM because of a limitation of measurements using fura-2. Addition of 100 μM GABA 10 min before starting the incubation reduced the $[Ca^{2+}]_i$ increased by DM (Fig. 6B).

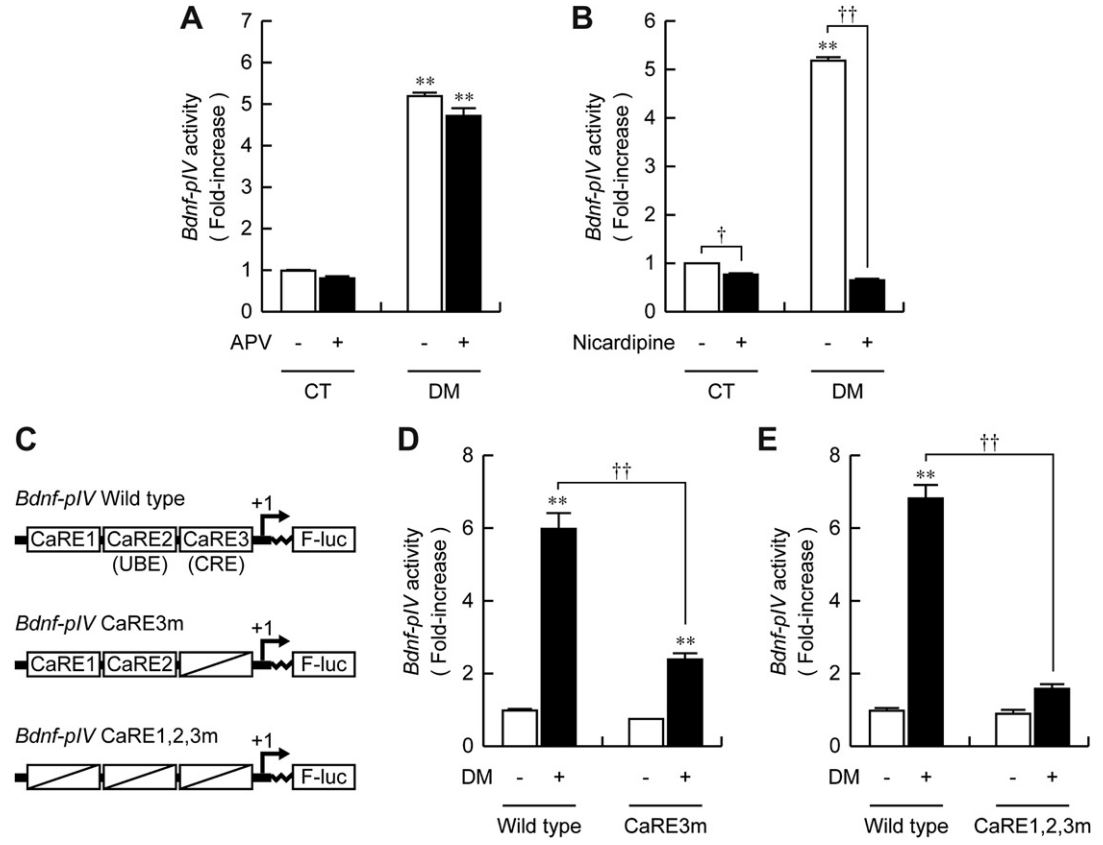

Fig. 5. The role of Ca^{2+} signaling and CaREs in DM-induced *Bdnf-pIV* activation. Rat cortical neurons were co-transfected with pGL4.12-*Bdnf-pIV* wild type and the internal control vector phRL-TK(−), and 2 days later pretreated with 200 μM APV (A) or 5 μM nicardipine (B) for 10 min before the treatment with 1 μM DM. Cellular extracts were prepared 6 h later. $^{**}p < 0.01$ compared with the control, $\dagger p < 0.05$ versus the control with no pretreatments, $\dagger\dagger p < 0.01$ versus DM with no pretreatments using a one-way ANOVA with the Scheffe's F test. The reporter plasmids, pGL4.12-*Bdnf-pIV* wild type, CaRE3m, and CaRE1,2,3m, are shown (C). Similarly, these plasmids and phRL-TK(−) were co-transfected into neurons, and at DIV6, the cells were treated with 1 μM DM for 6 h (D and E). The ratio of *Bdnf-pIV* activation relative to the control is shown. Values represent the mean ± S.E.M ($n = 3$). $^{**}p < 0.01$ compared with the control, $\dagger\dagger p < 0.01$ versus the wild type using a one-way ANOVA with the Scheffe's F test.

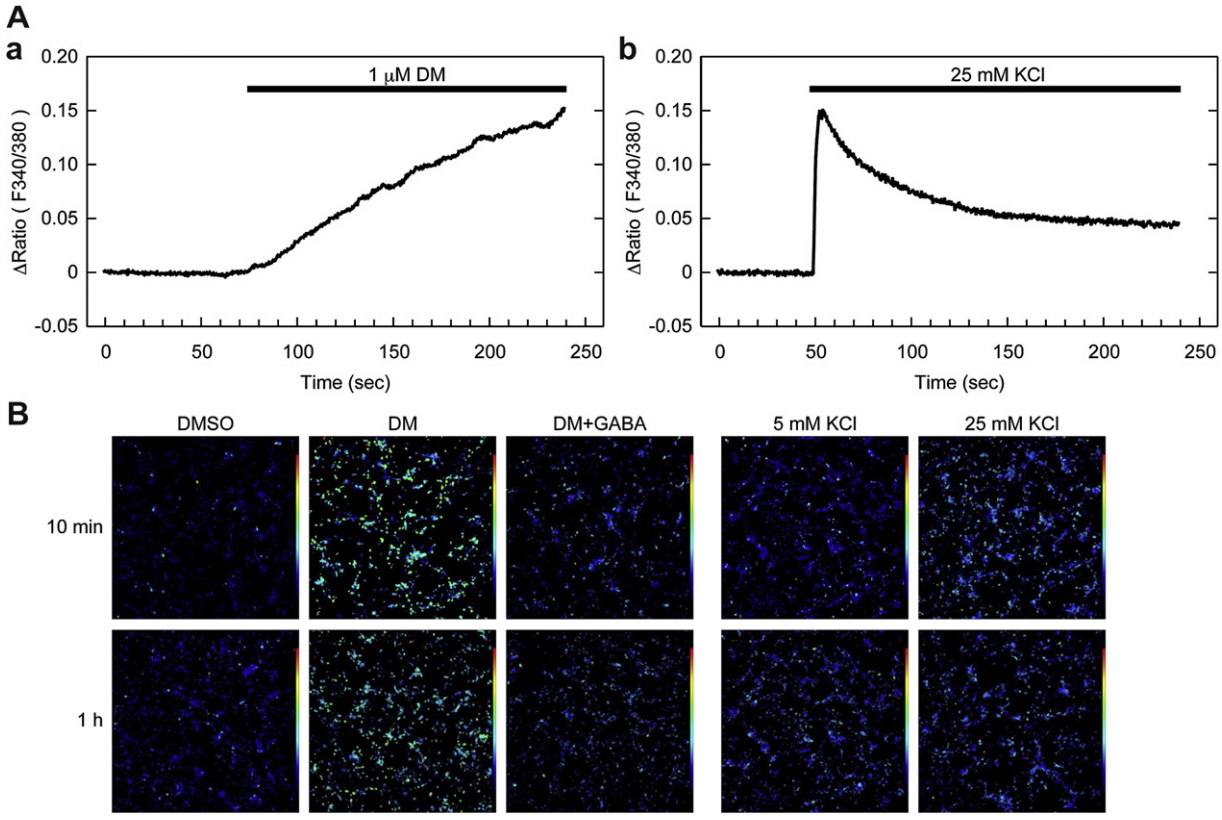

Fig. 6. Ca^{2+} influx into neurons induced by DM or depolarization. (A) One μM DM (a) or 25 mM KCl (b) was added to the culture medium and the change in $[Ca^{2+}]_i$ was monitored. The ratio of 510 nm emission fluorescence at 340 nm excitation to that at 380 nm excitation was used as an indicator of $[Ca^{2+}]_i$. The mean of the representative responses of Ca^{2+} influx in individual cells from the same culture dish (a, $n = 100$; b, $n = 20$) is shown. Each Ratio (F340/380) subtracted the value at time 0 and was termed "ΔRatio (F340/380)". These ΔRatio (F340/380) values were plotted to represent the change in $[Ca^{2+}]_i$. (B) Images of $[Ca^{2+}]_i$ after treatment with 1 μM DM or 25 mM KCl. One μM DM or 25 mM KCl was added to neurons and, subsequently, the $[Ca^{2+}]_i$ was monitored after the period indicated. GABA (100 μM)-pretreatment was performed 10 min before the addition of DM. The cells become brighter as Ca^{2+} influxes into neurons.

3.7. Prolonged activation of Bdnf-pIV during the culture with DM

Since the level of *Bdnf eIV-IX* mRNA was still increased after 24, 48 and 72 h (Imamura et al., 2006), we examined whether or not the activation of *Bdnf-pIV* was maintained after a prolonged DM exposure to a culture of cortical neuron. As shown in Fig. 7A, optimum activity was detected 3 and 6 h after the incubation with DM but this activation remained elevated even after 12 or 24 h though it was reduced to about one third of that at 3 or 6 h. After 5 μM nicardipine or 100 μM GABA was added 12 h after starting the incubation and the neurons were then cultured for another 6 h, the levels of *Bdnf-pIV* activity induced by DM were reduced (Fig. 7B).

4. Discussion

Because DM markedly induces BDNF expression (Imamura et al., 2006), we expected DM to have a neurotrophic effect. As demonstrated in this study, we showed the effects of DM on the survival and structure of cultured cortical neurons. The increases in neurite complexity and length were reduced by prior addition of GABA or nicardipine, indicating that the neuronal excitability and the influx of Ca^{2+} into neurons through L-VDCC are critical to the changes in structure of neurites. As BDNF has neurotrophic activity (Binder and Scharfman, 2004; Lipsky and Marini, 2007), it seems highly possible that endogenously synthesized BDNF, whose production could be stimulated by DM, is responsible for the neurotrophic effect of DM through the secretion of BDNF. In support of this notion, prior addition of TrkB/Fc reduced the increases in neurite complexity and length induced by DM.

Next, we investigated how the neurotrophic effect of DM is controlled in neurons. By using Bay K8644 or BAPTA-AM, we confirmed that extracellular and intracellular Ca^{2+} are essentially required for the increase in DM-induced *Bdnf eIV-IX* mRNA expression. The simultaneous addition of Bay K8644 with DM markedly enhanced the *Bdnf eIV-IX* mRNA expression, compared to the increase induced by Bay K8644 only. This effect of combined DM + Bay K8644 is likely due to an easier accessibility of Bay K8644 to sites within L-VDCC molecule, which could be induced by depolarization via the action of DM on VSSC. The same effect of BAPTA-AM or Bay K8644 was detected on the DM-induced activation of *Bdnf-pIV*, indicating that the increase in *Bdnf eIV-IX* mRNA expression induced by DM is controlled at the transcriptional level.

The selective activation of $GABA_A$ but not $GABA_B$ receptors markedly reduced the DM-induced increase in *Bdnf eIV-IX* mRNA expression. This result indicates that DM excites neurons through its action on Na^+ channels, whereas the activation of $GABA_A$ receptor decreases its excitability, resulting in a reduction in the DM-induced increase in *Bdnf eIV-IX* mRNA expression.

In the promoter analysis using the mutated *Bdnf-pIV*, we found that CRE is involved in the DM-induced activation of *Bdnf-pIV*. Other factors could also be involved because CaRE3m was not enough to reduce the activation. ERK/MAPK pathway induces the phosphorylation of CREB at Ser133, which can recruit the CREB-binding protein (CBP) to activate CRE-dependent transcription (Parker et al., 1996). However, it has recently become evident that TORC (transducer of regulated CREB) is dephosphorylated by calcineurin and then transported from the cytoplasm to the nucleus to activate CRE-dependent transcription independently of the

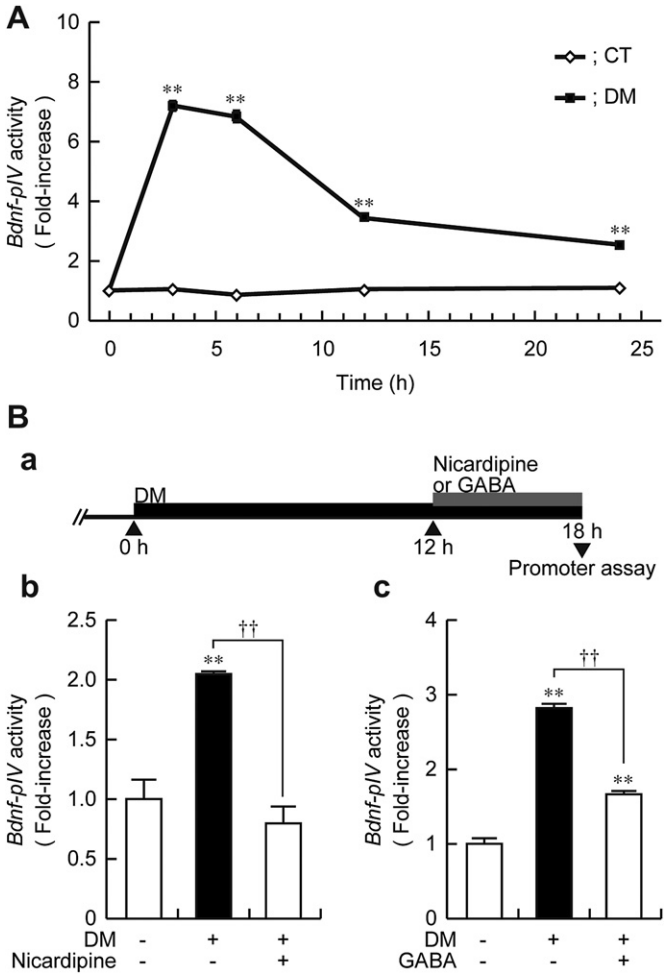

Fig. 7. Prolonged activation of *Bdnf-pIV* in rat cortical neurons induced by DM and the effect of nicardipine or GABA on it. (A) DM (1 μM) was added and the cells were further incubated for the periods indicated before cellular extracts were prepared to measure *Bdnf-pIV* activity using the Dual-luciferase assay. The ratio of *Bdnf-pIV* activity relative to the control (at time 0) is shown. $^{**}p < 0.01$ compared with the control at the same time point using a one-way ANOVA with the Scheffe's F test. (B) The cells were treated with 1 μM DM for 12 h, before 5 μM nicardipine or 100 μM GABA was added. Cells were incubated for another 6 h (a). The ratio of *Bdnf-pIV* activity relative to the control treated with vehicle only is shown (b and c). Values represent the mean ± S.E.M ($n = 3$–4). $^{**}p < 0.01$ compared with the control, $\dagger\dagger p < 0.01$ versus DM without nicardipine- or GABA-treatment using a one-way ANOVA with the Scheffe's F test.

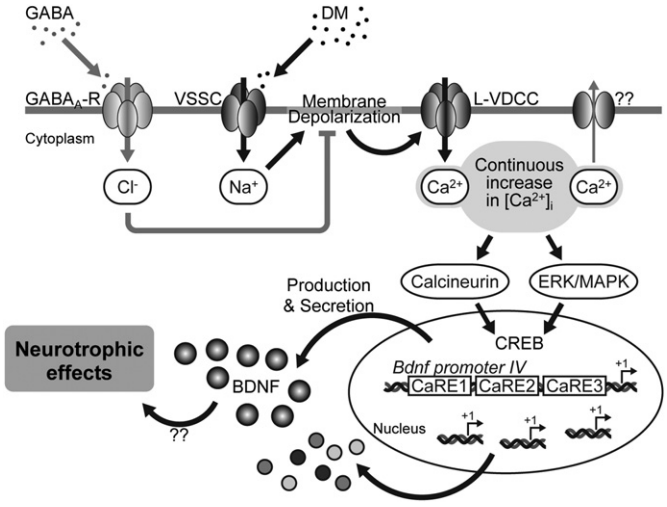

Fig. 8. Schematic model of neurotrophic effects of DM on neurons. Question marks (??) indicate mechanisms remained to be unsolved in this study.

phosphorylation of Ser133 (Conkright et al., 2003; Screaton et al., 2004). Supporting this idea, the calcineurin pathway is involved in the control of DM-induced *Bdnf eIV-IX* mRNA expression, in addition to the ERK/MAPK pathway. In this study, however, it is still unclear how the calcineurin and ERK/MAPK pathways cooperate to control the DM-induced transcription of *Bdnf-pIV* through CREB.

The addition of DM gradually increases $[Ca^{2+}]_i$, in contrast to the transient increase induced by depolarization. The most remarkable feature of the DM-induced increase was that the $[Ca^{2+}]_i$ remained elevated for at least 1 h after the incubation with DM. Since the $[Ca^{2+}]_i$ was high from 4 min to 1 h, it is likely that not only the influx but also the efflux of Ca^{2+} from neurons is accelerated by DM. Cellular toxicity, which could be induced by an excess flow of Ca^{2+} into neurons, was not detected, but rather, cell viability was enhanced by 1 μM DM even 24 h after the incubation. These results suggest that the turnover of intracellular Ca^{2+} could be accelerated in neurons stimulated with DM.

Consistent with the maintenance of $[Ca^{2+}]_i$, the *Bdnf-pIV* activity remained elevated for at least 24 h after the incubation with 1 μM DM. Since the half-life of the firefly luciferase protein is estimated at about 3 h (Thompson et al., 1991; Bronstein et al., 1994), it is unlikely that the increased level of *Bdnf-pIV* activity at 12 or 24 h is due to the luciferase synthesized immediately after the stimulation. That is, it is more likely that the Ca^{2+} signal-dependent activation of *Bdnf-pIV* continued even 24 h after the incubation. In support of this, addition of nicardipine or GABA reduced the DM-induced activation of *Bdnf-pIV* 12 h after the incubation of cortical neurons with DM, indicating that the Ca^{2+} influx via L-VDCC is continuously caused by DM. Taken together, it is evident that the strong, continuous *Bdnf eIV-IX* mRNA expression induced by DM is primarily caused by the continuous elevation of $[Ca^{2+}]_i$ and, thereby, the activation of *Bdnf-pIV*.

Although other genes were also activated by DM (see Tables S1, S2, and S3), it seems plausible that the enhanced expression of *Bdnf* mRNA is mainly responsible for the neurotrophic effects of DM because the changes in cell survival and structure induced by DM in the presence or absence of inhibitors showed a good correspondence with those in the DM-induced *Bdnf eIV-IX* mRNA expression or activation of *Bdnf-pIV* (Fig. 8). In any case, these characteristic neurotrophin-like effects of DM may provide a novel tool to analyze the intracellular reactions widely responsible for expressing neuronal functions including cell survival, neuronal differentiation and synaptic plasticity in terms of Ca^{2+} homeostasis and BDNF expression in neurons. Furthermore, DM may be a possible seed for developing drugs to treat neurodegenerative and psychiatric disorders.

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Appendix. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.neuropharm.2011.10.023.

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