学位論文

Latent process genes for cell differentiation are common decoders of neurite extension length

細胞分化の準備期に機能する Latent process 遺伝子群の同定と、

神経突起伸長レベルのデコーダーとしての機能解析

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1. Abstract

A latent process involving signal transduction and gene expression is needed as a preparation step for cellular function. We previously found that nerve growth factor (NGF)-induced cell differentiation has a latent process, which is dependent on ERK activity and gene expression and required for subsequent neurite extension. A latent process can be considered a preparation step that decodes extracellular stimulus information into cellular functions; however, molecular mechanisms of this process remain unknown. I identified *Metrnl*, *Dclk1*, and *Serpinb1a* as latent process (LP) genes that are induced during the latent process with distinct temporal expression profiles and are required for subsequent neurite extension in PC12 cells. The LP gene products showed distinct spatial localization. Overexpression of all LP genes together, but not of each gene separately, enhanced NGF-induced neurite extension. The LP genes showed distinct dependency on the duration of ERK activity, and they were also induced during the latent process of pituitary adenylate cyclase-activating polypeptide (PACAP)- and forskolin-induced cell differentiation. Regardless of neurotrophic factors, expression levels of the LP genes during the latent process (0-12 h), but not phosphorylation levels of ERK, always correlated with subsequent neurite extension length (12-24 h). Thus, the LP genes appeared to be the common decoders for neurite extension length regardless of neurotrophic factors, and they may function in distinct temporal and spatial manners during the latent process. Our findings provide molecular insight into the physiological meaning of the latent process as the preparation step for decoding information for future phenotypic change.

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4. Introduction

Cell fate, such as proliferation and differentiation, is determined by cell response to extracellular signals and requires a series of functionally distinct processes, including signal transduction, transcription, translation, and function of *de novo* proteins. Accordingly, sequential steps including signal transduction, transcription, and translation can be regarded as preparation steps for the future phenotype. Such a preparation step corresponds to a latent process in cell differentiation in PC12 cells (Chung et al., 2010) and to the G_1 phase in the cell cycle (Jones and Kazlauskas, 2001). Conceivably, the future phenotype is predetermined during the preparation step, such as the latent process.

PC12 cells, an adrenal chromaffin cell line, are a well-characterized model of nerve cells, responding to nerve growth factor (NGF) by differentiating into a sympathetic neuron-like phenotype (Burstein et al., 1982; Greene and Tischler, 1976). This differentiation is characterized by the extension of neuritis, the cessation of mitosis, and the acquisition of a neuronal phenotype such as electrical excitability and production of neurotransmitters. NGF induces differentiation via the receptor tyrosine kinase, TrkA, which activates downstream signaling molecules including, phosphatidylinositol 3-kinase (PI3K), cAMP-dependent protein kinase (PKA) and Ras/Raf/MEK/ERK (Segal and Greenberg, 1996). Treatment of PC12 cells with epidermal growth factor (EGF), which also stimulates another receptor tyrosine kinase, fails to induce differentiation but maintains PC12 cells proliferation (Huff et al., 1981). The distinct biological responses induced by NGF compared to EGF is intriguing since both growth factors activate receptor tyrosine kinases that are coupled to a similar set of

downstream signaling molecules. The ability of NGF to trigger neuronal differentiation instead of proliferation is thought to depend on its ability to activate ERK for long, sustained periods (Cowley et al., 1994; Marshall, 1995; Sasagawa et al., 2005; Vaudry et al., 2002b). These studies show that the temporal dynamics of intracellular signaling molecules are an important factor for cell fate decision.

On the other hand, in addition to NGF, cAMP-elevating agents, such as pituitary adenylate cyclase-activating polypeptide (PACAP) or forskolin, are capable of inducing differentiation and neurite extension (Deutsch and Sun, 1992; Richter-Landsberg and Jastorff, 1986). Although the primary effecter for cAMP is PKA, PACAP initiated a cAMP-dependent, PKA-independent activation of ERK (Gerdin and Eiden, 2007; Ravni et al., 2008).

The role of each signaling molecules in triggering PC12 cell differentiation is well established, the transcriptional program that is activated by those signaling, and is ultimately responsible for acquisition of a future neuronal phenotype, has not been as well characterized. The transcription of genes responsible for neuronal phenotype can be regarded as decoding process of information of future phenotype.

We previously found that NGF-dependent differentiation of PC12 cells can be divided into two processes: a latent process and a subsequent neurite extension process (Fig. 1A) (Chung et al., 2010). This finding suggested a timing-dependent action of NGF required for cell differentiation, proposing that NGF stimulation is required to induce two temporally separated differentiation processes: a first stimulation-driven latent and a second stimulation-driven extension processes. Prominent neurite extension was observed only during the extension process but not during the latent process. However, ERK and transcription activity during the latent process were required for

subsequent neurite extension. Therefore, the latent process corresponds to a preparation step for neurite extension. Continuous stimulation with NGF was not necessary to trigger the latent process; a pulse stimulation of only 1 h was sufficient to trigger the process. The latent process activity for the subsequent neurite extension was sustained for 12 h after stimulation and eventually decreased. The extension process showed rapid neurite extension and required ERK and PI3K activity but not transcription. Continuous stimulation with NGF during the extension process was required for the full extension of a neurite. We previously found candidate genes that are essential to the latent process. To identify genes specifically involved in the latent process, but not other differentiation processes, we used diverse patterns of stimulations to narrow down the candidate genes. We obtained total RNA samples for microarray assay at 3 h after stimulation because it was supposed that genes regulated at the 3 h time point should involve genes essential for the latent process. We used pulse stimulation with NGF or PACAP and step stimulation with NGF as positive controls and pulse stimulation with insulin as a negative control, since PACAP and NGF, but not insulin, can serve as a latent process-inducing stimulations. We selected genes that were regulated by these positive stimulants but not by insulin. Further, we also used inhibitors to identify genes that were specifically regulated by ERK, but not PI3K, because ERK, but not PI3K, are required for the latent process. Forty seven candidate genes were obtained by these assays.

In the present study, I checked NGF responsiveness and temporal expression patterns of candidate genes and examined what gene is involved in NGF-induced neurite extension by siRNA-mediated knockdown experiments. I identified three genes, *Metrnl*, *Dclk1*, and *Serpinb1a*, as the latent process (LP) genes that have induced expression during the latent process and are required for subsequent neurite extension. I

investigated which upstream factor is involved in the expression of the LP genes and the subcellular localization pattern of their products. The LP genes show distinct dependency of their expression on the duration of ERK activity and distinct spatial pattern. Overexpression of all LP genes together, but not that of each LP gene separately, cooperatively enhanced NGF-induced neurite extension. I examined the relationship between the latent and extension process by quantifying the LP genes expression levels during the latent process and neurite extension levels during the extension process, respectively. Neurotrophic factors that were able to trigger the latent process induced the expression of the LP genes. The expression levels of the LP genes during the latent process were positively correlated with the neurite extension length during the extension process, whereas the phosphorylation levels of ERK, which is an upstream regulator of the LP genes, was not correlated. Given that expression of the LP genes occurred during the latent period (0-12 h) and before the extension period (12-24 h), my findings demonstrate that the LP genes are common decoders of neurite extension length regardless of neurotrophic factors and cooperative enhancers of neurite extension.

5. Material and Methods

5-1. Cell culture

PC12 cells (kindly provided by Masato Nakafuku, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio) were cultured in a humidified 5% CO_2 atmosphere at 37°C in complete medium, Dulbecco modified Eagle medium supplemented with 5% horse serum, and 10% bovine calf serum (Nichirei, Tokyo, Japan). For stimulation, PC12 cells were plated on poly-L-lysine–coated 96-well plates (1.0×10^4 cell/well) in the complete medium for 24 h and then treated for the first stimulation with the complete medium in the presence or absence of the indicated doses of NGF (R&D Systems, Minneapolis, MN), PACAP (Sigma, Zwijndrecht, The Netherlands), forskolin (Sigma), EGF (Roche, Mannheim, Germany), and insulin (Sigma). For the second stimulation, PC12 cells first treated each neurotrophic factor for 12 h or 1 h followed by an 11-h interval, were uniformly switched to complete medium containing 50 ng/mL of NGF. Inhibitors for mRNA synthesis (50 nM 5,6-dichloro-1-8-D-ribofuranosylbenzimidazole (DRB) [Sigma]), TrkA (200 nM K252a [Sigma]), MEK (50 μM U0126 [Promega, Madison, WI]), or PKA (50 μM H-89 [Sigma]) were used as indicated in the respective figure legends for Fig. 5, 7, 8, 9 and 17. To wash out stimulants or inhibitors, the culture medium was replaced with an equal volume of complete medium four times.

5-2. Quantitative analysis of neurite length

PC12 cells (0.5×10^4 cells/well) were fixed with 10% formalin solution (Wako, Osaka, Japan) for 10 min. Cells were washed with phosphate-buffered saline (PBS) and incubated with 1 μg/mL Hoechst solution (Life Technologies, Carlsbad, CA) and 1 μg/mL CellMask (Life Technologies) in PBS for 1 h at room temperature, and then washed with PBS. Images were captured on a CellWoRx (Thermo Fisher Scientific, Rockford, IL). Using the CellMask signal as the neuronal cell image and the Hoechst signal as the nuclear image, the lengths of the neurites were measured by the NeuroTracer, NIH ImageJ plug-in (Pool et al., 2008), except that GFP-Mem, GFP containing a signal for localization to cellular membranes, was used as the neuronal cell image in plasmid-transfected cells. The length of neurites of cells treated with each stimulation condition was represented as the average neurite length of cells. At least three experiments were performed for each stimulation condition and their mean value was calculated.

5-3. qRT-PCR analysis

For reverse transcription-polymerase chain reaction (RT-PCR), total RNA was prepared from PC12 cells using Agencourt RNAdvance Tissue Kit according to the manufacturer's instructions (Beckman Coulter, Brea, CA). RNA samples were reverse transcribed by High Capacity RNA-to-cDNA Kit (Applied Biosystems, Carlsbad, CA) and the resulting cDNAs were used as templates for the quantitative real-time polymerase chain reaction (qRT-PCR). qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems); the primers are shown in Table 1. As an internal control for normalization, the β-actin transcript was similarly amplified using

the primers. Each primer set was designed using Primer Express software (Applied Biosystems). qRT-PCR was conducted using a 7300 Real Time PCR System (Applied Biosystems), and the data were acquired and analyzed by a 7300 System SDS software version 1.3.1.21 (Applied Biosystems). Three experiments of independent samples were performed for each primer set, and their mean value was calculated.

5-4. siRNAs and transfections

Twenty-four hours after plating, the cells were transfected with siRNAs by using Lipofectamin RNAiMAX transfection reagent according to the manufacturer's instructions (Life Technologies). As a positive control, siRNAs targeting *TrkA* were used. Block-iT Alexa Fluor Red Fluorescent Oligo (Life Technologies) with no predicted target site served as a negative control. Before stimulation, the transfected cells were incubated for 12 h. Target sequences for RNAi were designed by siDirect2, a highly effective and target-specific siRNA design software (Naito et al., 2009). The target sequences of siRNAs used are indicated in Table 1. The siRNA duplexes were chemically synthesized (Sigma).

5-5. Immunoblotting

Cell lysates were subjected to standard sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). After fractionation by SDS-PAGE and transfer to nitrocellulose membranes, the blots were reacted with antibodies for phosphorylated ERK1/2 (1:2000 dilution; Cell Signaling Technology [CST], Danvers, MA, #9101),

phosphorylated CREB (1:2000 dilution; CST, #9198), pan ERK1/2 (1:2000 dilution; CST, #9102), SERPINB1a (1:1000 dilution; Santa Cruz Biotechnology, sc-34305), DCLK1-long (1:1000 dilution; abcam, ab31704), or hemagglutinin (HA) epitope tag (1:1000 dilution; CST, #3724) followed by horseradish peroxidase-conjugated rabbit or goat IgG (GE Healthcare, Buckinghamshire, England). Chemiluminescence was detected with Immobilon Western (Millipore, Billerica, MA). The resulting image was captured on a luminescent image analyzer LAS-4000 (FUJIFILM, Tokyo, Japan). Signal intensity was quantified using Phoretix 1D (TotalLab Ltd, Newcastle upon Tyne, UK).

5-6. Rescue and overexpression experiment

The coding regions of rat *Metrnl*, *Carp/Ania-4*, and *Serpinb1a* cDNA, which I cloned from total RNA, were cloned into the vector pIRES-AcGFP1 (Clontech, Mountain View, CA) at the *Nhe*I and *Xho*I sites. I obtained a novel sequence of *Metrnl* by cloning and sequence determination. The novel sequence data of *Metrnl* have been deposited with the DDJB nucleotide sequence data banks and are available under the accession number AB646250. To trace the neurites of the transfected cells, AcGFP1 was replaced with GFP-Mem obtained from pAcGFP1-Mem (Clontech) in order to have high resolution of plasma membrane for detecting the leading edge of a neurite. As a control, a plasmid bearing only GFP-Mem was transfected. Silent point mutations in the siRNA-targeted regions were introduced by overlapping PCR (*Metrnl*, 5′-GAGAAGACCGGCGAGCTCAGG-3′; *Carp/Ania-4*, 5′-CTGATCGAGGTTAACGGCACA-3′; *Serpinb1a*, 5′-GCTCAACAAAAAGAATACCAA-3′; underlining indicates the nucleotides

mutated) to produce the rescue constructs with KOD -Plus- Mutagenesis Kit (TOYOBO, Osaka, Japan). The cells were co-transfected with the siRNA and each expression construct, or transfected with each expression construct by Lipofectamin 2000 reagent, according to the manufacturer's instructions (Life Technologies). The cells were fixed and stained as described in the Quantitative analysis of neurite length, except that the transfected cells were stained with anti-GFP antibody (Roche, Cat# 11814460001) in Can Get Signal (TOYOBO) for 1 h at room temperature and stained with Alexa Fluor 488-conjugated goat anti-mouse IgG (Life Technologies) in PBS for 1 h at room temperature. Using the GFP signal as the neuronal cell image and the Hoechst signal as the nuclear image, the lengths of the neurites were measured as described in the Quantitative analysis of neurite length.

5-7. Immunofluorescence staining for analysis of localization

To determine the subcellular localization of LP gene products, two tandem copies of the HA-tag sequence was engineered at the C terminus of METRNL, the N terminus of SERPINB1a, and the N and C terminus of CARP/ANIA-4 by Mutagenesis Kit for immunofluorescent detection using antibodies to the HA tag. A glycine-glycine linker was inserted between HA tag and LP gene sequences. The cells were transfected as described in Rescue and Overexpression experiment. For immunofluorescence staining, cells were fixed as described in the Quantitative analysis of neurite length, permeabilized by treatment with methanol for 30 min at 4°C. The cells were stained as described in the Rescue and overexpression experiment, except that anti-HA antibody (1:1600 dilution, CST, #3724) and Alexa Fluor 546-conjugated goat anti-rabbit IgG

(Life Technologies) were used as primary and fluorescent secondary antibody, respectively. For the analysis of localization of endogenous DCLK1-long, anti-DCLK1-long antibody was used as primary antibody.

6. Results

6-1. Identification of the LP genes expressed during the latent process and required for subsequent neurite extension

I aimed to identify the LP genes from among 47 candidates that were selected in our previous study (Chung et al., 2010). Among these 47 candidate genes, the primers of 30 genes could be designed (Table 1) and the expression of these genes was examined by using qRT-PCR. Twenty-two of the 30 genes were induced during the latent process in response to NGF stimulation (Fig. 1B-D). Temporal expression patterns of these genes fell into three groups: (1) transient expression with a peak at 3 h after the stimulation (four genes), (2) transient and sustained expression with a peak at 3 h and a duration of 12 h, respectively (12 genes), and (3) sustained expression with a duration of 24 h (six genes).

I examined whether 22 genes are essential for the neurite extension. I tested the effect of specific knockdown of 22 genes on NGF-induced neurite extension (Fig. 2A-G). The siRNAs targeting 3 of 22 genes significantly inhibited neurite extension; these genes were *Metrnl*, *Dclk1*, and *Serpinb1a*. I confirmed that expression of the three genes was suppressed by the addition of the siRNAs by qRT-PCR (Fig. 3A). Some of the siRNAs targeting other genes seemed to inhibit the neurite extension; however, this inhibition was not statistically significant. To confirm the specificity of the siRNAs, rescue experiments were performed by co-transfection with each siRNA and each plasmid construct expressing a siRNA-resistant version of *Metrnl* (see following text) or *Dclk1* (*Carp/Ania-4*, see following text) or *Serpinb1a* (Fig. 3B). *Metrnl* and *Dclk1*

rescue constructs partially rescued the inhibited neurite extension caused by the siRNA, while the *Serpinb1a* rescue construct did not show any statistically significant effect. The partial and nonsignificant effect of LP gene rescue constructs may have occurred because of the distinct expression profiles, e. g. distinct temporal patterns and intensity of the expressions, between endogenous and the expressed genes. Taken together, I identified the three genes as the LP genes because they were induced during the latent process and were essential for neurite extension. However, since the expression of some of the other 19 genes was not suppressed by siRNAs (Fig. 3A), I cannot exclude the possibility that some of them are also LP genes.

The gene product of *Metrnl* is a protein akin to METEORIN, a secreted protein expressed in undifferentiated neural progenitors (Nishino et al., 2004). I obtained a novel sequence of *Metrnl* by cloning and sequence determination for rescue and overexpression experiments (Fig. 4). *Dclk1* encodes a microtubule-associated protein and plays a role in neurogenesis and neuronal migration (Shu et al., 2006; Vreugdenhil et al., 2007). *Dlck1* has many alternative splicing variants including *Carp*/*Ania-4*, *Dclk1-short* and *Dclk1-long* (Schenk et al., 2007; Vreugdenhil et al., 1999). In this study, I used the common siRNA and primers for those variants of *Dclk1* except that cDNA of *Carp*/*Ania-4* was used for rescue and overexpression experiments (see also section 6-5). *Serpinb1a* is a member of a subgroup of the serine protease inhibitors (serpins) superfamily that is important in regulating several biologic processes, including neurogenesis (Parmar et al., 2002; Silverman et al., 2001).

I examined whether the LP genes are also required for PACAP- and forskolin-induced latent process. Although NGF-induced latent process required transcriptional activity, whether PACAP- and forskolin-induced latent process requires it

is unknown. I examined and confirmed that transcriptional activity is required for PACAP- and forskolin-induced latent process (Fig. 5). Unlike NGF, the restricted inhibition of transcriptional activity for pulse stimulation with PACAP and forskolin did not markedly suppressed neurite extension (Fig. 5B-D; treatment pattern 1 vs 2), whereas the transcriptional inhibition for the whole latent process showed a prominent suppression of neurite extension (Fig. 5B-D; treatment pattern 1 vs 6). The transcriptional inhibition for the extension process showed a weaker suppression of neurite extension than that for the latent process (Fig. 5B-D; treatment pattern 4 vs 6). Surprisingly, the transcriptional inhibition from 3 to 12 h during NGF, PACAP, and forskolin- induced latent process caused prominent suppression of neurite extension (Fig. 5B-D; treatment pattern 1 vs 3). These results represent that transcriptional activity is also required for PACAP and forskolin-induced latent process. To evaluate the knockdown effect of PACAP- or forskolin-induced expression of the LP genes on neurite extension, the siRNA-treated cells were treated with PACAP or forskolin for 12 h to induce the latent process and then with NGF for an additional 12 h to induce neurite extension. The siRNAs targeting the LP genes significantly suppressed the expression of the LP genes (Fig. 6A,B) and inhibited the PACAP- and forskolin-induced neurite extension, although the siRNA targeting *Metrnl* did not significantly inhibit neurite extension in response to forskolin (Fig. 6C,D). These results indicate that the LP genes are commonly essential for the NGF-, PACAP-, and forskolin-induced latent process.

6-2. Expression of LP genes in response to pulse or step stimulation with NGF

The time courses of *Metrnl*, *Dclk1*, and *Serpinb1a* expression in response to step stimulation with NGF are classified into temporal expression pattern groups 1, 2, and 3, as described at the last section, respectively (Fig. 1B-D and 7A-D). Because pulse stimulation with NGF induces the latent process similarly to a step stimulation with NGF (Chung et al., 2010), I examined whether pulse stimulation with NGF can also induce expression of the LP genes (Fig. 7A-D) and found that it could (Fig. 7B-D). This indicated that pulse stimulation with NGF is as efficient at triggering expression pattern of the LP genes as step stimulation with NGF. We previously found that transcriptional activity is required in the initial 3 h after stimulation (Chung et al., 2010) and I confirmed it (Fig. 5B). Consistent with this notion, expression of all LP genes increased within 3 h, which is the crucial phase of transcription. The peak level of *Metrnl* expression by pulse stimulation with NGF, however, was significantly higher than that by step stimulation with NGF, suggesting that NGF stimulation after 1 h may negatively regulate the expression of *Metrnl* (Fig. 7B).

6-3. Requirement of ERK activity for induction and maintenance of the expression of LP genes

Persistent activation of ERK is required for the NGF-induced differentiation of PC12 cells (Cowley et al., 1994; Marshall, 1995; Sasagawa et al., 2005; Vaudry et al., 2002b). We previously found that ERK activity is essential for the latent process (Chung et al., 2010). Therefore, I examined ERK activation by step and pulse stimulations with NGF and found that activation of ERK is similarly sustained for at least 12 h by both types of stimulation (Fig. 7E,F).

I next examined whether ERK activity is required for expression of LP genes. Prior addition of U0126, a MEK inhibitor, almost completely inhibited ERK activation (Fig. 8A,B) and concomitantly suppressed LP gene expression (Fig. 8C-E). I investigated whether sustained ERK activation is needed for the expression of LP genes by the addition of U0126 at 1 or 3 h after NGF stimulation. The expression of *Metrnl* was not suppressed (Fig. 8C); however, when ERK activity was inhibited after 1 h, the peak level of *Metrnl* at 3 h was significantly higher than that in the absence of U0126. This result suggests that the expression of *Metrnl* after 1 h may be negatively regulated by sustained ERK activity. The expression of *Dclk1* was suppressed when ERK activity was inhibited after 1 h, whereas it was not suppressed when ERK activity was inhibited after 3 h (Fig. 8D). The expression of *Serpinb1a* was suppressed when ERK activity was inhibited after 1 h, whereas it was suppressed only at 24 h after the NGF stimulation when ERK activity was inhibited after 3 h (Fig. 8E). I confirmed the inhibition of ERK activity by the addition of U0126 at 1 h after the NGF stimulation (Fig. 8B). Taken together, expression of all LP genes showed distinct dependency on the duration of ERK activity. ERK activation during the initial 1 h was sufficient to induce *Metrnl* expression. In contrast, the expression of *Dclk1* required ERK activation for 3 h and expression of *Serpinb1a* required ERK activation for longer than 3 h. Sustained ERK activation is required not only for induction but also for maintenance of the expression of *Dclk1* and *Serpinb1a*. I also examined the expression of the LP genes by adding K252a, an inhibitor of the neurotrophin receptor Trk, and found that like U0126, K252a completely suppressed LP gene expression (Fig. 8F-H) and ERK activation by NGF (Fig. 8B), indicating that NGF- and TrkA-mediated expression of LP genes mainly depends on ERK activity. In contrast, the addition of H-89, a protein kinase A (PKA)

inhibitor, did not inhibit the expression of the LP genes under the conditions in which phosphorylation of CREB (Fig. 9A-D), one of the PKA substrates, was inhibited, indicating that PKA was not involved in the expression of the LP genes. This result is consistent with the previous report that PKA is not necessary for the induction of NGF-mediated gene expression of *Egr1* and *GAP-43* (Ginty et al., 1991) or neurite extension (Yao et al., 1998), while ERK is necessary for both (Cowley et al., 1994; Marshall, 1995). I also confirmed that U0126 inhibited NGF-induced neurite extension and H-89 did not (Fig. 10).

I next investigated how LP genes are transcriptionally regulated. I examined the effect of siRNAs-mediated knockdown of the immediate early genes, such as *c-Fos* and *c-Jun* (Eriksson et al., 2007), on NGF-induced expression of the LP genes (Fig. 11). The expression of the LP genes was not affected under the conditions in which the expression of *c-Fos* and *c-Jun* were suppressed by 64% at 1 h (*c-Fos*) and 51% at 1 h (*c-Jun*), respectively (Fig. 11A-E). I further examined the effect of siRNA-mediated knockdown of *c-Fos* and *c-Jun* on NGF-induced neurite extension (Fig. 11F). The no effect of them on neurite extension was observed in this condition although the knockdowns of *c-Fos* and *c-Jun* were thought to decrease the percentage of cells with neurite (Eriksson et al., 2007). In addition, I examined the knockdown effects of other members of the immediate early genes, such as *Egr1* (Harada et al., 2001; Ravni et al., 2008), *Fra1* and *JunB* (Mullenbrock et al., 2011), on NGF-induced expression of the LP genes (Fig. 11G-L). Under the conditions in which the expression of *Egr1*, *Fra1* and *JunB* were suppressed by 53% at 1 h (*Egr1*), 67% at 3 h (*Fra1*), and 72% at 1 h (*JunB*), respectively, *Dclk1* expressions were inhibited (Fig. 11K). Moreover, when the expression of *Fra1* was suppressed, *Serpinb1a* expression was also inhibited (Fig. 11L).

These results indicate that *Egr1*, *Fra1* and *JunB* are required for *Dclk1* expression and that *Fra1* is required for *Serpinb1a* expression. This also highlights that expressions of LP genes were differently regulated by the immediate early genes.

6-4. Subcellular localization of the LP gene products

To investigate the potential functions of the LP genes, I analyzed the localization of the LP gene products, METRNL, CARP/ANIA-4, and SERPINB1a, in PC12 cells. Commercially purchased SERPINB1a antibody was available for Western blotting but not for immunostaining (Fig. 12). I raised rabbit polyclonal antibodies against the LP gene products, but the antibodies did not specifically detect the endogenous proteins (data not shown). Therefore, I transfected a plasmid bearing hemagglutinin (HA)-tagged LP genes into PC12 cells and analyzed the localization of the LP gene products (Fig. 13). METRNL showed diffusible distribution throughout the cell body except for the nucleus before NGF stimulation (Fig. 13A) and localized at the terminuses of neurites after NGF stimulation (Fig. 13B, arrowhead). CARP/ANIA-4 showed diffusible localization throughout the cell body including the nucleus before NGF stimulation (Fig. 13C), but was not markedly localized at the terminuses of neurites after NGF stimulation (Fig. 13D, arrowhead). SERPINB1a showed diffusible localization throughout the cell body including the nucleus before NGF stimulation (Fig. 13E) and localized at the terminuses of neurites after NGF stimulation (Fig. 13F, arrowheads). I confirmed the specificity of anti-HA antibody for the HA-tagged METRNL and SERPINB1a by Western blotting (Fig. 14). Since the product size of *Carp/Ania-4* is small (approx. 10 kDa), I couldn't detect the band of CARP/ANIA-4. In

addition, I used a commercially purchased DCLK1 antibody which detects two splicing variant products of *Dclk1* (*Dclk1-long*A and B) for immunostaining (Fig. 15A). DCLK1-longs showed diffusible localization throughout the cell body including the nucleus before NGF stimulation (Fig. 15B) and localized at the terminuses of neurite after NGF stimulation (Fig. 15C, arrowhead). The distinct localization of METRNL, DCLK1(CARP/ANIA-4 and DCLK1-long) and SERPINB1a suggest that the LP gene products are involved in distinct functions and that at the same time the latent process accompanied the three distinct functions mediated by the LP gene products, some of which may need the latent period to be completed. In addition, the localization of METRNL, DCLK1-long and SERPINB1a at the terminuses of neurites after the NGF stimulation suggests that the LP gene products are also involved in the neurite extension process.

6-5. Overexpression of all LP genes cooperatively enhances NGF-induced neurite extension

I examined whether overexpression of the LP genes enhance neurite extension. I used *Carp*/*Ania-4*, one of the alternative splice variants of *Dclk1* (Schenk et al., 2007; Vreugdenhil et al., 1999), for the overexpression experiment because *Carp*/*Ania-4* but not *Dclk1-short*, another alternative splice variant, was commonly induced by NGF, PACAP, and forskolin (Fig. 16). Overexpression of each LP gene, all LP genes, and control showed similar background levels of neurite length before NGF stimulation, indicating that overexpression of the LP genes themselves did not induce neurite extension (Fig. 17A). Overexpression of all LP genes showed a significant enhancement

of NGF-induced neurite extension compared to the control, while overexpression of each LP gene separately did not show a significant enhancement of NGF-induced neurite extension. Unlike the non-transfected conditions, greater neurite extension was observed during the first 12 h than the latter even in the control plasmid-transfected cells (Fig. 17A). Therefore, I cannot conclude that the co-overexpression of the LP genes is sufficient to bypass the latent process. I further confirmed the involvement of each LP gene in the cooperative enhancement of NGF-induced neurite extension by testing the effect of specific knockdown of the LP genes on the cooperative neurite extension (Fig. 17B). I found that siRNA targeting each LP gene significantly inhibited the cooperative neurite extension, but the genes' effects were partially restored by the expression of siRNA-resistant constructs. This indicates that each LP gene is indispensable for the cooperative enhancement of NGF-induced neurite extension.

6-6. Induction of the LP genes and neurite extension length by various neurotrophic factors

I measured the LP gene expression during the latent period (0-12 h) and neurite extension length during the extension period (12-24 h) in response to other neurotrophic factors, including PACAP, forskolin, epidermal growth factor (EGF), and insulin (Fig. 18). I used NGF as the second stimulation to evaluate the effect of the neurotrophic factors on the latent process. The step stimulation with PACAP and forskolin, but not EGF or insulin, induced neurite extension length (cf. neurite extension length [#] in Fig. 18A; Fig. 18B), and PACAP and forskolin were more potent for neurite extension length than NGF. In addition, the pulse stimulations with PACAP and forskolin, but not EGF or

insulin, similarly induced neurite extension length (Fig. 18B). This result indicates that PACAP and forskolin can induce the latent process.

I examined whether the neurotrophic factors induce the expression of LP genes. Step stimulation with PACAP and forskolin, which triggered the latent process, also induced LP gene expression (Fig. 18C). Likewise for neurite extension length, PACAP and forskolin were more potent for induction of LP gene expression than NGF. In contrast, step stimulation with EGF and insulin, which did not trigger the latent process, did not induce LP gene expression. Similar results were obtained when pulse stimulation with neurotrophic factors was used (Fig. 18C).

Taken together, these results indicate that PACAP and forskolin are capable of inducing both the latent process and the expression of LP genes, whereas EGF and insulin are not. They also suggest that LP genes may trigger the PACAP- and forskolin-dependent latent process that is required for neurite extension.

6-7. Expression of the LP genes, but not ERK phosphorylation, correlates with neurite extension length

I examined the quantitative relationship between the LP genes and neurite extension length. I measured the time course of the expression of LP genes, ERK phosphorylation, and neurite extension length in a dose-dependent manner for NGF, PACAP, and forskolin (Fig. 19). ERK phosphorylation showed a graded response to NGF (Fig. 19A). While LP gene expression showed a switch-like response; higher concentrations of NGF induced LP gene expression, whereas lower concentrations did not (Fig. 19B,C). In contrast, PACAP induced the expression of LP genes in a

dose-dependent manner, which appeared as a graded response (Fig. 19B,C). Similarly, forskolin induced the LP gene expression in a dose-dependent manner, which appeared as a graded response (Fig. 19B,C). Interestingly, although PACAP and forskolin induced smaller phosphorylation levels of ERK than NGF, PACAP and forskolin induced larger expression levels of the LP genes than NGF (Fig. 19A,B). This led us to examine the dependency of LP gene expression on ERK activity in response to PACAP and forskolin. The addition of U0126 inhibited LP gene expression induced by NGF (Fig. 8C-E), whereas U0126 inhibited only the expression of *Metrnl* but not that of *Dclk1* or *Serpinb1a* induced by PACAP and forskolin (Fig. 20A-F). This indicates that PACAP and forskolin induced expression of *Dclk1* and *Serpinb1a* in an ERK-independent manner, whereas NGF induced expression of *Dclk1* and *Serpinb1a* in an ERK-dependent manner. Although the expression of *Metrnl* depended on ERK activity, PACAP and forskolin induced larger *Metrnl* expression and smaller ERK phosphorylation, compared with NGF (Fig. 19A,B and C), suggesting that *Metrnl* expression is regulated not only by ERK but also by other signaling molecules at least in response to PACAP and forskolin. I also examined the effect of H-89 on the PACAP- or forskolin-induced expression of the LP genes (Fig. 20G-L). The expression of *Metrnl* was partially suppressed, while that of *Dclk1* was not affected and that of *Serpinb1a* was enhanced. Taken together with the effect of H-89 on NGF-induced LP gene expression (Fig. 9), the expression of the LP genes show a distinct PKA dependency. Furthermore, addition of U0126 has been reported to inhibit PACAP- and forskolin-induced neurite extension (Hansen et al., 2003; Ravni et al., 2008), which was also confirmed in our experiment (Fig. 10). This indicates that ERK activity is also essential for PACAP- and forskolin-induced neurite extension. However, phosphorylation levels of ERK do not

seem to correlate with expression of the LP genes or neurite extension (see following text). It is noteworthy that, despite the different dependencies on ERK phosphorylation, the shape of time courses of the LP genes appeared similar regardless of the neurotrophic factors and the concentrations, an exception being that *Metrnl* and *Dclk1* expression induced by PACAP and forskolin showed more sustained expression than that induced by NGF.

Pulse stimulation with these neurotrophic factors induced phosphorylation and expression patterns similar to the step stimulations, although the expression levels induced by pulse stimulation were slightly weaker than those induced by step stimulation (Fig. 19E-H).

The time course of the neurite extension depends on the first stimulation (Fig. 21A). I defined the effect of the first stimulation on the neurite extension length during 12-24 h as the neurite extension length because the neurite extention length, which is a difference of neurite extension length during 12-24 h between treated and untreated cells with the first stimulation, was the most potent among those during 12-18, 12-24 and 12-36 h (Fig. 21B). The neurite extensions during 12-36 h of cells which were treated with the first stimulation were as long as that of untreated cells. Furthermore, I found that neurite extension was observed only during the late phase (18-24 h) but not during the early phase (12-18 h) of the extension period (Fig. 22), indicating that the effect of the LP genes for neurite extension was evoked with a 6-h lag during the extension period.

The dose responses of the neurite extension length induced by both the step and pulse stimulations with the neurotrophic factors appeared similar to those of expression levels of the LP genes but not those of phosphorylation levels of ERK (Fig. 19C,D and

G,H). This result led us to examine the quantitative relationship of ERK phosphorylation and LP gene expression with the neurite extension length in response to all stimulation patterns used; step and pulse stimulations with NGF, PACAP and forskolin and step stimulation with EGF and insulin. Despite the different shapes of the time courses of the LP genes, the integrated expression levels of each LP gene during the latent process were positively correlated with the neurite extension lengths during the extension period (*Metrnl*, r = 0.81; *Dclk1*, r = 0.74; and *Serpinb1a*, r = 0.76; Fig. 23A-C). In contrast, the integrated phosphorylation levels of ERK during the latent process were not correlated with the neurite extension lengths (r = 0.20; Fig. 23D). When the peak amplitudes of the LP genes and ERK phosphorylation were used instead of their integrated levels, similar results were obtained (Fig. 23E-H). Thus, the LP genes appeared not only to be qualitatively required for the latent process, but also to always quantitatively show a correlation with neurite extension length regardless of the neurotrophic factors. Given that expression of the LP genes occurred during the latent period (0-12 h) and neurite extension was particularly induced during the late extension period (18-24 h), information of neurite extension length is likely to be decoded by the expression levels of the LP genes before the extension process starts. Despite ERK being an essential molecule for neurite extension, phosphorylation levels of ERK were not correlated with neurite extension length. This means that essential molecules for neurite extension such as ERK are not necessarily decoders of information of neurite extension length. In addition, I measured the time course of the expression of the immediate early genes, such as *c-Fos*, *c-Jun* and *Egr1*, in a dose response manner for NGF, PACAP and forskolin and in response to EGF and insulin and measured the integrated expression levels of them during the latent process (Fig. 24).The dose responses of the neurite

extension length induced with the neurotrophic factors appeared slightly similar to those of expression levels of *c-Fos* but not those of expression levels of *c-Jun* and *Egr1*. I also examined whether their expression levels during the latent process were correlated with the neurite extension lengths during the extension period (Fig. 25). In the knockdown experiment, *c-Fos* expression did not affect the LP gene expression and neurite extension (Fig. 11C-F), but it showed a correlation with neurite extension length ($r = 0.72$; Fig. 25A). The expression of *c-Jun* and *Egr1* showed no and a weak correlation with neurite extension length, respectively ($r = -0.17$ and 0.52; Fig. 25B,C). I observed the immediate early gene expression only in response to step stimulation because their expression have an acute and high peak at 1 h. These results represent that the expression levels of stimulant-responsive genes did not always correlate with neurite extension length.

7. Discussion

I identified *Metrnl*, *Dclk1*, and *Serpinb1a* as LP genes. Overexpression of all LP genes but not expression of each LP gene individually showed a significant enhancement of neurite extension. The LP genes, *Metrnl1*, *Dclk1*, and *Serpinb1a*, appeared to be cooperative enhancers of neurite extension. The expression levels of the LP genes during the latent process were positively correlated with neurite extension length during the extension process. Although ERK activity was required for LP gene expression, ERK activity during the latent process was not correlated with neurite extension length during the extension process. Regardless of the neurotrophic factors and ERK dependency, the expression levels of LP genes always correlated with neurite extension lengths. Because expression of LP genes occurs before neurite extension, they are considered common decoders of information of neurite extension length regardless of neurotrophic factors and upstream signaling pathway (Fig. 26).

How the LP genes function during the latent process and regulate subsequent neurite extension remains unknown. Several early studies of relevant proteins suggest their potential roles, but a role for *Metrnl* has not yet been reported. However, the N-terminus of METRNL shows homology with that of METEORIN, a secreted protein that is involved in the development of the nervous system and in glial cell differentiation and axonal extension (Nishino et al., 2004; Surace et al., 2009). METRNL might be involved in neurite extension as a secreted protein in a similar manner. It has been reported that *Dclk1* is one of the NGF-responsive genes whose expression is enhanced by SH2B1b, an adaptor protein for TrkA in PC12 cells (Chen et al., 2008), and that *Dclk1* was induced during the NGF-induced cell differentiation in

PC12 cells (Dijkmans et al., 2009; Dijkmans et al., 2008). *Dclk1-long*, a product of the alternative splice variants of *Dclk1*, is a microtubule-associated active protein kinase expressed in neurites (Burgess and Reiner, 2000). *Carp/Ania-4* has been reported to be involved in apoptosis and tubulin polymerization *in vitro* (Schenk et al., 2007) and in seizures and long-term potentiation *in vivo* (Schenk et al., ; Vreugdenhil et al., 1999; Wibrand et al., 2006). Thus, the DCLK1 proteins might direct the latent process of neural cell differentiation by controlling tubulin formation or by modulating cell viability and subsequent synaptic plasticity (Kawaai et al., 2010; Shu et al., 2006; Vreugdenhil et al., 2007). Expression of *Serpinb1a* has also been reported as induced in response to NGF- and PACAP-induced cell differentiation in PC12 cells (Ravni et al., 2008). The serpins are a superfamily of proteins with a diverse set of functions, including inhibition of serine proteases (Irving et al., 2000; Silverman et al., 2001). *Serpinb1a* belongs to clade B of the superfamily and encodes neutrophil elastase inhibitor (Benarafa et al., 2002; Remold-O'Donnell et al., 1992). Possible biologic roles of *Serpinb1a* in cancer metastasis and the immune system were reported recently (Tseng et al., 2009), but a role for SERPINB1a in neurogenesis is unclear. Neuroserpin, another clade, is implicated in the regulation of neurite extension in NGF-induced differentiation in PC12 cells (Parmar et al., 2002), and SERPINB1a might similarly contribute to neurite extension in cell differentiation. In this study, I identified three genes, *Metrnl*, *Dclk1*, and *Serpinb1a*, as the LP genes that are required for the latent process of cell differentiation in PC12 cells. *Metrnl*, *Dclk1*, and *Serpinb1a* are involved in different biological processes. Given the LP genes' different temporal expression patterns, the distinct spatial localization of their products, and the cooperativity for

enhancement of neurite extension, our results suggest that at least three temporally, spatially, and functionally distinct steps are cooperatively required for the latent process.

Although the expression pattern of *Metrnl in vivo* is unknown, it has been reported that *Dclk1* was expressed in the active region of neurogenesis such as the external granule layer and internal granule layer in the cerebellum and the subventricular and ventricular zones in the neocortex (Shu et al., 2006). In contrast, the expression of *Carp/Ania-4* is highly up-regulated by kainite-induced seizures in the hippocampus (Vreugdenhil et al., 1999) and by adrenalectomy-induced apoptosis in the dentate gyrus (Schenk et al., 2007). *Serpinb1a* was identified as a striatally expressed gene from a genome-scale mapping of expression for a mouse brain section (Chin et al., 2007). Taken together, the expression patterns of the LP genes in the brain suggest they have potential roles in neurogenesis and higher order brain function. Further study will clarify the *in vivo* role of the LP genes in the latent process in cell differentiation and brain functions.

It is possible that other LP genes are yet to be identified. Gene expression accompanied by cell differentiation induced by NGF, PACAP, forskolin, and dibutyryl cAMP has been studied intensively (Dijkmans et al., 2008; Ng et al., 2009; Ravni et al., 2008; Vaudry et al., 2002a), and the neurotrophic factor-responsive genes that were highlighted potentially involve LP genes. LP genes might be some of the many genes whose expression is induced by neurotrophic factors and correlated with neurite extension length. It has been reported that transcriptional activation has a ripple effect (Ebisuya et al., 2008). Thus, it is conceivable that the genes show similar expression patterns. The requirement for neurite extension and the correlation of expression levels during the latent process with neurite extension length are necessary to identify LP

genes. Although ERK is required for neurite extension, the phosphorylation levels of ERK did not correlate with neurite extension length. It is possible that ERK activity might have a nonlinear relationship with neurite extension length.

Although ERK regulated expression of the LP genes, at least by stimulation with NGF, ERK itself did not act as a decoder. *Metrnl* depends on ERK activity regardless of neurotrophic factors. *Dclk1* and *Serpinb1a* were induced by NGF in an ERK-dependent manner, PACAP and forskolin induction occurred in an ERK-independent manner. These results show that expression of all the LP genes correlates with neurite extension length despite a difference in the time course of the LP genes. This implies that the LP genes share common upstream molecules and may be an initial decoder for neurite extension length. One candidate for the common upstream regulators of the LP genes is a protein of immediate early genes (Hazzalin and Mahadevan, 2002). Although it has been reported that *c-Fos* and *c-Jun* expression are required for NGF-induced neurite extension (Eriksson et al., 2007), those expression were not needed for the expression of the LP genes. It has been reported that *Egr1* is required for NGF- and PACAP-induced PC12 differentiation (Harada et al., 2001; Ravni et al., 2008) and that AP-1 family members, such as *Fra1* and *JunB*, bind to regulatory region of the preferentially NGF-induced genes including *Dclk1* (Mullenbrock et al., 2011). Consistent with this, I confirmed that *Egr1*, *Fra1* and *JunB* are involved in regulation of *Dclk1* expression and that *Fra1* is involved in regulation of *Serpinb1a* expression. This result suggests that the LP genes do not share a common immediate early gene(s) for their expression. It is possible that other immediate early genes might be a common upstream regulator(s) for the LP genes. Correlation of the expression

levels of the immediate early genes with neurite extension length and involvement in LP gene induction is necessary to address this issue in the future.

In addition, I also have to consider the importance of the temporal pattern of expression. In this study, I tried to inhibit or restore functions of genes which have a stimulation-responsive expression, but, these procedures were not adjusted to inhibit them during a restricted phase or to recapitulate the endogenous expression pattern. I recall the partial and nonsignificant effect of LP gene rescue constructs (Fig. 3B and 17B). Opposite to past reports, in our experiment, inhibition of *c-Fos* and *c-Jun* did not affect NGF-induced neurite extension, which may due to the timing of assay after transfection. Molecularly and temporally specific analysis will be needed to discuss a function of some genes during the latent process.

It has also been shown that PC12 cells, primed by NGF pretreatment for 1 week, do not require new RNA synthesis to regenerate neurites and to develop neurites more rapidly than those without NGF pretreatment (Burstein and Greene, 1978; Greene et al., 1982). Although the time scale is different between the latent process in this study (12 h) and the priming effect in the previous study (1 week), both processes similarly require de novo gene expression. Urokinase plasminogen activator receptor (UPAR)/plasminogen activator, urokinase receptor (PLAUR) has been identified as one of the essential genes for the priming effect (Farias-Eisner et al., 2001; Farias-Eisner et al., 2000) and is likely to be important for NGF-induced differentiation in PC12 cells (Chen et al., 2008). *Plaur* was selected as one of the candidate LP genes in this study; however, siRNA targeting of *Plaur* did not show an inhibitory effect (Fig. 2A). However, I cannot exclude the possibility that *Plaur* is an LP gene because I did not succeed in the suppression of mRNA of *Plaur* by siRNA (Fig. 3A).

The 22 genes that I selected as candidate LP genes were classified into three groups in terms of a temporal pattern (Fig. 1B-D). The expression pattern of each LP gene belonged to a distinct group. The expression of LP genes depends on ERK activity with a distinct duration, which may lead to a distinct temporal pattern of expression. The dependency on ERK activation duration might be caused not only by direct regulation by ERK, such as phosphorylation of a target factor, but also by the widespread intracellular environment created by the nuclear localization of ERK (von Kriegsheim et al., 2009). Sustained ERK activation is required not only for induction but also for maintenance of *Dclk1* and *Serpinb1a* expression. Since NGF induces sustained ERK activation and stabilizes specific mRNAs (Murphy et al., 2002), a balance between transcription activity and stabilization of mRNA by ERK might cause the temporal pattern seen with LP gene expression. To our surprise, the temporal patterns of LP gene expression induced by PACAP and forskolin were similar to that induced by NGF despite the differences in ERK dependency. An intriguing possibility is that some mechanisms ensure the robustness of the temporal expression pattern even if upstream signaling pathways are different. By that type of mechanism, LP genes are likely to serve as common decoders of neurite extension length despite upstream dependency.

8. Conclusion

The aim of this thesis is to disclose the underlying mechanism of latent process for cell differentiation from the point of view what genes expression during the latent process are responsible for triggering cell differentiation and acquisition of future neuronal phenotype, e. g. neurite extension, during extension process. I found that the expression levels of three genes, *Metrnl*, *Dclk1* and *Serpinb1a*, during the latent process are positively correlated with neurite extension levels during extension process. I proposed that these genes, referred as the latent process (LP) genes, are regarded as decoders of information of cell differentiation.

On the other hand, some upstream factors should encode information of cell differentiation which the LP genes decode. By elucidating those upstream regulators of the expression of the LP genes, the encoding mechanism of information of neurite extension length will be revealed. Furthermore, although the temporal dynamics of intracellular signaling molecules is an important factor for cell differentiation in PC12 cells, it is unclear whether the temporal expression patterns of the LP genes are the important factor. It is interesting to address these issues in the future.

In the future, we will use the LP genes as common decoders of neurite extension length and model the neurotrophic factor-dependent expression of the LP genes to elucidate the mechanism of specific induction of cell fate decisions in PC12 cells.

9. Figures

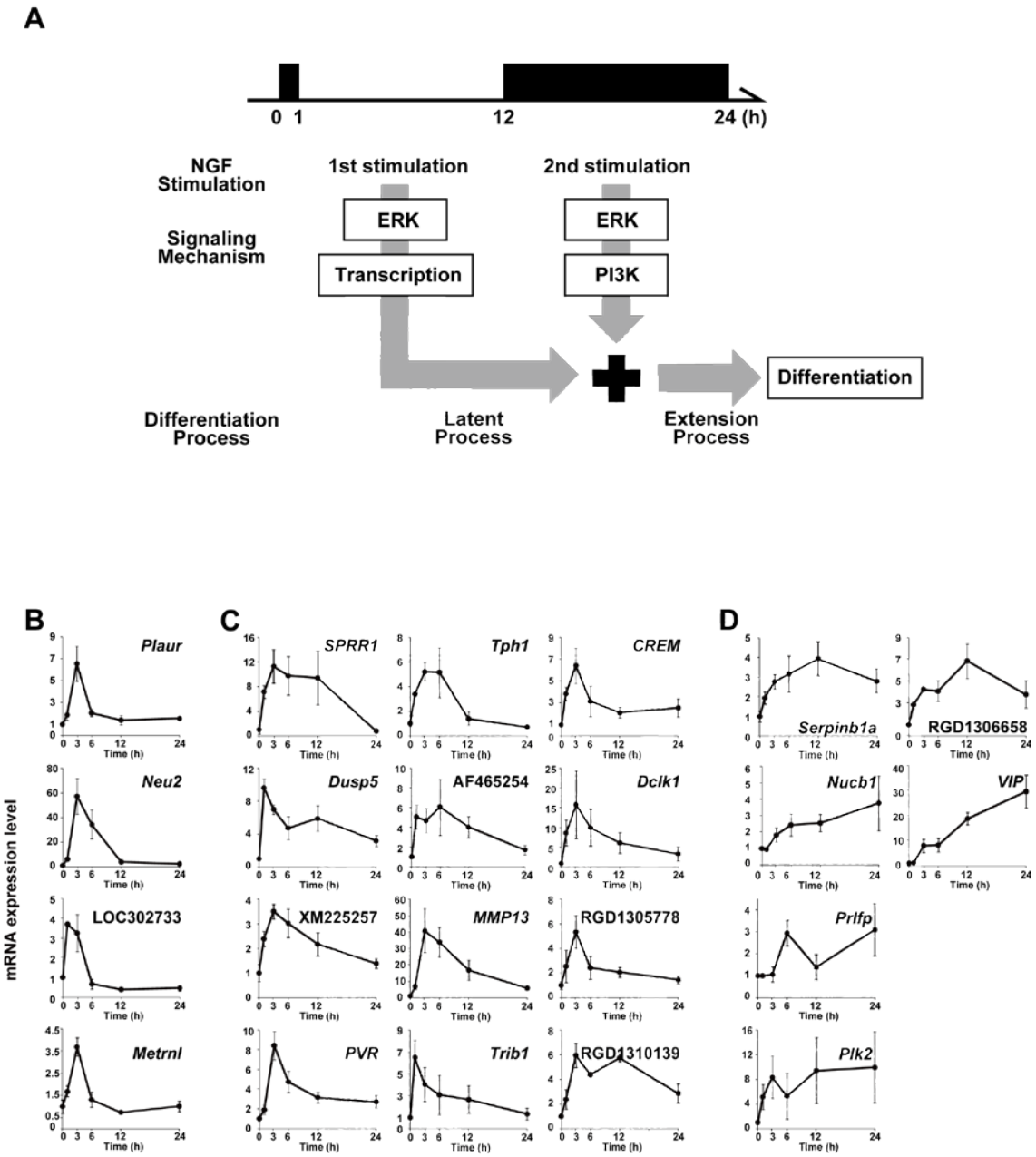


Fig. 1. Cell differentiation in PC12 cells possesses a latent process prior to neurite extension and the time profiles of the expression of the LP gene candidates

(A) We previously reported that NGF induces cell differentiation via two temporally and mechanistically distinct processes, the latent process and the extension process (Chung et al., 2010). Neurite extension occurs only in the extension process, not in the latent process. The latent process is driven by the first transient NGF stimulation via ERK and

transcription activities, whereas the extension process is driven by the second sustained NGF stimulation via ERK and PI3K activities. In this study, the genes essential for the latent process were identified from the candidates obtained in a comparative microarray (Chung et al., 2010). (B-D) Time courses of expression of 22 candidate LP genes in response to NGF (50 ng/mL). Temporal expression patterns were categorized into three groups: (1) transient expression with a peak at 3 h (four genes; B); (2) transient and sustained expression with a peak at 3 h and a duration of 12 h, respectively (12 genes; C); and (3) sustained expression with a duration of 24 h (six genes; D).

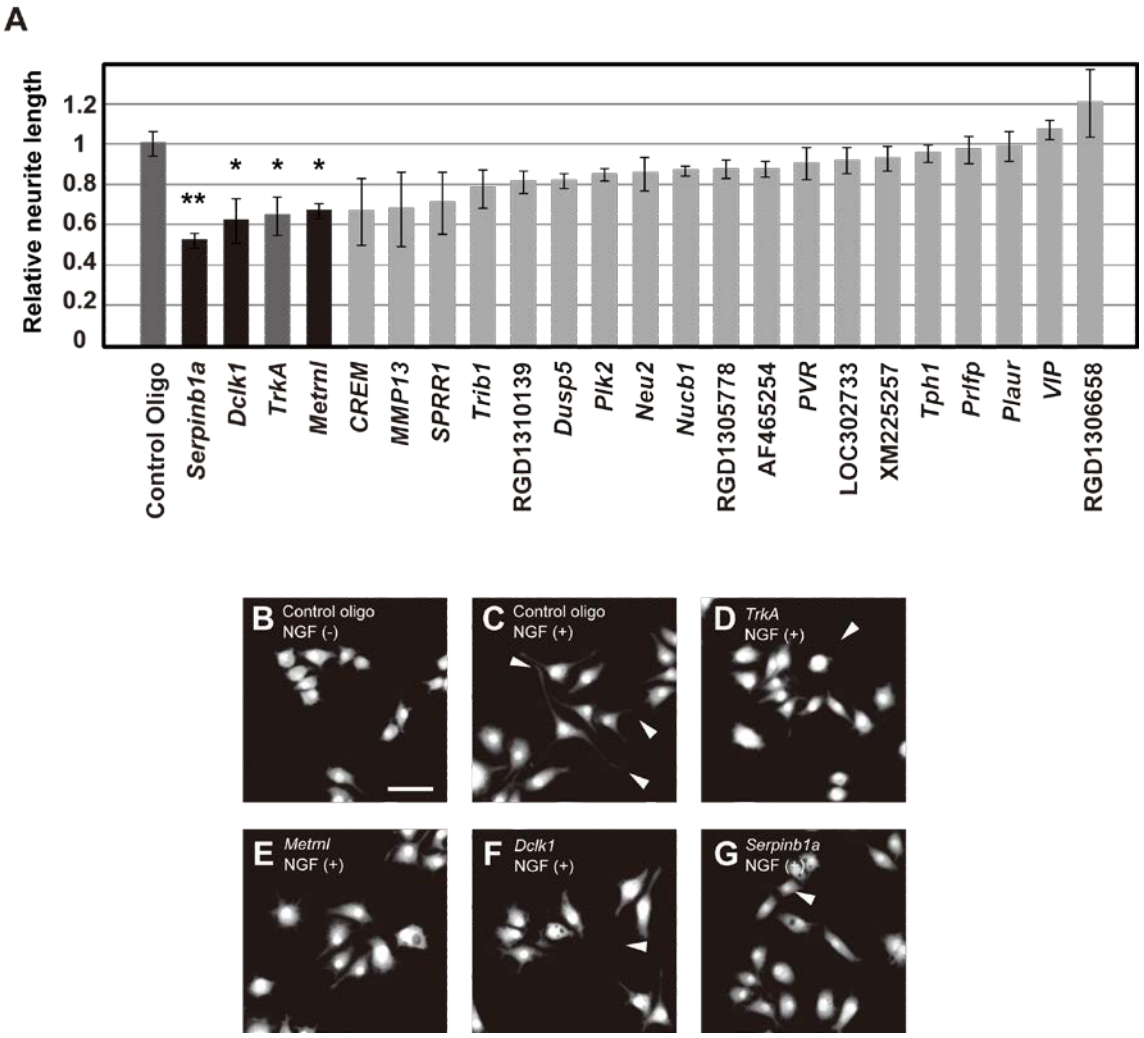


Fig. 2. Effects of siRNA-mediated knockdown of LP gene candidates on NGF-induced neurite extension

(A) PC12 cells were treated with the indicated siRNA for 12 h and then subjected to differentiation by NGF (50 ng/mL) for 24 h. The relative neurite lengths of the cells transfected with the indicated siRNA compared to that of cells transfected with negative control siRNA are shown. As a positive control, siRNAs targeting *TrkA* was used. The siRNA targeting *Metrnl*, *Dclk1*, and *Serpinb1a* significantly inhibited neurite extension. The values are shown as means ± s.e.m. (n = 3). The Student *t* test was used to evaluate the statistical significance of differences (**$P < 0.01$,*$P < 0.05$). See also Fig. 3A and Table 1. (B-G) The transfected cells with the indicated siRNA were incubated in the absence (B) or the presence (C-G) of NGF (50 ng/mL) for 24 h. Bar length (see B), 50 μm. The terminuses of neurites are highlighted with arrowheads.

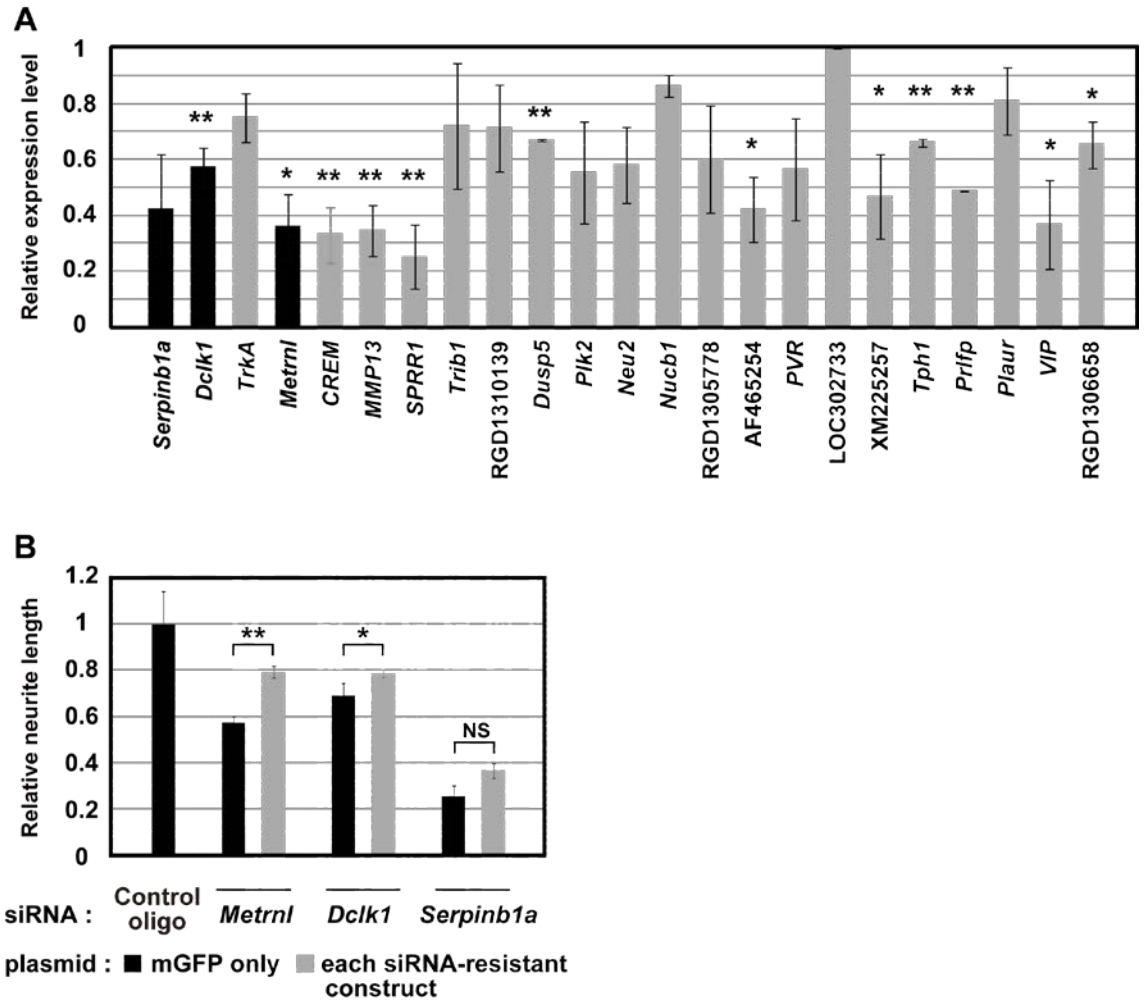


Fig. 3. The effect of siRNA-mediated knockdown of the LP genes candidates on their expression levels and the rescue experiment

(A) The effect of indicated siRNA-mediated knockdown of the LP candidate genes on NGF-induced expression level in PC12 cells at 3 h after treatment. (B) The rescue experiment by co-expression of the siRNA-resistant constructs. Twelve hours after transfection, the cells were treated with complete medium supplemented with 50 ng/mL NGF for 24 hr. The Student *t* test was used to evaluate the statistical significance of differences (**$P < 0.01$,*$P < 0.05$).

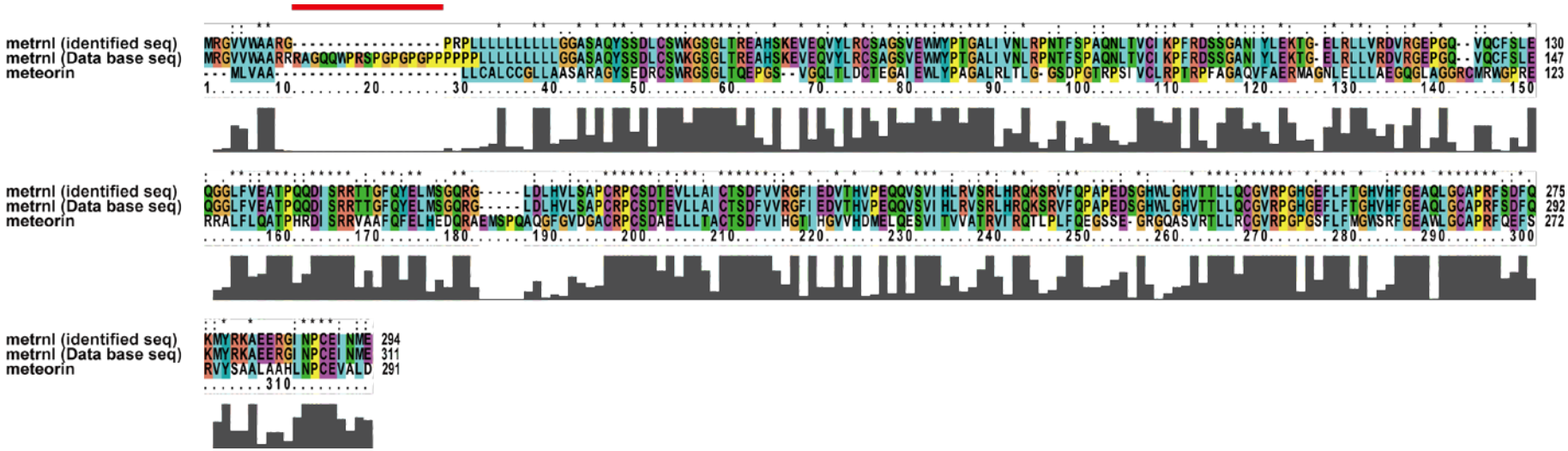


Fig. 4. The identification of a novel sequence of METRNL

The comparison of amino acid sequence of METRNL cloned and data base sequence and METEORIN sequence. The result of multiple sequence alignment by ClustalX was shown. Seventeen amino acids at neighboring N-terminal residues of data base sequence were deleted in cloned sequence (Red bar). The novel mRNA sequence data of *Metrnl* have been deposited with the DDJB nucleotide sequence data banks and are available under the accession number AB646250.

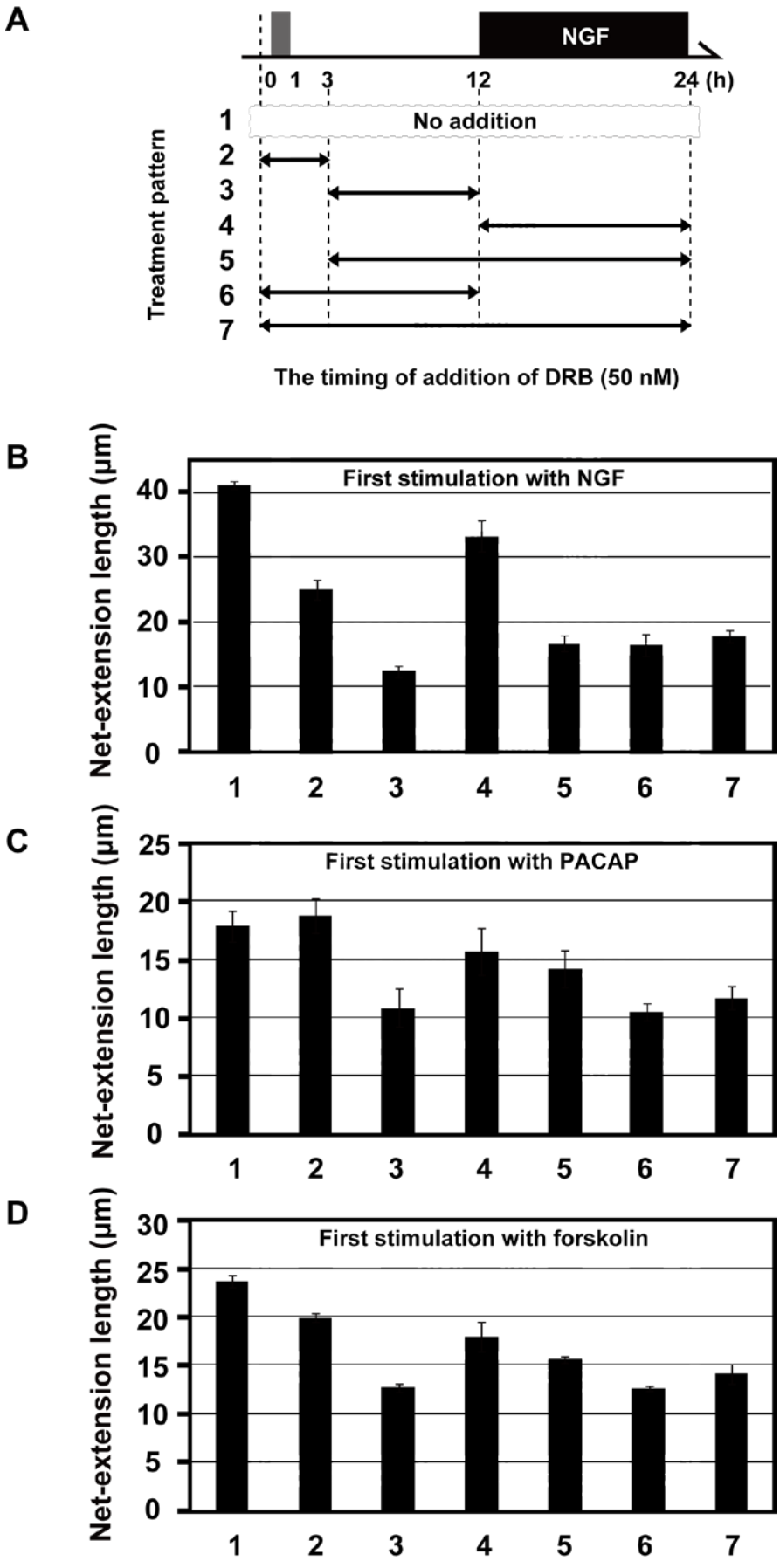

A
NGF
0 1 3 12 24 (h)
No addition
Treatment pattern
1
2
3
4
5
6
7
The timing of addition of DRB (50 nM)
B
First stimulation with NGF
Net-extension length (μm)
0 10 20 30 40
1 2 3 4 5 6 7
C
First stimulation with PACAP
Net-extension length (μm)
0 5 10 15 20 25
1 2 3 4 5 6 7
D
First stimulation with forskolin
Net-extension length (μm)
0 5 10 15 20 25 30
1 2 3 4 5 6 7

Fig. 5. The effect of inhibition of transcriptional activity on neurite extension following NGF-, PACAP- and forskolin-induced latent process

(A) Stimulation scheme: For discontinuous stimulation, PC12 cells were exposed to NGF or PACAP or forskolin for 1 h; then NGF-containing medium was washed out and replaced with NGF-free medium. After 12 h, cells were re-exposed with NGF for 12 h. Arrows represent the timing of the addition of 5,6-dichloro-1-8-D-ribofuranosylbenzimidazole (DRB), a reversible transcription inhibitor. Seven treatment patterns were performed. PC12 cells in treatment pattern 2, 6 and 7 were treated in the presence of DRB (50 nM) at 30 min before the first stimulation. PC12 cells in treatment pattern 3 and 5, and 4 were treated in the presence of DRB (50 nM) at 3 h and 12 h after the first stimulation, respectively. PC12 cells in treatment pattern 2, 3 and 6 were switched to complete medium without DRB at 3 or 12 h. (B-D) The effect of inhibition of transcriptional activity on net-neurite extension lengths following NGF (B)-, PACAP (C)- and forskolin (D)-induced latent process. PC12 cells were fixed at 24 h and analyzed for neurite extension. Note that background neurite length was subtracted from each neurite length at 24 h to indicate a net-extension length.

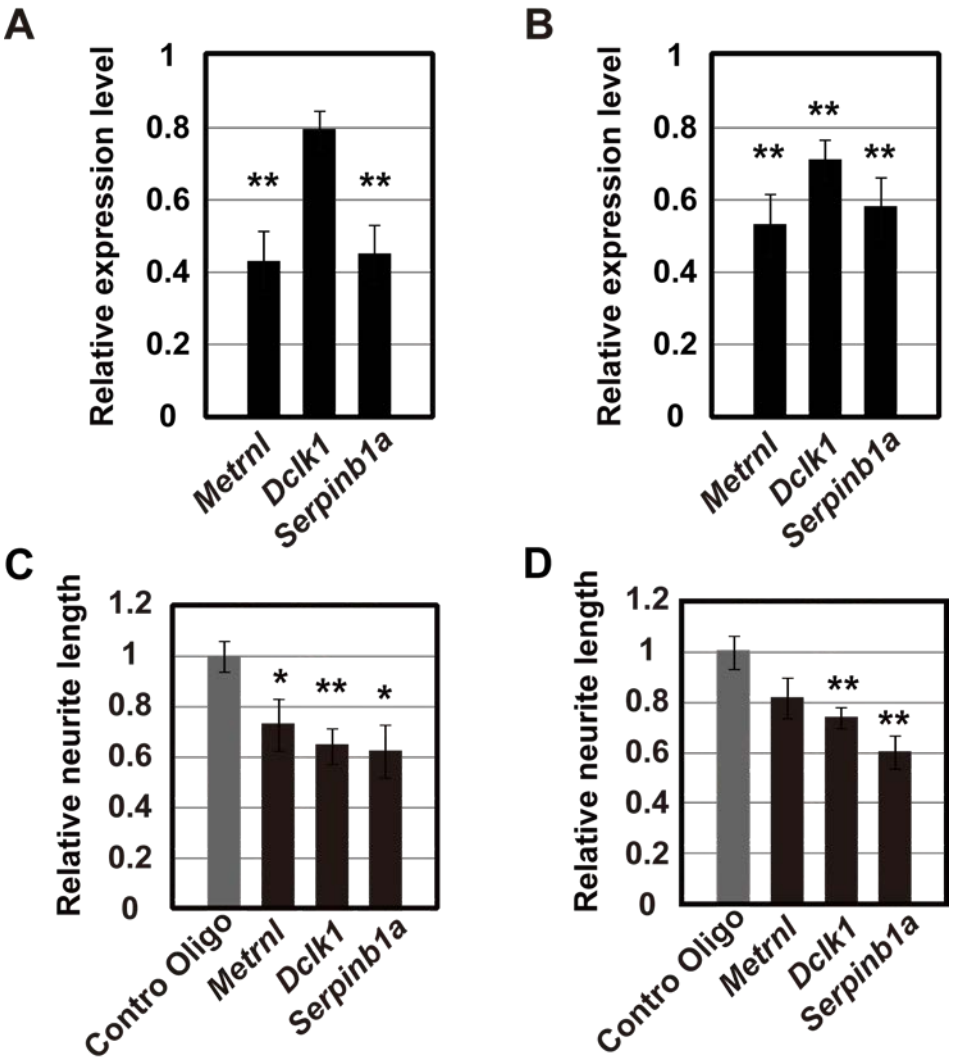


Fig. 6. Effects of siRNA-mediated knockdown of LP genes on PACAP- and forskolin-induced latent process

(A, B) PC12 cells were treated with the indicated siRNAs for 12 h followed by PACAP (100 nM) or forskolin (10 nM) treatment. The effect of the indicated siRNA-mediated knockdown of the LP genes on PACAP (A)- or forskolin (B)-induced expression level in PC12 cells at 3 h after treatment. (C, D) PC12 cells were treated with the indicated siRNAs for 12 h followed by PACAP or forskolin treatment for 12 h and then treated with NGF for additional 12 h. The effect of the indicated siRNA-mediated knockdown of the LP genes on neurite extension following PACAP (C)- or forskolin (D)-induced latent process. The values are shown as means ± s.e.m. (n = 3). The Student *t* test was used to evaluate the statistical significance of differences (***P* < 0.01,**P* < 0.05).

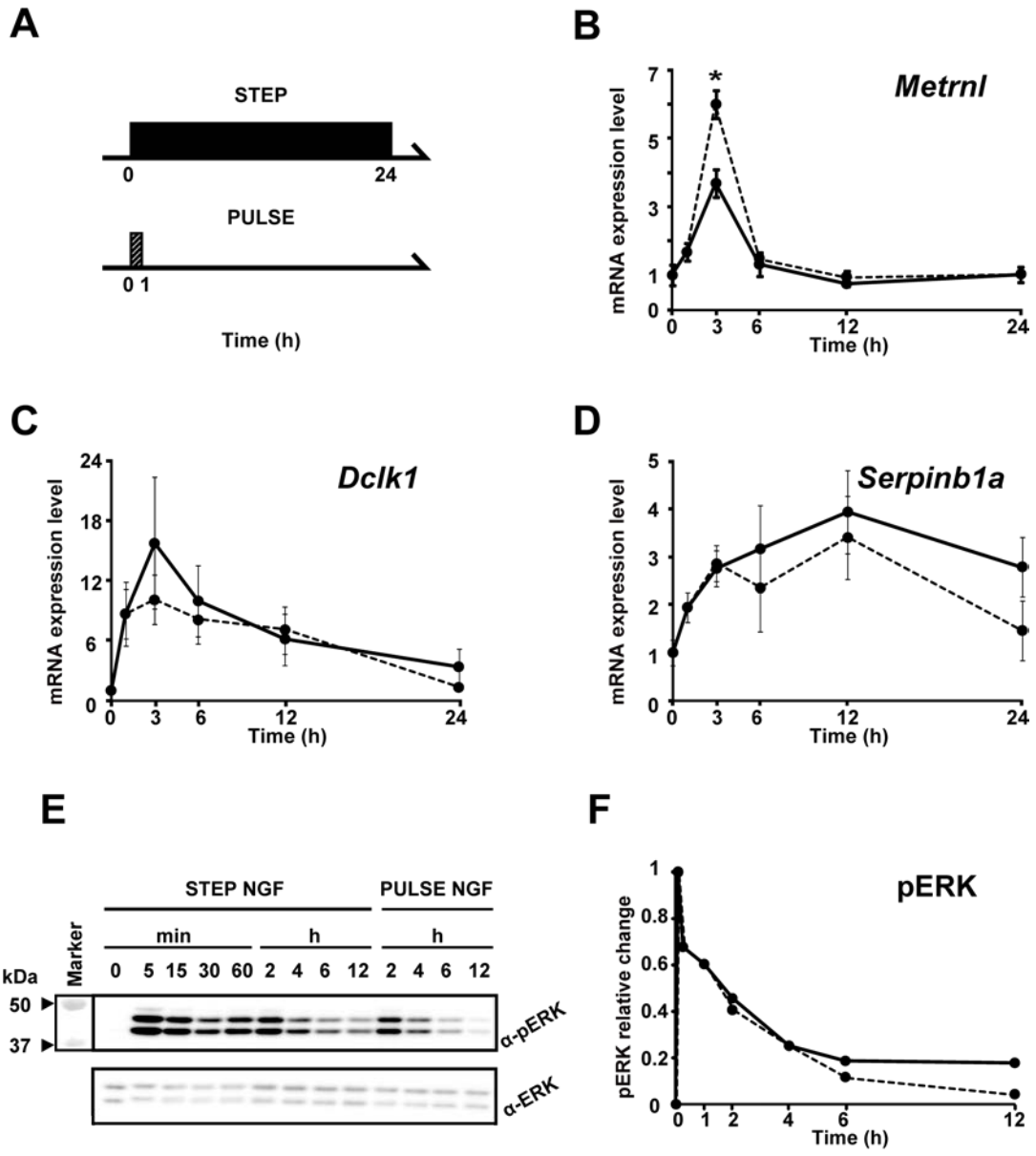


Fig. 7. The time course of expression of the LP genes and ERK phosphorylation in response to pulse or step stimulation with NGF

(A) Stimulation scheme: step (upper) and pulse (lower) stimulation by NGF (50 ng/mL). For step stimulation, PC12 cells were continuously exposed to NGF. For pulse stimulation, cells were exposed to NGF for 1 h; then NGF-containing medium was washed out and replaced with NGF-free medium. (B-D) Time courses of expression of *Metrnl* (B), *Dclk1* (C), and *Serpinb1a* (D) were determined by qRT-PCR at 1, 3, 6, 12, and 24 h of pulse (dashed lines) and step (solid lines) stimulation with NGF. (E) ERK phosphorylation (42/44 kDa) in response to step or pulse stimulation with NGF (50

ng/mL) was measured by Western blotting. As a control, pan-ERK (42/44 kDa) was measured to evaluate the amount of ERK. Representative bands in SDS-PAGE are shown. (F) The time courses of ERK phosphorylation normalized by the amount of ERK. The details of quantification are described in Materials and methods. The Student *t* test was used to evaluate the statistical significance of differences in expression levels at 3 h after pulse and step stimulation (*$P < 0.05$ in panel B).

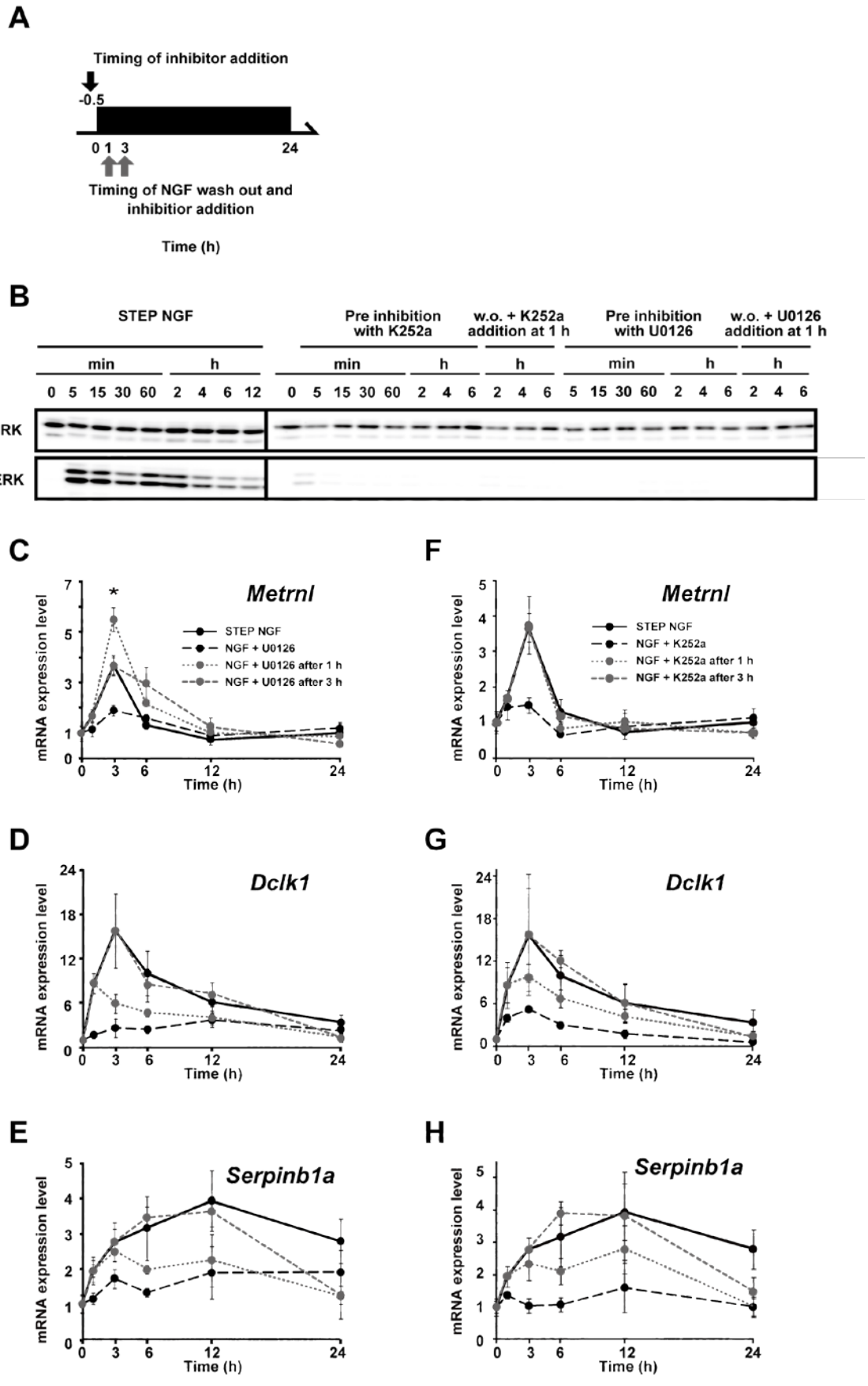

A
Timing of inhibitor addition
-0.5
0 1 3
24
Timing of NGF wash out and inhibitior addition
Time (h)
B
STEP NGF
Pre inhibition with K252a
w.o. + K252a addition at 1 h
Pre inhibition with U0126
w.o. + U0126 addition at 1 h
min
h
0 5 15 30 60 2 4 6 12
α-ERK
α-pERK
C
Metrnl
STEP NGF
NGF + U0126
NGF + U0126 after 1 h
NGF + U0126 after 3 h
mRNA expression level
Time (h)
D
Dclk1
E
Serpinb1a
F
Metrnl
STEP NGF
NGF + K252a
NGF + K252a after 1 h
NGF + K252a after 3 h
G
Dclk1
H
Serpinb1a

Fig. 8. The time course of expression of the LP genes in response to NGF in the presence of U0126 or K252a

(A) Stimulation scheme: arrows represent the timing of the addition of U0126 or K252a and that of NGF withdraw accompanied by the addition of U0126 or K252a. (B) ERK phosphorylation in response to step stimulation with NGF (50 ng/mL) in the presence or absence of K252a (200 nM), a TrkA inhibitor, or U0126 (50 μM), an ERK inhibitor, were measured by Western blotting. (C-H) The effect of U0126 and K252a on the expression of the LP genes. (C-E) PC12 cells were treated in the absence (black solid lines) or the presence of U0126 (50 μM; black dashed lines) at 30 min before stimulation with NGF (50 ng/mL) or stimulated with NGF followed by withdrawal of NGF and the addition of U0126 at 1 h (gray dotted lines) or 3 h (gray dashed lines) after stimulation. Time courses of expression of *Metrnl* (C), *Dclk1* (D), and *Serpinb1a* (E) were determined by qRT-PCR. The values are shown as the means ± s.e.m.. The Student *t* test was used to evaluate the statistical significance of differences in expression levels at 3 h in the absence of U0126 and when U0126 was added after 1 h (*$P < 0.05$ in panel C). (F-H) PC12 cells were treated in the absence (black solid lines) or the presence of K252a (200 nM; black dashed lines) at 30 min before stimulation with NGF (50 ng/mL) or treated with NGF followed by withdrawal of NGF and the addition of K252a at 1 h (gray dotted lines) or 3 h (gray dashed lines) after stimulation. Time courses of expression of *Metrnl* (F), *Dclk1* (G), and *Serpinb1a* (H) were measured by qRT-PCR.

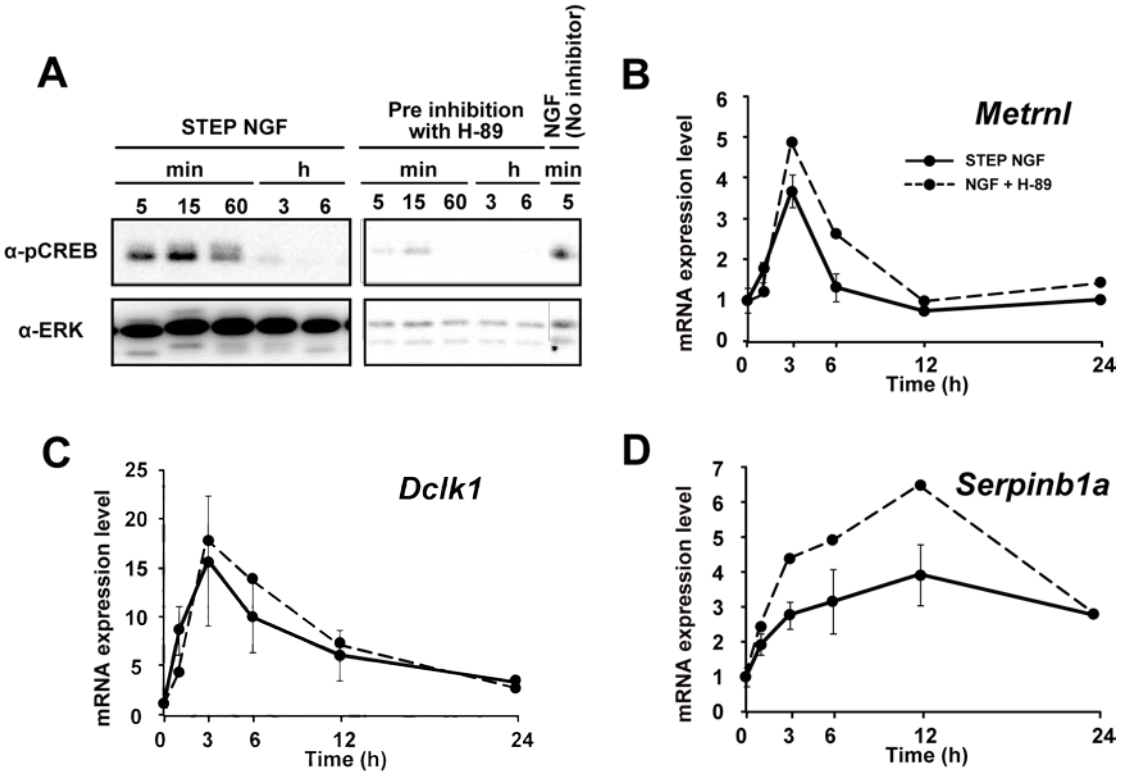


Fig. 9. The time course of expression of the LP genes in response to NGF in the presence of H-89

(A) CREB phosphorylation (43 kDa) in response to step stimulation with NGF (50 ng/mL) in the presence or absence of H-89 (50 μM), a PKA inhibitor, were measured by Western blotting. (B-D) The effect of H-89 on the expression of the LP genes. Time course of expression of *Metrnl* (B), *Dclk1* (C), and *Serpinb1a* (D) were measured by qRT-PCR. PC12 cells were treated with NGF (50 ng/mL) in the absence (solid lines) or the presence of H-89 (50 μM; dashed lines). H-89 was applied at 30 min before stimulation with NGF. The values in the presence of inhibitor are representative of duplicate values.

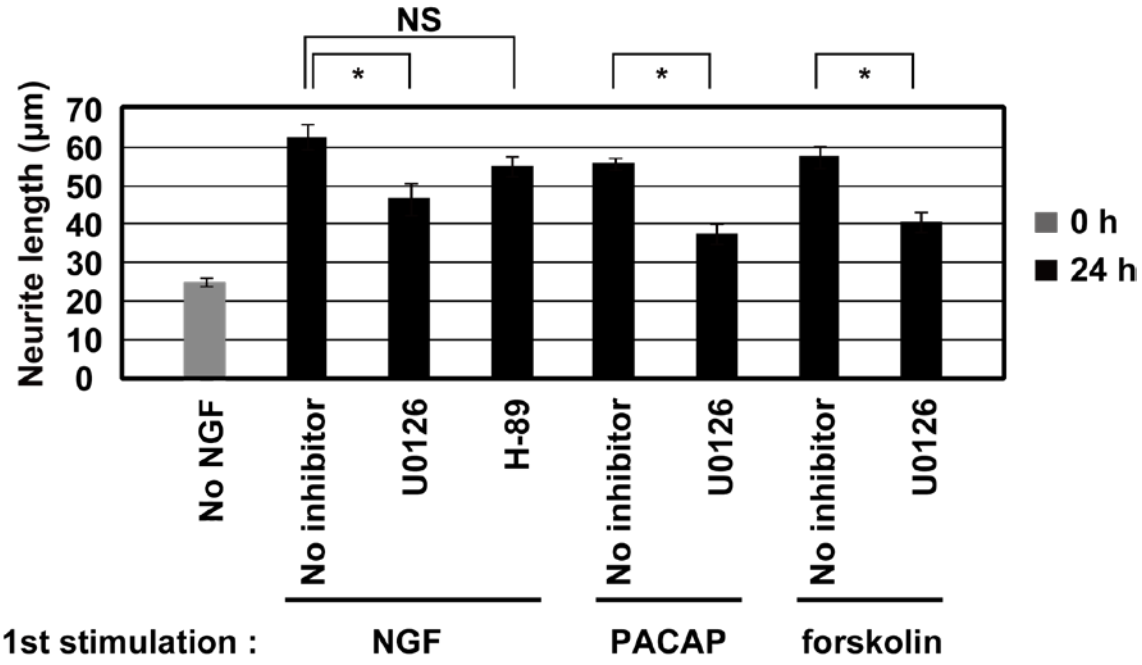


Fig. 10. The effect of inhibitors on NGF-, PACAP- and forskolin-induced neurite extension

PC12 cells were treated in the absence or the presence of U0126 (50 μM) or H-89 (50 μM) at 30 min before step stimulation with NGF (50 ng/mL), PACAP (100 nM), or forskolin (10 nM). At 12 h after start of stimulation, PC12 cells were uniformly switched to complete medium containing 50 ng/mL of NGF. The values are shown as the means ± s.e.m. The Student *t* test was used to evaluate the statistical significance of differences (*$P < 0.05$; NS, not significant).

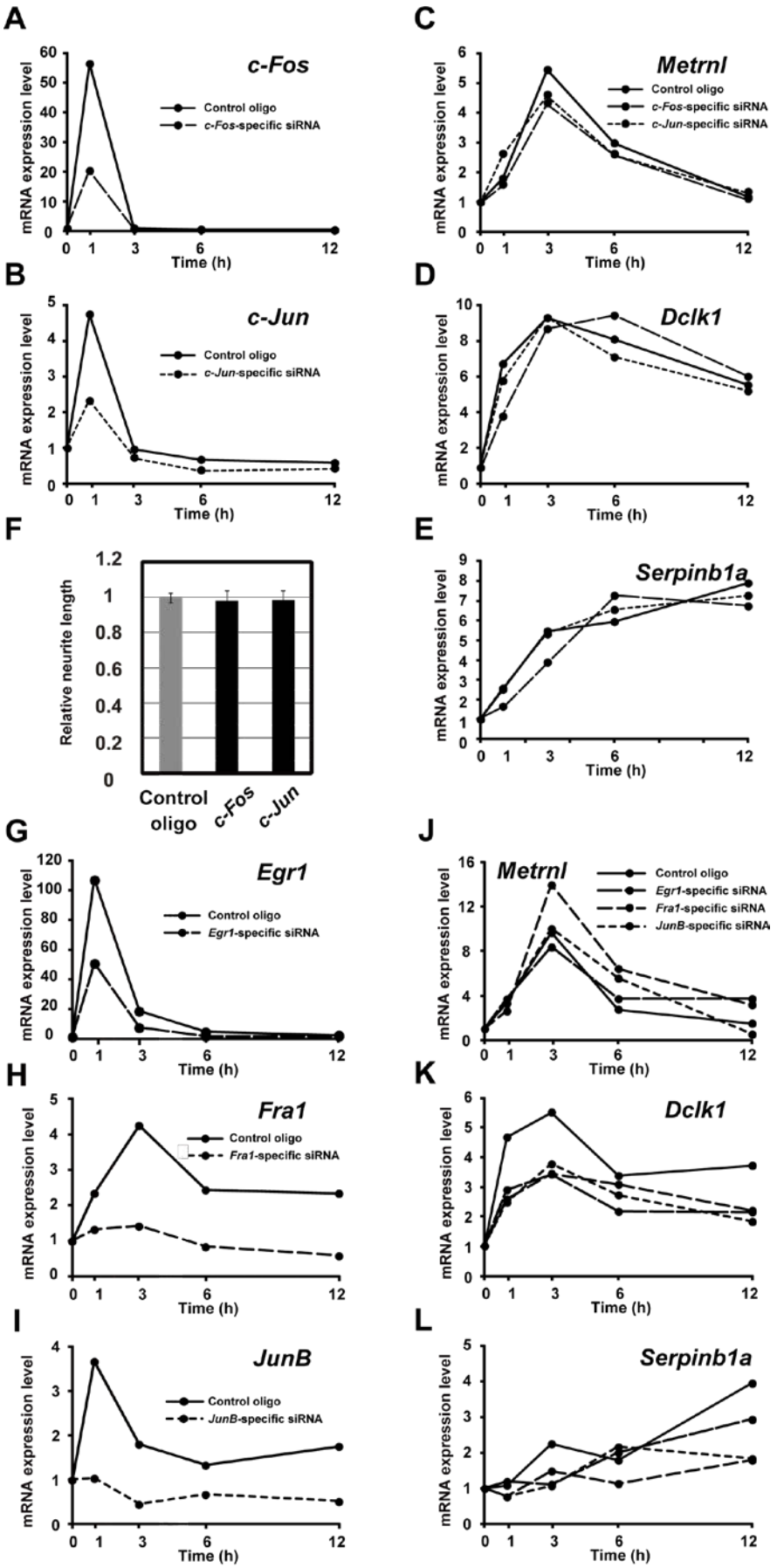

A
c-Fos
Control oligo
c-Fos-specific siRNA
mRNA expression level
Time (h)
B
c-Jun
Control oligo
c-Jun-specific siRNA
C
Metrnl
Control oligo
c-Fos-specific siRNA
c-Jun-specific siRNA
D
Dclk1
E
Serpinb1a
F
Relative neurite length
Control oligo
c-Fos
c-Jun
G
Egr1
Control oligo
Egr1-specific siRNA
H
Fra1
Control oligo
Fra1-specific siRNA
I
JunB
Control oligo
JunB-specific siRNA
J
Metrnl
Control oligo
Egr1-specific siRNA
Fra1-specific siRNA
JunB-specific siRNA
K
Dclk1
L
Serpinb1a

Fig. 11. The effect of the immediate early genes on the expression of the LP genes and neurite extension

(A-E) PC12 cells were treated with *c-Fos*- or *c-Jun*-specific siRNA for 12 h and then treated with NGF (50 ng/mL). The effect of *c-Fos*- or *c-Jun*-specific siRNA on time course of the expression of *c-Fos* (A), *c-Jun* (B), *Metrnl* (C), *Dclk1* (D), and *Serpinb1a* (E). (F) PC12 cells were treated with *c-Fos*- or *c-Jun*-specific siRNA for 12 h and then subjected to differentiation by NGF (50 ng/mL) for 24 h. The relative neurite lengths of the cells transfected with the indicated siRNA compared to that of cells transfected with negative control siRNA are shown. The values are shown as means ± s.e.m.. (G-L) PC12 cells were treated with *Egr1*-, *Fra1*- or *JunB*-specific siRNA for 12 h and then treated with NGF (50 ng/mL). The effect of *Egr1*-, *Fra1*- or *JunB*-specific siRNA on time course of the expression of *Egr1* (G), *Fra1* (H), *JunB* (I), *Metrnl* (J), *Dclk1* (K), and *Serpinb1a* (L). Each mRNA expression was determined by qRT-PCR. The values are representative of duplicate values.

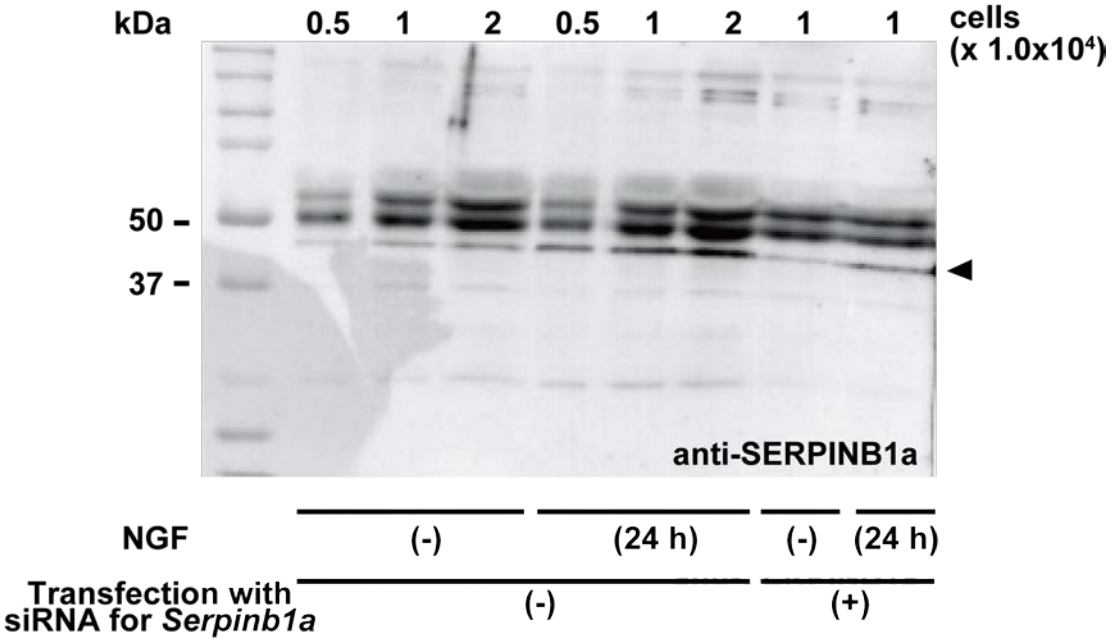


Fig. 12. The specificity of the antibody for SERPINB1a

SERPINB1a (42 kDa, arrowhead) were detected by the purchased antibody (sc-34305). The amount of SERPINB1a was increased by NGF stimulation for 24 h, which were knocked down by the transfection of siRNA for *Serpinb1a*. The loading volumes were 0.5, 1.0, and 2.0 x 10^4 cells / well as indicated.

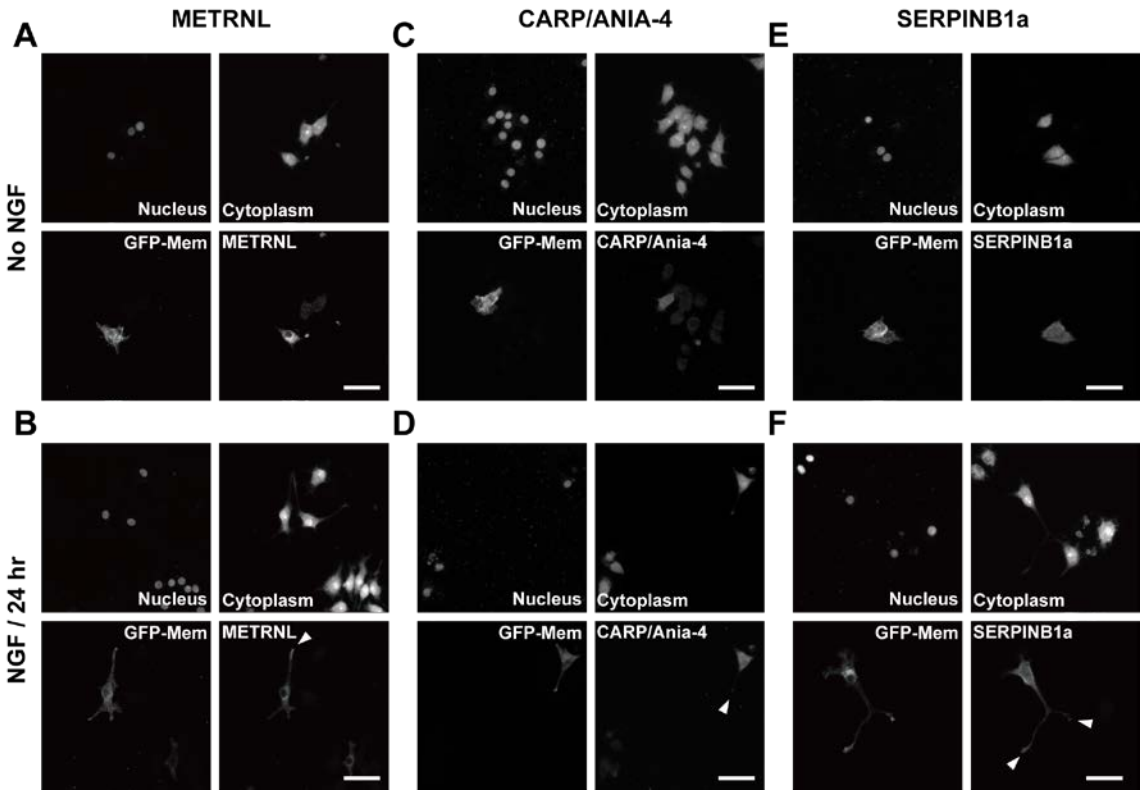


Fig. 13. Subcellular localization of the LP gene products

(A-F) The subcellular localization of HA-tagged LP gene products in transformed PC12 cells. PC12 cells were transfected with a construct of the indicated HA-tagged LP gene for 24 h, and incubated with no stimulant (A, C and E) or treated with NGF (50 ng/mL) for 24 h (B, D and F). The exogenous HA-tagged LP gene products were detected with anti-HA antibody. The transfected cells express membrane-located GFP (GFP-Mem). Nucleus and cytoplasm were stained with Hoechst and CellMask, respectively. The terminuses of neurites are highlighted with arrowheads. Bar length, 50 μm.

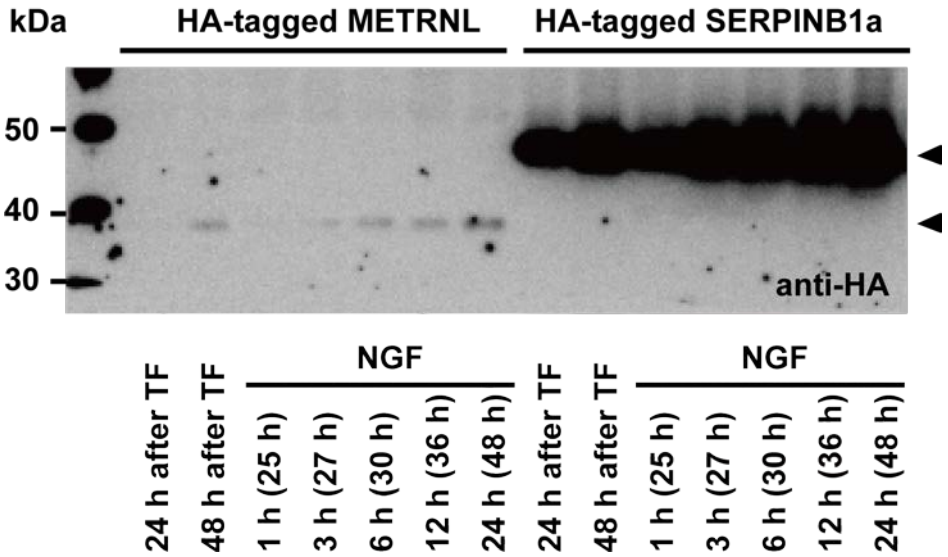


Fig. 14. The HA-tagged METRNL and SERPINB1a were specifically detected by anti-HA antibody.

The HA-tagged METRNL (lower arrowhead) and SERPINB1a (upper arrowhead) were specifically detected by anti-HA antibody. At 24 h after the transfection (TF), the cells were treated with NGF or not. The time after the transfection are shown in parentheses. The amount of the HA-tagged METRNL and SERPINB1a were increased in time- and NGF-dependent manner.

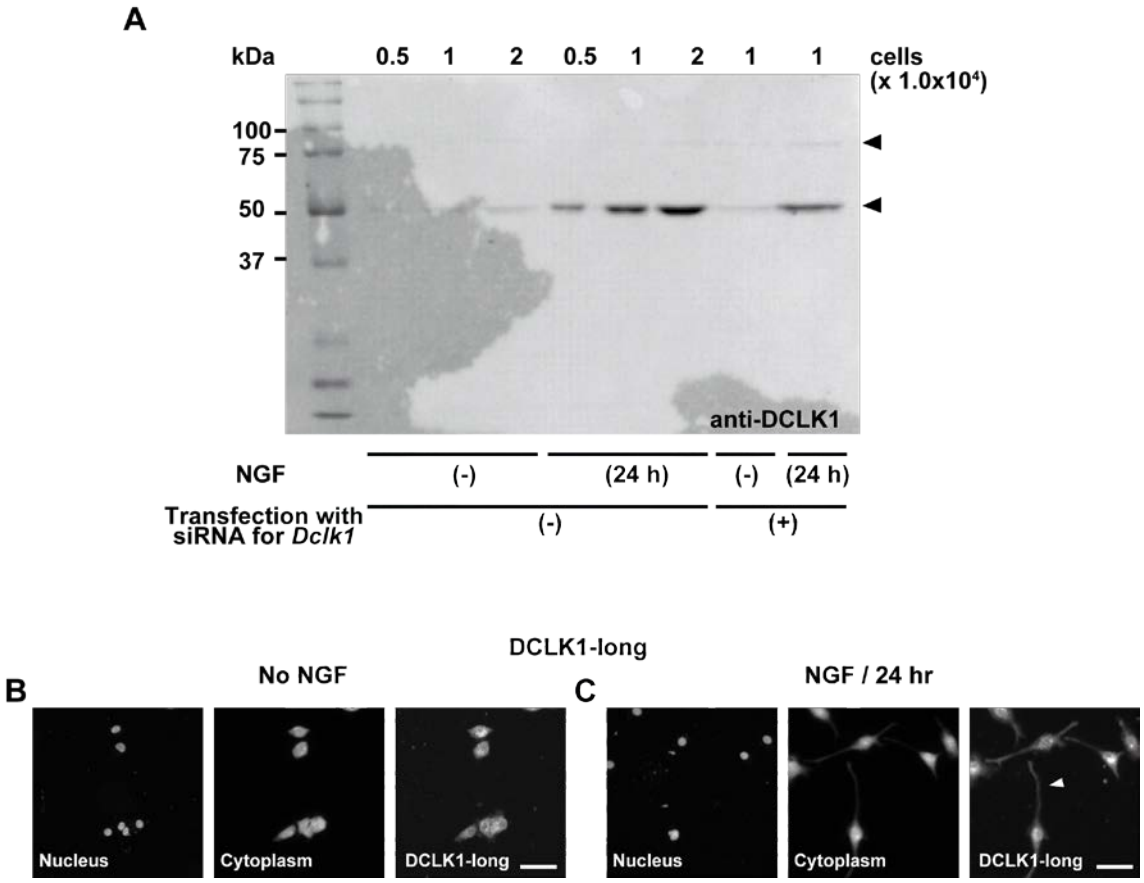


Fig. 15. The specificity of the antibody for DCLK1-long and subcellular localization of DCLK1-long

(A) Two *Dclk1-long* products (47 kDa, lower arrowhead; 82 kDa, upper arrowhead) were specifically detected by the purchased antibody (ab31704). The amount of Dclk1-long product (47 kDa) was increased by NGF stimulation for 24 h. The effect of the siRNA for *Dclk1* on the increased amount of product by NGF was weak or not detected. The loading volumes were 0.5, 1.0, and 2.0 x 10^4 cells / well as indicated. (B, C) PC12 cells were incubated in the absence of NGF (B) or presence of NGF for 24 h (C). The endogenous DCLK1-long was detected with anti-DCLK1-long antibody. Nucleus and cytoplasm were stained with Hoechst and CellMask, respectively. The terminuse of neurite is highlighted with arrowhead. Bar length, 50 μm.

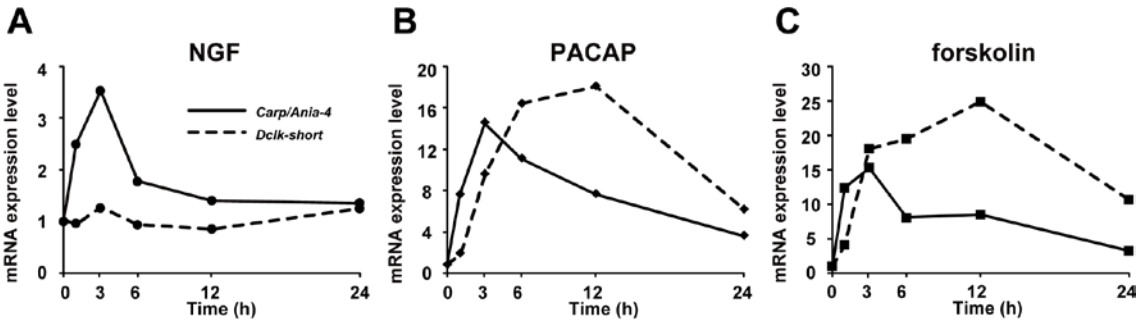


Fig. 16. The expression profile of *Dclk1* splicing variant, *Carp/Ania-4* and *Dclk-short*, in response to NGF, PACAP, and forskolin

(A-C) Time course of expression of *Carp/Ania-4* (solid lines) and *Dclk-short* (dashed lines) in response to step stimulation with NGF (50 ng/mL; A), PACAP (100 nM; B), and forskolin (10 nM; C).

Specific expression of the splicing variants was determined by qRT-PCR with the specific primer of *Carp/Ania-4* (forward; 5′-CCAGTAGGTATTGTGGCCAACTT-3′, reverse; 5′-CGTATGTTCCAGGATGAAATGC-3′) and *Dclk-short* (forward; 5′-CTCTGCTCTGATTGGAGGGAGTT-3′, reverse; 5′-AGCACAGCAGTGAATGGAGACA-3′). The values are representative of duplicate values.

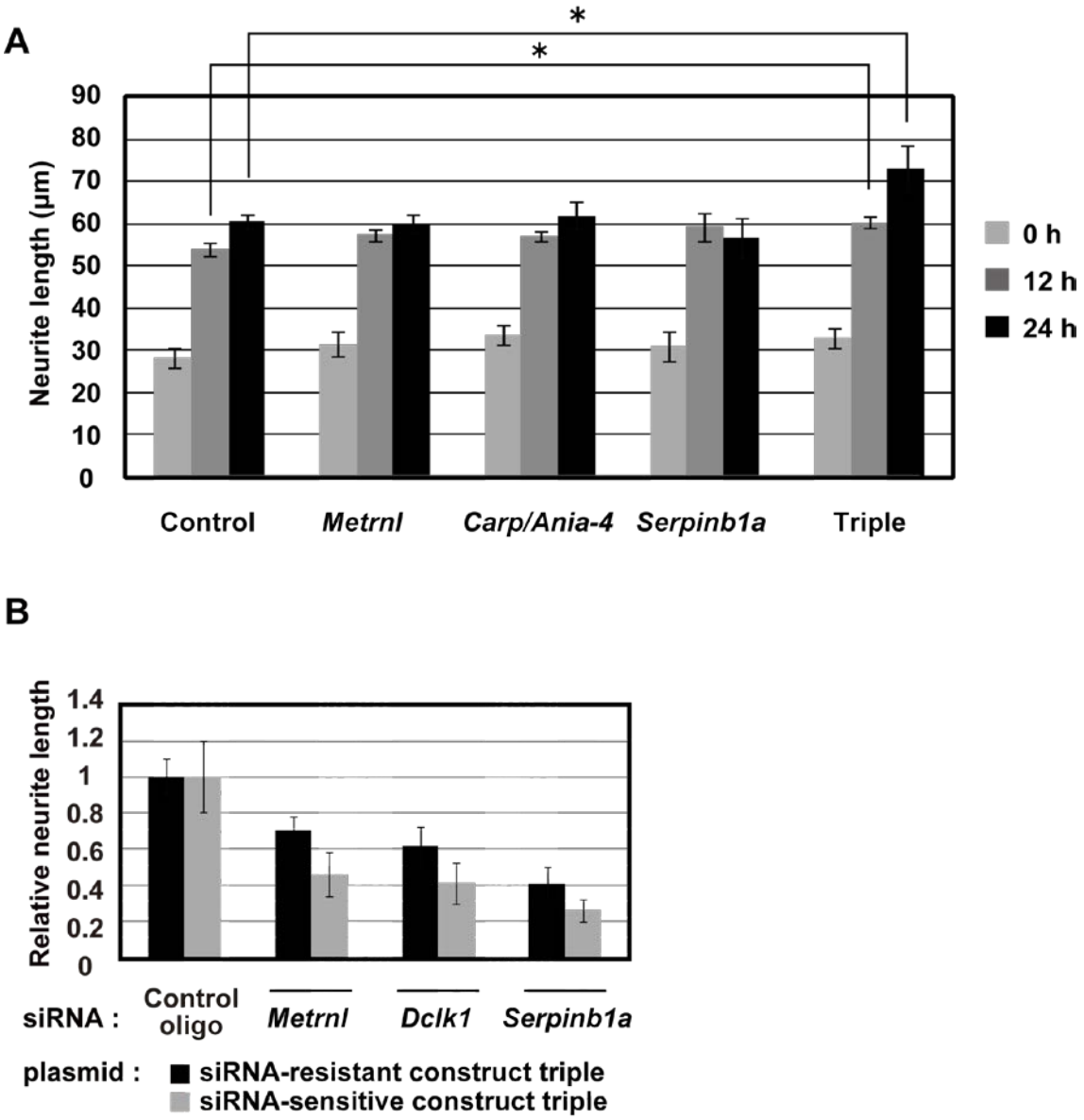


Fig. 17. The roles of the LP genes in neurite extension in PC12 cells

(A) The effect of overexpression of the LP genes on the neurite extension. *Metrnl*, *Carp/Ania-4*, and *Serpinb1a* and all LP genes (triple) were expressed in PC12 cells for 48 h after transfection, respectively, and then subjected to differentiation by NGF (50 ng/mL). GFP-Mem, membrane-located GFP, was co-expressed to mark transfected cells, or used as a control. Neurite lengths of the GFP-Mem-positive cells were measured before (0 h) and at 12 h and 24 h after NGF. Overexpression of all LP genes together showed a significant enhancement of NGF-induced neurite extension compared to the control. The values are shown as means ± s.e.m.. The Student *t* test was used to evaluate

the statistical significance of differences (*$P < 0.05$). (B) The effect of siRNA-mediated knockdown of each LP gene on cooperative enhancement of NGF-induced neurite extension of the triple transfected cells. The cells were transfected with each siRNA together with all siRNA-resistant constructs of LP genes or all siRNA-sensitive (wild type) constructs of LP genes. Twelve hours after transfection, the cells were treated with NGF (50 ng/mL) for 24 h and assayed for neurite extension.

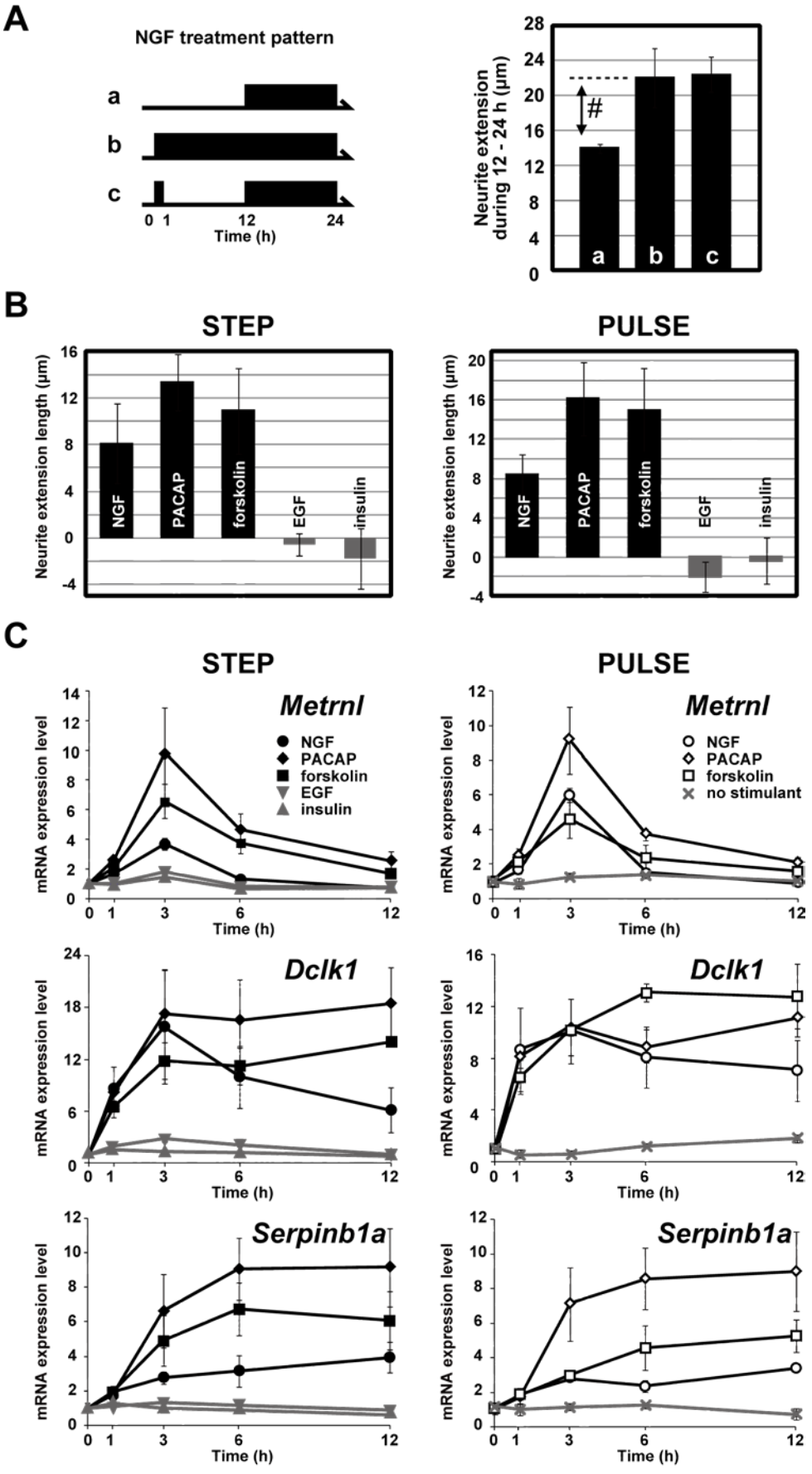
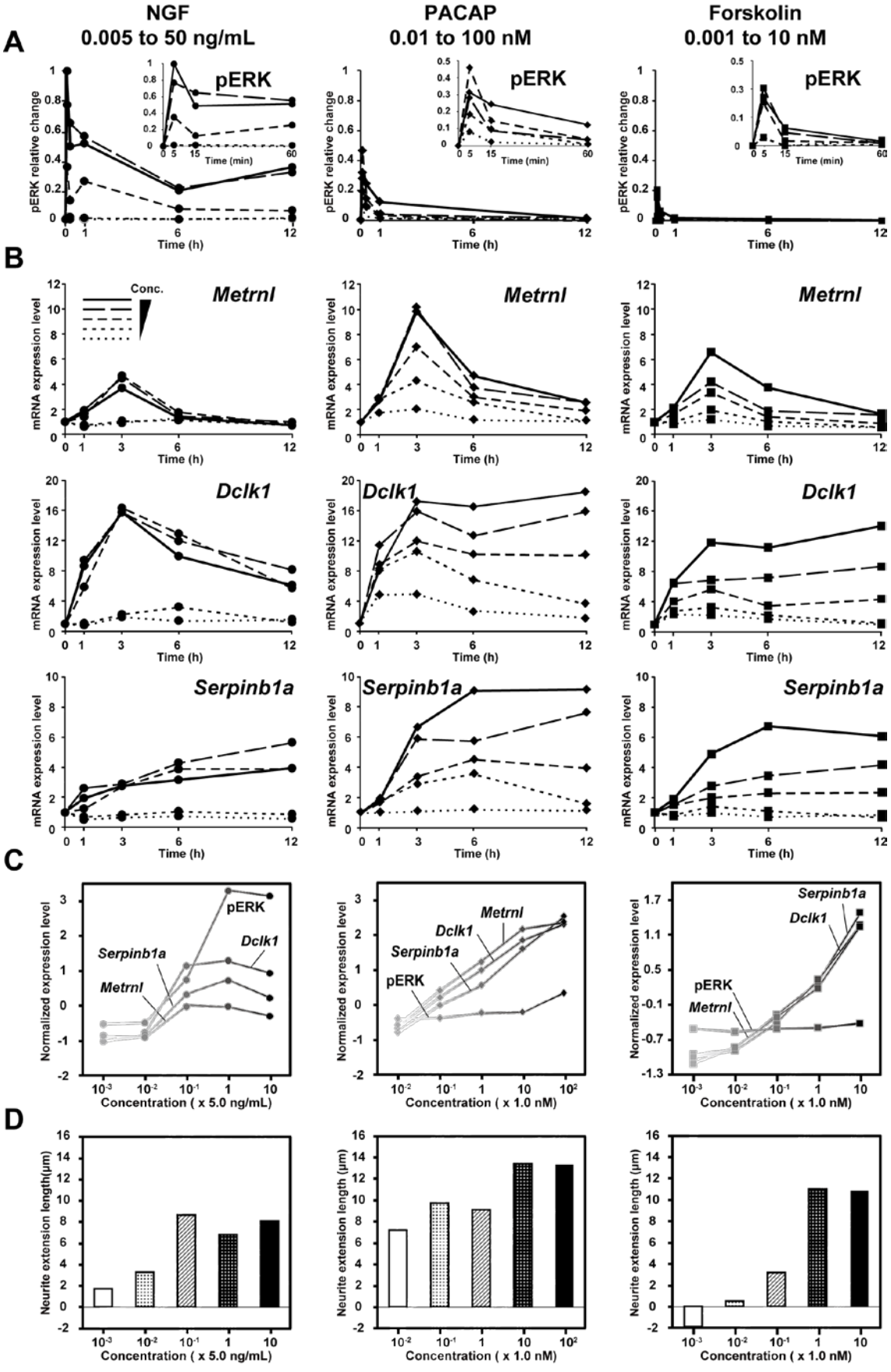

A
NGF treatment pattern
a
b
c
0 1 12 24
Time (h)
Neurite extension during 12 - 24 h (µm)
#
a
b
c
B
STEP
PULSE
Neurite extension length (µm)
NGF
PACAP
forskolin
EGF
insulin
C
STEP
PULSE
Metrnl
Dclk1
Serpinb1a
mRNA expression level
Time (h)
NGF
PACAP
forskolin
EGF
insulin
no stimulant

Fig. 18. Induction of the LP gene expression during the latent process and neurite extension during the extension period

(A) Scheme of NGF treatment pattern (left) and neurite length extended during the extension period (12-24 h; right). Neurite extension length (#) was measured by subtraction of neurite length extended during the extension process (12-24 h) of untreated cells by the first stimulation from which the neurite length extended during the extension process (12-24 h) of the indicated cells. The values are shown as the means ± s.e.m.. (B) Neurite extension length induced by the indicated neurotrophic factors. NGF was used as the second stimulation to evaluate the effect of the neurotrophic factors on the latent process. Step (left) or pulse (right) stimulation with NGF (50 ng/mL), PACAP (100 nM), forskolin (10 nM), EGF (50 ng/mL), and insulin (10 nM) was used as the first stimulation. The values are shown as the means ± s.e.m.. (C) The time courses of expression of the LP genes in response to step (left) and pulse (right) stimulation with NGF (circle), PACAP (diamond), forskolin (square), EGF (inverted triangle), and insulin (triangle). Gene expression was quantified by qRT-PCR. The values are shown as the means ± s.e.m..


A
NGF
0.005 to 50 ng/mL
PACAP
0.01 to 100 nM
Forskolin
0.001 to 10 nM
pERK
pERK relative change
Time (min)
Time (h)
B
Metrnl
Dclk1
Serpinb1a
Conc.
mRNA expression level
C
Normalized expression level
Concentration (x 5.0 ng/mL)
Concentration (x 1.0 nM)
D
Neurite extension length(μm)

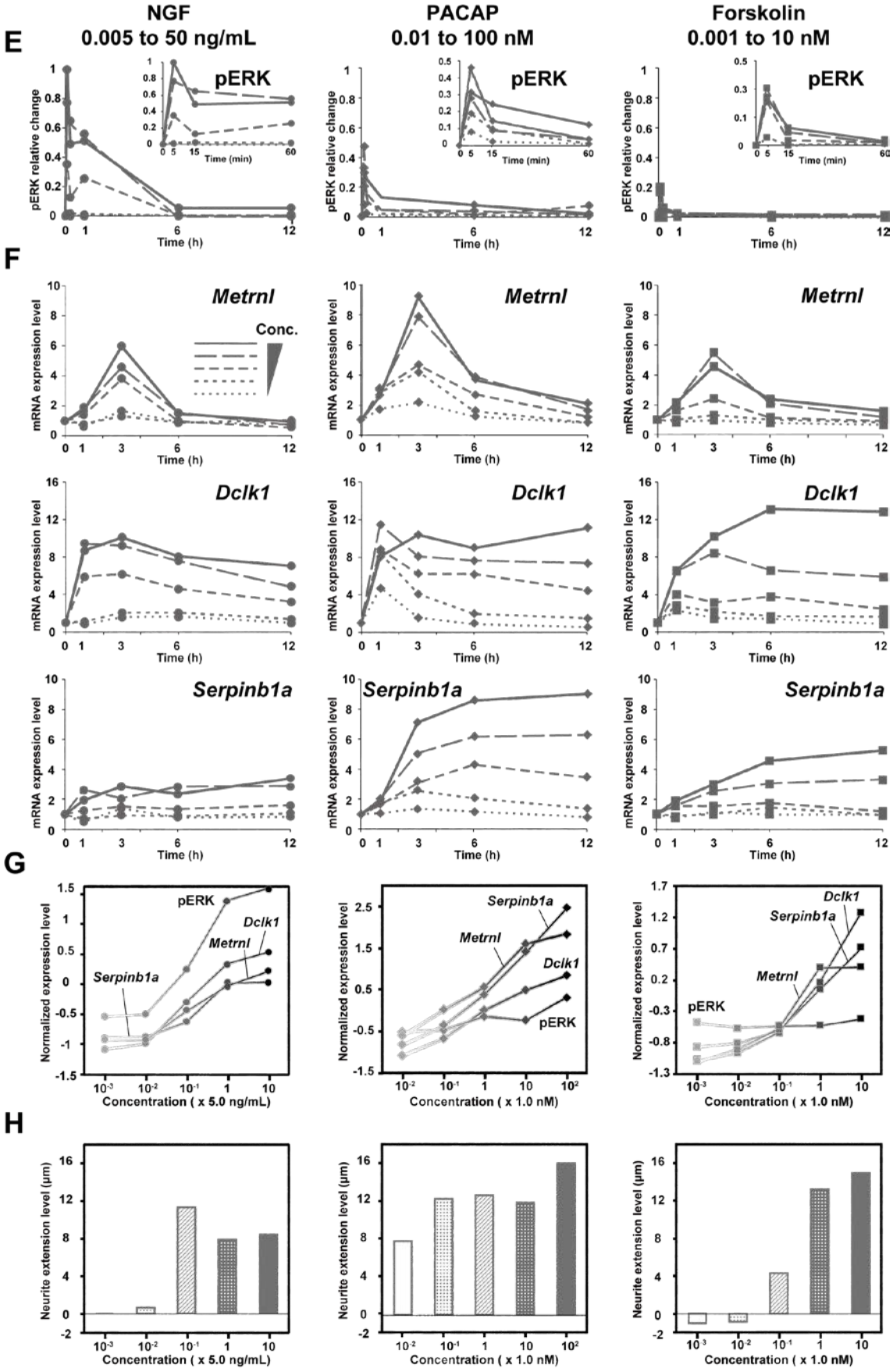

E
NGF
0.005 to 50 ng/mL
PACAP
0.01 to 100 nM
Forskolin
0.001 to 10 nM
pERK
pERK relative change
Time (min)
Time (h)
F
Metrnl
Dclk1
Serpinb1a
Conc.
mRNA expression level
G
Normalized expression level
Concentration (x 5.0 ng/mL)
Concentration (x 1.0 nM)
H
Neurite extension level (μm)

Fig. 19. Temporal pattern of pERK and LP genes during the latent period and neurite extension length during extension period

(A) The time course of ERK phosphorylation (pERK) in response to step stimulation with NGF (0.005, 0.05, 0.5, 5, 50 ng/mL), PACAP (0.01, 0.1, 1, 10, 100 nM), and forskolin (0.001, 0.01, 0.1, 1, 10 nM). The concentrations (from lower to higher) of NGF (circle), PACAP (diamond), and forskolin (square) are indicated by the interval of dashed lines (from dotted to solid). The phosphorylation levels of ERK were quantified by Western blotting. The values are representative of duplicate values. Insets represent the time courses from 0 to 60 min. (B) The time course of expression of the LP genes in response to step stimulation with NGF, PACAP, and forskolin. Their expressions were quantified by qRT-PCR. The values represent means of triplicate. (C) Step stimulation with NGF, PACAP, and forskolin dose response of the integrated levels of ERK phosphorylation (0-12 h), and those of the expression of the LP genes (0-12 h). The values represent the normalized integrated levels (mean 0 and variance 1) of ERK phosphorylation and those of the expression of the LP genes. (D) Step stimulation with NGF, PACAP, and forskolin dose response of the neurite extension lengths. The values represent means of triplicate. The time courses of ERK phosphorylation (E) and LP genes (F) in response to pulse stimulation with NGF, PACAP, and forskolin. (G) Pulse stimulation with NGF-, PACAP-, and forskolin-dependent dose response of the normalized integrated levels (mean 0 and variance 1) of ERK phosphorylation (0-12 h) and those of the expression of the LP genes (0-12 h). (H) Pulse stimulation with NGF-, PACAP-, and forskolin-dependent dose response of the neurite extension lengths. (E-H) The symbols and the colors of the symbols represent the condition of stimulation as described in A-D.

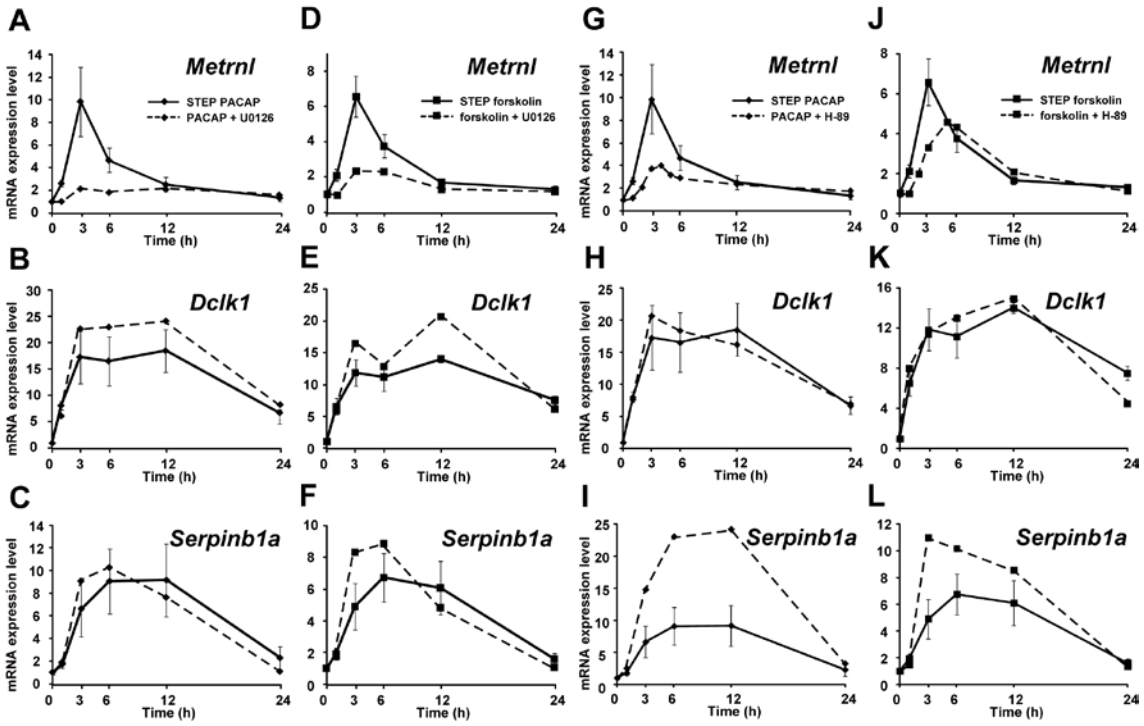


Fig. 20. The effect of inhibition of ERK or PKA on the time course of the LP genes in response to PACAP or forskolin.

(A-F) PC12 cells were treated in the absence (solid lines) or the presence (dashed lines) of U0126 (50 μM) at 30 min before stimulation with PACAP (100 nM) or forskolin (10 nM). The values in the presence of inhibitor are representative of duplicate values.

(G-L) PC12 cells were treated in the absence (solid lines) or the presence (dashed lines) of H-89 (50 μM) at 30 min before stimulation with PACAP (100 nM) or forskolin (10 nM). The values in the presence of inhibitor are representative of duplicate values.

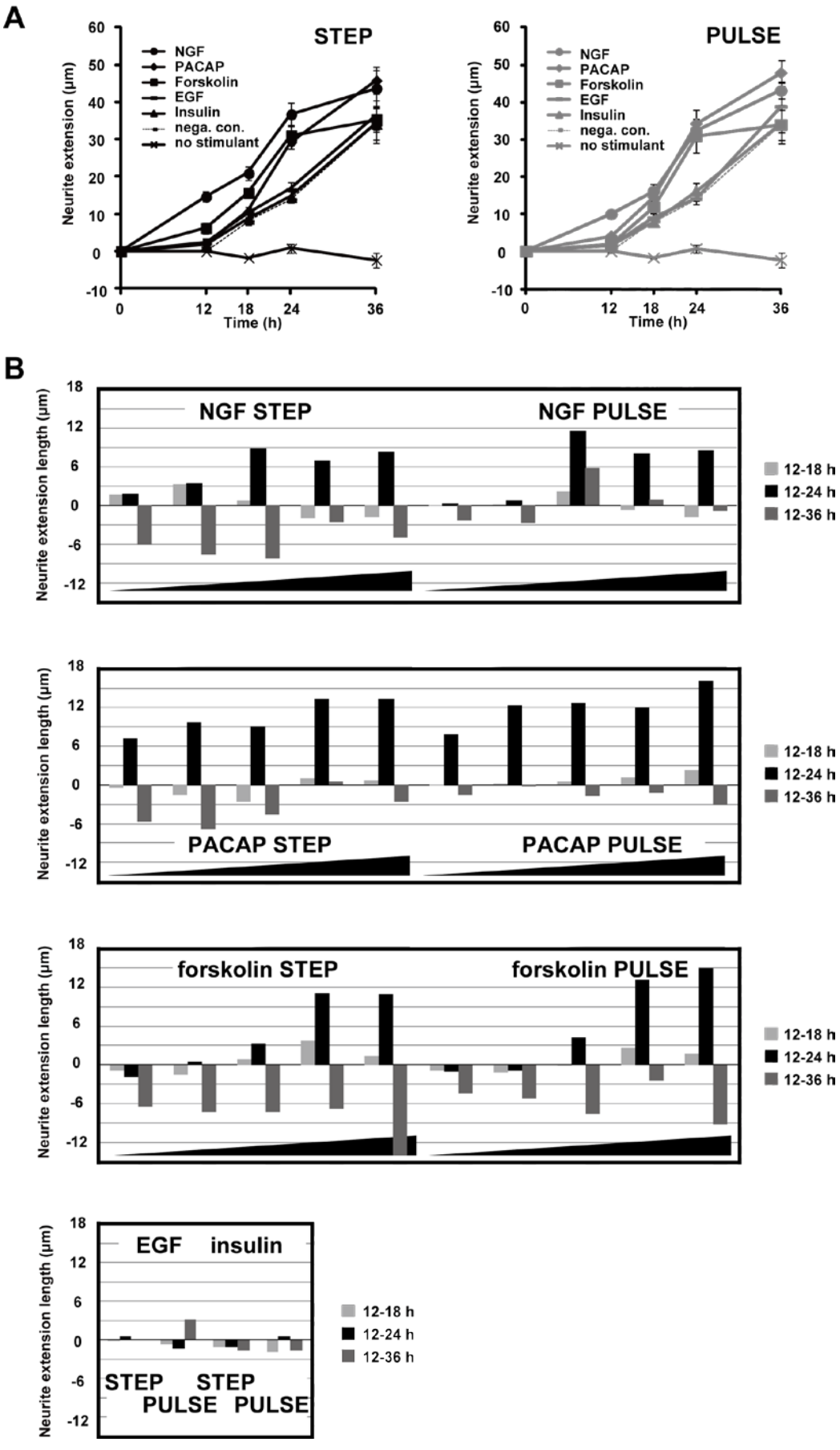

A
STEP
PULSE
NGF
PACAP
Forskolin
EGF
Insulin
nega. con.
no stimulant
Neurite extension (µm)
Time (h)
B
NGF STEP
NGF PULSE
PACAP STEP
PACAP PULSE
forskolin STEP
forskolin PULSE
EGF
insulin
STEP
PULSE
12-18 h
12-24 h
12-36 h
Neurite extension length (µm)

Fig. 21. The first stimulation-dependent dose response of neurite extension
(A) The time courses of neurite extension. Step (left) or pulse (right) stimulation with NGF (50 ng/mL), PACAP (100 nM), forskolin (10 nM), EGF (50 ng/mL) and insulin (10 nM) were used as the first stimulation (0-12 h) followed by the second stimulation of step stimulation with NGF (50 ng/mL). Note that background neurite length was subtracted for each point. The values are shown as the means ± s.e.m. (B) The first stimulation-dependent dose response of neurite extension length during 12-18, 12-24 and 12-36 h. Step (left) or pulse (right) stimulation with NGF (0.005, 0.05, 0.5, 5, 50 ng/mL), PACAP (0.01, 0.1, 1, 10, 100 nM), forskolin (0.001, 0.01, 0.1, 1, 10 nM), EGF (50 ng/mL) and insulin (10 nM) were used as the first stimulation (0-12 h) followed by the second stimulation of step stimulation with NGF (50 ng/mL). Note that the value of negative control (untreated cells with the first stimulation) was subtracted from each point.

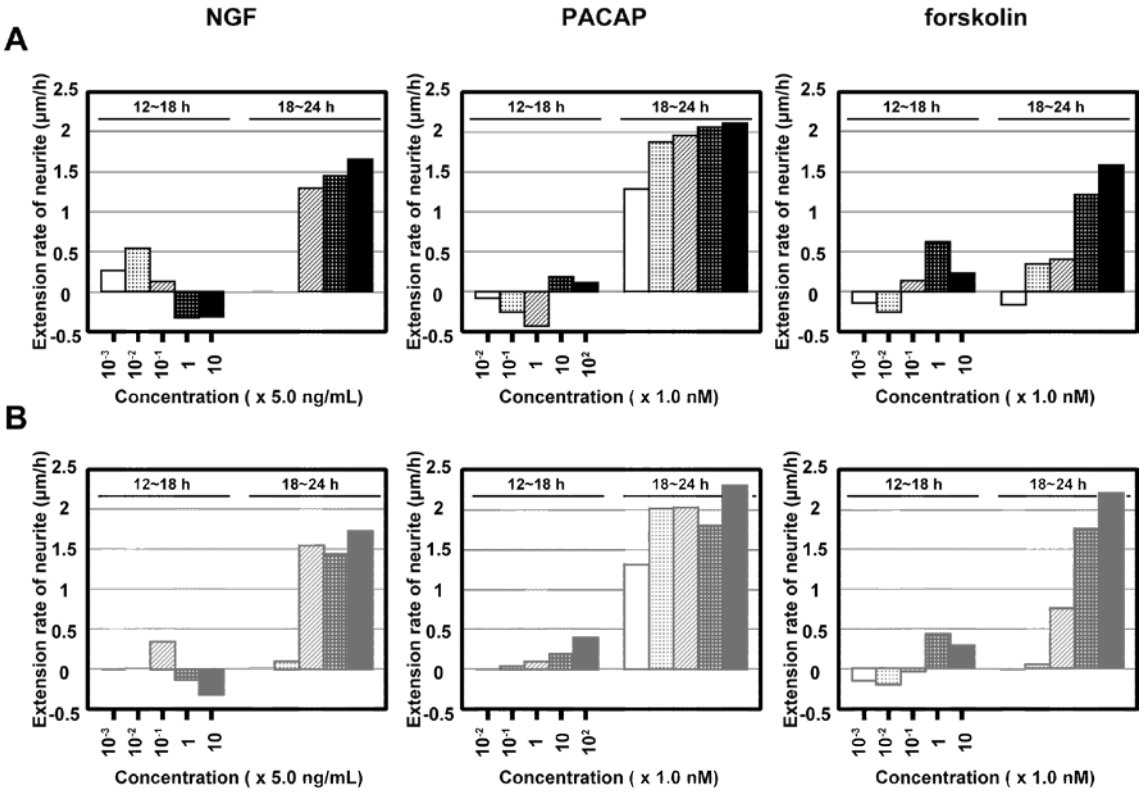


Fig. 22. The rates of neurite extension length during the extension period

(A, B) The rates of neurite extension length during the early phase (12-18 h) and the late phase (18-24 h) of the extension period were plotted as the NGF-, PACAP-, and forskolin-dependent dose response. (A) Step stimulation. (B) Pulse stimulation. The color code that represents conditions of stimulation is the same as those in Fig. 19D,H.

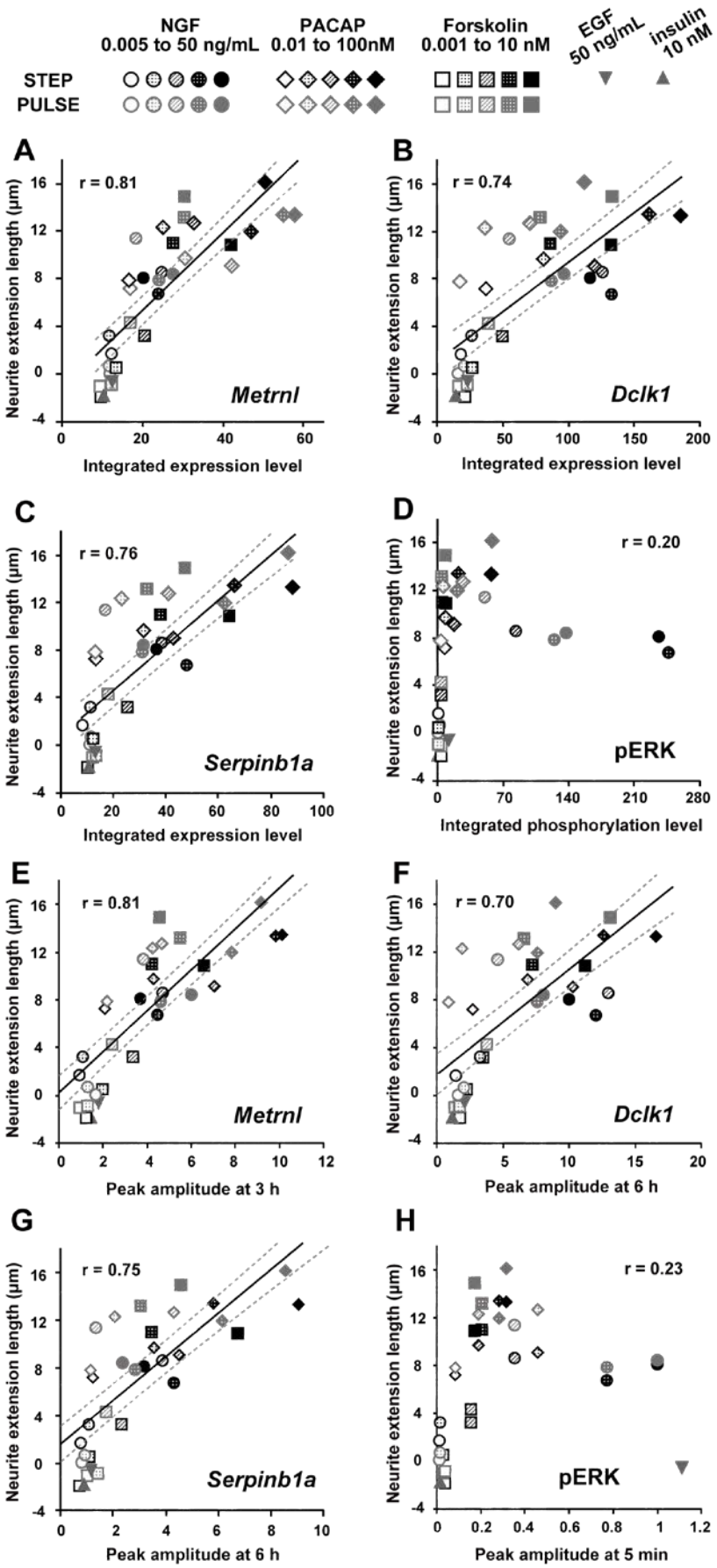

NGF
0.005 to 50 ng/mL
PACAP
0.01 to 100nM
Forskolin
0.001 to 10 nM
EGF
50 ng/mL
insulin
10 nM
STEP
PULSE
A
r = 0.81
Metrnl
Neurite extension length (µm)
Integrated expression level
B
r = 0.74
Dclk1
Neurite extension length (µm)
Integrated expression level
C
r = 0.76
Serpinb1a
Neurite extension length (µm)
Integrated expression level
D
r = 0.20
pERK
Neurite extension length (µm)
Integrated phosphorylation level
E
r = 0.81
Metrnl
Neurite extension length (µm)
Peak amplitude at 3 h
F
r = 0.70
Dclk1
Neurite extension length (µm)
Peak amplitude at 6 h
G
r = 0.75
Serpinb1a
Neurite extension length (µm)
Peak amplitude at 6 h
H
r = 0.23
pERK
Neurite extension length (µm)
Peak amplitude at 5 min

Fig. 23. Correlation of the phosphorylation levels of ERK and the expression levels of the LP genes with the neurite extension lengths

(A-D) A scatter plot of a mean of the neurite extension length (12-24 h) of each individual condition of stimulation versus a mean of the integrated levels of *Metrnl* (A), *Dclk1* (B), and *Serpinb1a* (C) expressions and ERK phosphorylation (D) (0-12 h) of the same individual condition. PC12 cells are treated with a pulse and step stimulation with NGF (0.005, 0.05, 0.5, 5, 50 ng/mL), PACAP (0.01, 0.1, 1, 10, 100 nM), and forskolin (0.001, 0.01, 0.1, 1, 10 nM) and with a step stimulation with EGF (50 ng/mL) and insulin (10 nM). Each Pearson's correlation coefficient (r) is indicated (*Metrnl*, 0.81; *Dclk1*, 0.74; *Serpinb1a*, 0.76; pERK, 0.20). (E-H) A scatter plot of a mean of the neurite extension length of each individual condition of stimulation versus a mean of the peak amplitudes of expression of *Metrnl* at 3 h (E), *Dclk1* at 6 h (F), and *Serpinb1a* at 6 h (G) and ERK phosphorylation at 5 min (H) of the same individual condition. Each Pearson's correlation coefficient (r) is indicated (*Metrnl*, 0.81; *Dclk1*, 0.70; *Serpinb1a*, 0.75; pERK, 0.23). Regression lines (lines) and 95% confidence limits (dashed lines) for *Metrnl*, *Dclk1*, and *Serpinb1a* are depicted.

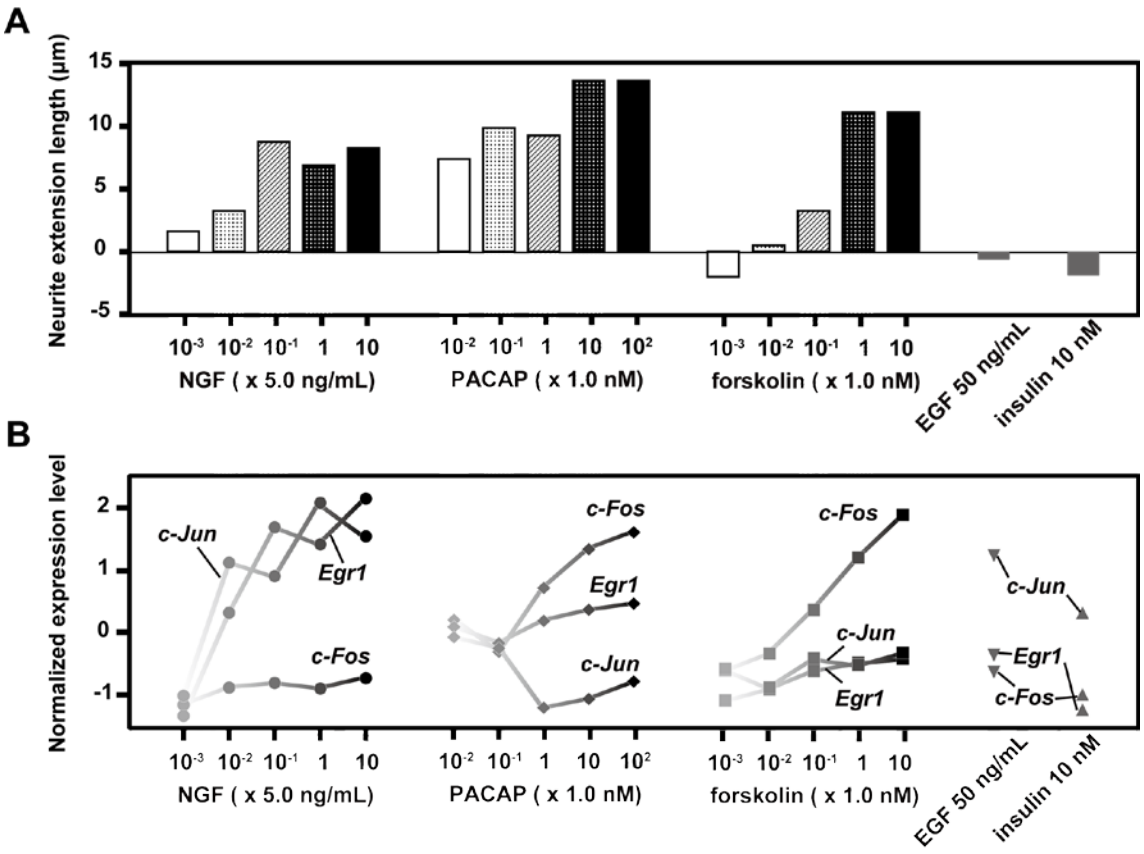


Fig. 24. The first stimulation-dependent dose response of the expression of *c-Fos*, *c-Jun* and *Egr1*

(A) Step stimulation with NGF, PACAP, forskolin, EGF and insulin dose response of the neurite extension lengths. The values represent means of triplicate. A is the same as Fig. 19D and indicated to be compared with B. (B) Step stimulation with NGF, PACAP, forskolin, EGF and insulin dose response of the integrated levels of the expression of *c-Fos*, *c-Jun* and *Egr1* (0-12 h). The values represent the normalized integrated levels (mean 0 and variance 1) of the expression of them.

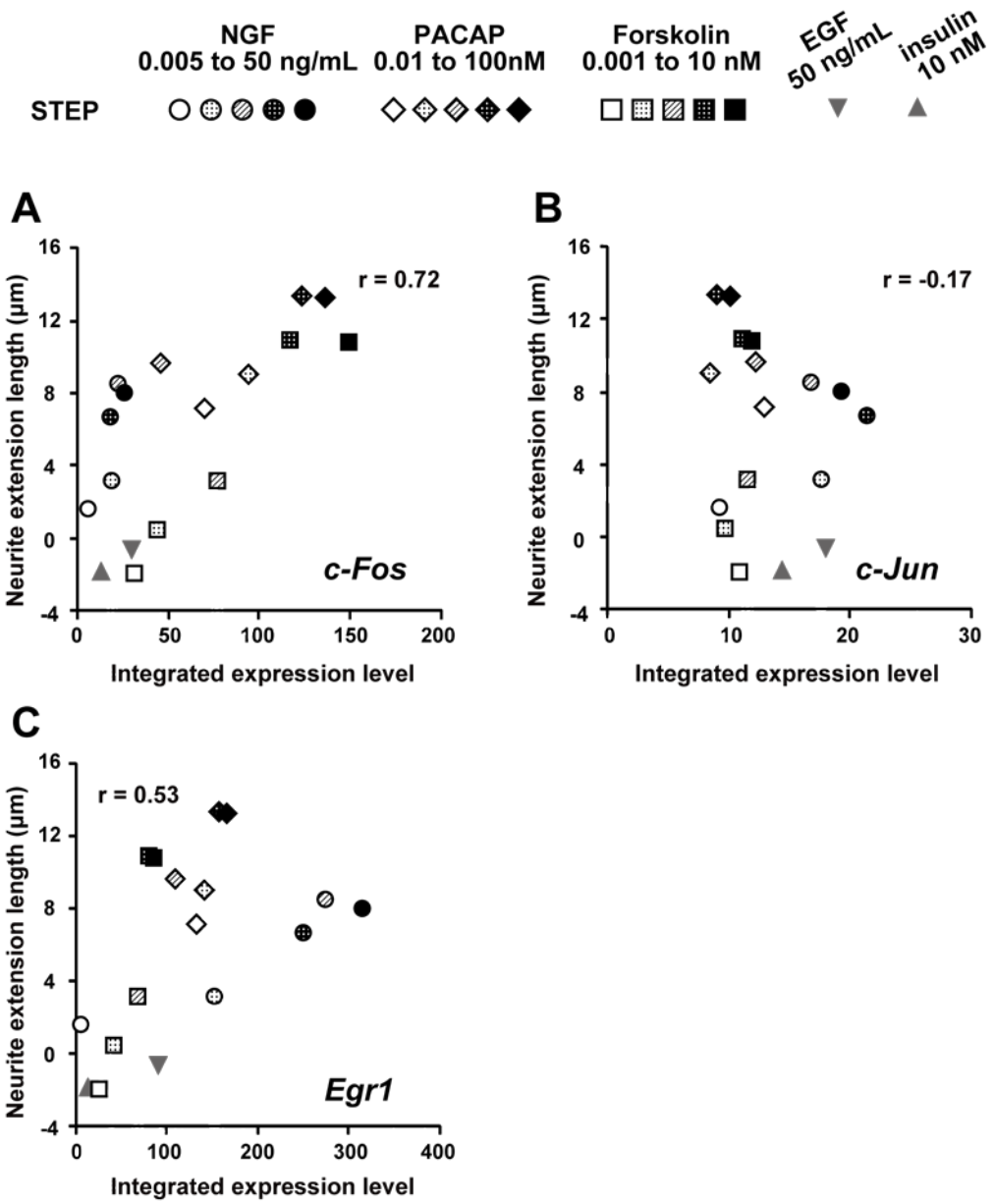


Fig. 25. Correlation of the expression levels of the immediate early genes with the neurite extension lengths

(A-C) A scatter plot of the neurite extension length (12-24 h) versus the integrated levels of *c-Fos* (A), *c-Jun* (B) and *Egr1* (C) expressions (0-12 h). PC12 cells are treated with a step stimulation with NGF (0.005, 0.05, 0.5, 5, 50 ng/mL), PACAP (0.01, 0.1, 1, 10, 100 nM), and forskolin (0.001, 0.01, 0.1, 1, 10 nM) and with a step stimulation with EGF (50 ng/mL) and insulin (10 nM). Each Pearson's correlation coefficient (r) is indicated (*c-Fos*, 0.72; *c-Jun*, -0.17; *Egr1*, 0.53).

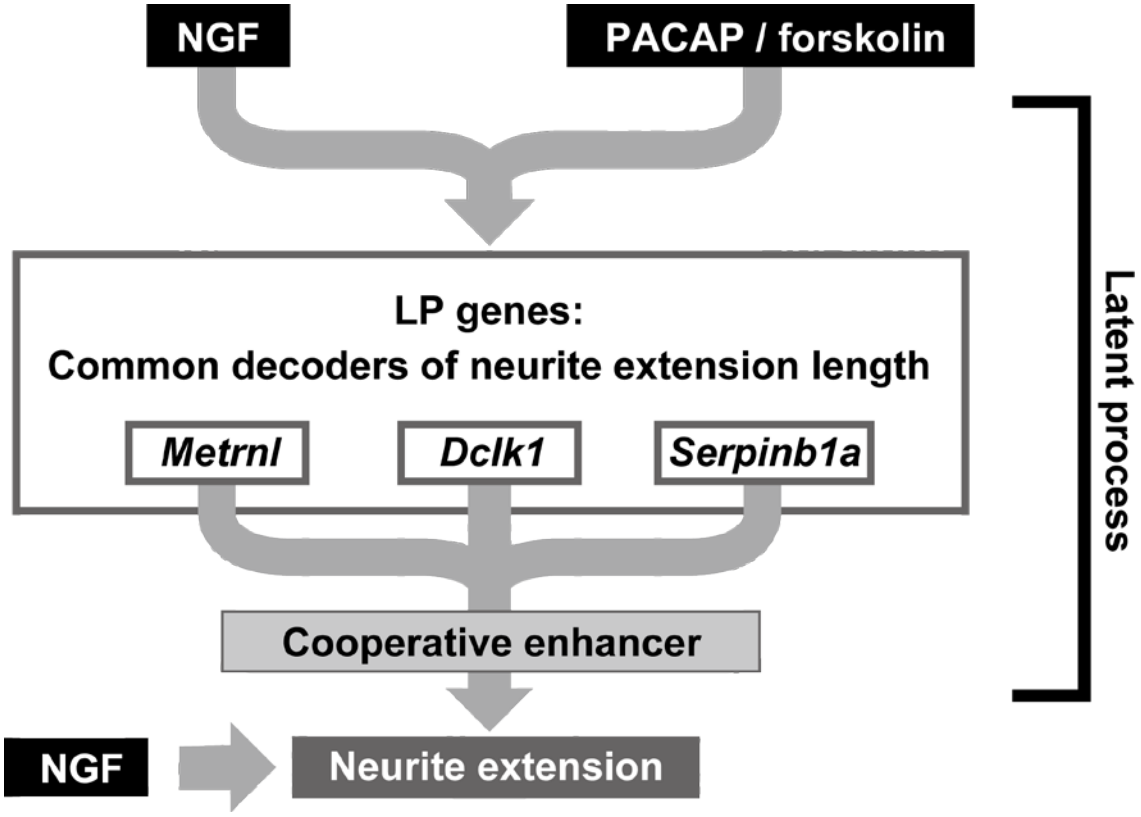


Fig. 26. The roles of LP genes as common decoders of neurite extension length

The expression of the LP genes, *Metrnl*, *Dclk1*, and *Serpinb1a*, during the latent process decodes information for neurite extension length before neurite extension starts. The LP genes cooperatively enhance NGF-induced neurite extension. Thus, the LP genes are common decoders of neurite extension length and cooperative enhancers of neurite extension.

10. Table

Table 1. The primer sequences used for qRT-PCR and the siRNA oligo sequences used for siRNA-mediated knockdown in PC12 cells

Gene	F/R[1]	Primers for qRT-PCR	G/P[2]	siRNA oligo sequences
Plasminogen activator, urokinase receptor (Plaur)	F	GGCTGGACCCAGGAACTTTT	G	GGAACGAGCACCUUUGGAUGU
	R	CGCCTGTCCTCAAAGATGGA	P	AUCCAAAGGUGCUCGUUCCCC
Sialidase 2 (Neu2)	F	CGAAGAGGGAGCTACAATGGA	G	CUAGUUUUUGGAUGAAUAUUA
	R	GGCTTGGGTCACCACTTCCT	P	AUAUUCAUCCAAAAACUAGGC
LOC302733	F	TGCACCCTATCAGCAGCTACA	G	CAUUGACACUGCACAAUGUGC
	R	CCTCGGGTCTGAAAGGGTAGA	P	ACAUUGUGCAGUGUCAAUGCA
Meteorin, glial cell differentiation regulator-like (Metrnl)	F	CGGCCCAACACCTTCTCA	G	GAAAAAACUGGAGAACUAAGA
	R	CCCCAGAGGAGTCCCTGAA	P	UUAGUUCUCCAGUUUUUUCCA
Small proline-rich protein 1 (SPRR1)	F	CCATAGCCAAGCCTGAAGATCT	G	CAUUGAAUGGCUAAUCUUUCC
	R	AGGCAATGGGACTCATAAGCA	P	AAAGAUUAGCCAUUCAAUGAG
Dual specificity phosphatase 5 (Dusp5)	F	GGAAGCTGTTGGTGTAAGGAGAA	G	GAAUUUCGGCCAACUUUGUCU
	R	GCCCTCGGTGAGCAAGAA	P	ACAAAGUUGGCCGAAAUUCAA
XM225257	F	CCCTGGTGAAGCCGTCAT	G	GUCCUCACCUGAUAAGCUUUC
	R	CATGTCCATGTGAGGTCACTCCTA	P	AAGCUUAUCAGGUGAGGACCC
Poliovirus receptor (PVR)	F	ATGAGTGTCAGATTGCCACGTT	G	GCAUUGUAUUCUGGAAAUACA
	R	TCGGGCGAACACCTTCAG	P	UAUUUCCAGAAUACAAUGCCU
Tryptophan hydroxylase 1 (Tph1)	F	CATAACCAGCGCCATGAATG	G	CAGAAUUUGAGAUUUUUGUGG
	R	CTGGGCCACCTGCTGACT	P	ACAAAAAUCUCAAAUUCUGAG
AF465254	F	GCTGATAACTCCGTTTCTCCTATGA	G	GGUUUCAACAGAUUCUUCAAA
	R	TCGTAGGAACAGTCCCAAGAACTA	P	UGAAGAAUCUGUUGAAACCUG
Matrix metallopeptidase 13 (MMP13)	F	ACGTTCAAGGAATCCAGTCTCTCT	G	GCUAUAUCUACUUUUUCAAUG
	R	GGATAGGGCTGGGTCACACTT	P	UUGAAAAAGUAGAUAUAGCCA
Tribbles homolog 1 (Trib1)	F	CGGCTCTTCAAGCAAATTGTT	G	GAAGAUACGCACAUGAUAAAG
	R	CCCAGCACAATGGCTGACT	P	UUAUCAUGUGCGUAUCUUCUA
cAMP responsive element modulator (CREM)	F	CCTTGCCCCAAGTCACATG	G	CCUUUAUUGCCAUAAAGCAGA
	R	AGCAGTAGTAGGAGCTCGGATCTG	P	CCUUUAUUGCCAUAAAGCAGA
Doublecortin-like kinase 1 (Dclk1)[3]	F	GGCTATTGTCAGGTCA	G	CUCAUAGAAGUUAAUGGAACC
	R	AGTGGAGAGCTGACTG	P	UUCCAUUAACUUCUAUGAGUU
RGD1305778	F	CGCTGGGATCGTCTGCAT	G	GGUCAAAUGGGCAUUUUCUGU
	R	GTTGTGATGTAATACGCAATGATGAC	P	AGAAAAUGCCCAUUUGACCCG
RGD1310139	F	CTGAAATGCAACAGTTATCGACATC	G	GUAUAGAUCGAAUAACUCAAA
	R	GCCGAGATAGCCAGTTTAGGAA	P	UGAGUUAUUCGAUCUAUACUG
Serine proteinase inhibitor, clade B, member 1a (Serpnb1a)	F	TGGGTGTGGTGGACAGCAT	G	GCUGAAUAAGAAAAACACAAA
	R	CTCCCACATCCCCTTGAAGTAG	P	UGUGUUUUUCUUAUUCAGCCG
Nucleobindin 1 (Nucb1)	F	CGGGACCTAGAGCTGCTGAT	G	CAAAUAAACAUUAGCAUAUCU
	R	TCGTAGCGTTTGAACTCTTCATG	P	AUAUGCUAAUGUUUAUUUGUG

Prlfp	F	ACGAGGCAGAGATGCAACTGT	G	GUAUACUUACCUUGAUUUUCG
	R	GGTTTGACACCACCATCAGAAG	P	AAAAUCAAGGUAAGUAUACUC
Polo-like kinase 2 (Plk2)	F	GCCCCACACCACCATCA	G	CUGAUAAAGCCUUAAUGAUGC
	R	GGTCGACTATAATCCGCGAGAT	P	AUCAUUAAGGCUUUAUCAGAC
RGD1306658	F	GGTGGGAAGCCTTGCTTAGA	G	CAGAAUUAUGGAAUAAAAUAU
	R	GTCGTTGACCCCATGCATACT	P	AUUUUAUUCCAUAAUUCUGAU
Vasoactive intestinal polypeptide (VIP)	F	GCAAACGAATCAGCAGTAGCAT	G	CUUUAAAAAAUAUAUUUAAUG
	R	ATCTGTGAAGACTGCATCAGAGTGT	P	UUAAAUAUAUUUUUUAAAGAA
TrkA	F	AGAGTGGCCTCCGCTTTGT	G	AUGUGGACAGAGGAGCAAATT
	R	ATTGGAGGAGAGATTCAGGTGACT	P	UUUGCUCCUCUGUCCACAUTT
c-Fos	F	CAACGAGCCCTCCTCTGACT	G	AAUAAACUCCAGUUUUUCCUU
	R	TGCCTTCTCTGACTGCTCACA	P	GGAAAAACUGGAGUUUAUUUU
c-Jun	F	GGCTGTTCATCTGTTTGTCTTCAT	G	AAAUUAUAUACUUUAUUACAA
	R	CTGCGGGCGCTGGAT	P	GUAAUAAAGUAUAUAAUUUUU
Egr1	F	CATGAACGCCCGTATGCTT	G	GACUUAAAGGCUCUUAAUAAC
	R	GCTCATCCGAGCGAGAAAAG	P	UAUUAAGAGCCUUUAAGUCCU
Fra1	F	CGCCCAGTGCCTTGTATCTC	G	UAUAUCAAAGCAUAACAUGUU
	R	TGCAGTGCTTCCGGTTCA	P	CAUGUUAUGCUUUGAUAUAGA
JunB	F	GGCTTTGCGGACGGTTTT	G	UUAUAUUCAAUAUGAAUUCAG
	R	GGCGTCACGTGGTTCATCT	P	GAAUUCAUAUUGAAUAUAAUA
ACTB (β-actin)	F	CCCGCGAGTACAACCTTCT	G	No oligo
	R	CGTCATCCATGGCGAACT	P	No oligo

Primer sets and siRNA oligo sequences were designed as described in Materials and methods.

[1]F/R: the direction of the primer, forward primer (F) or reverse primer (R).

[2]G/P: the direction of the siRNA oligo sequences, guide (G) or passenger (P).

[3]The primer and siRNA of *Dclk1* are designed for common region of its splicing variants.

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