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

Kanako Watanabe[1], Yuki Akimoto[1], Katsuyuki Yugi[1], Shinsuke Uda[1], Jaehoon Chung[1,*], Shinichi Nakamuta[2], Kozo Kaibuchi[2] and Shinya Kuroda[1,3,4,‡]

[1]Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan
[2]Department of Cell Pharmacology, Nagoya University, Graduate School of Medicine 65 Tsurumai, Showa-ku, Nagoya, Aichi 466-8550, Japan
[3]CREST, Japan Science and Technology Corporation, Bunkyo-ku, Tokyo 113-0033, Japan
[4]Department of Computational Biology, Graduate School of Frontier Sciences, University of Tokyo, Hongo 7-3-1, Bunkyo-ku, Tokyo 113-0033, Japan

*Present address: Nomura Research Institute Ltd, Seoul Branch, 11th Floor, Youngpoong Building, 33 Seorin-Dong Chongro-ku, Seoul 110-752, Korea
‡Author for correspondence (skuroda@bi.s.u-tokyo.ac.jp)

Summary

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 as a preparation step that decodes extracellular stimulus information into cellular functions; however, molecular mechanisms of this process remain unknown. We identified *Metrnl*, *Dclk1* and *Serpinb1a* as genes that are induced during the latent process (LP) with distinct temporal expression profiles and are required for subsequent neurite extension in PC12 cells. The LP genes showed distinct dependency on the duration of ERK activity, and they were also induced during the latent process of PACAP- and forskolin-induced cell differentiation. Regardless of neurotrophic factors, expression levels of the LP genes during the latent process (0–12 hours), but not phosphorylation levels of ERK, always correlated with subsequent neurite extension length (12–24 hours). Overexpression of all LP genes together, but not of each gene separately, enhanced NGF-induced neurite extension. The LP gene products showed distinct spatial localization. Thus, the LP genes appear to be the common decoders for neurite extension length regardless of neurotrophic factors, and they might 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.

Key words: Cell differentiation, ERK, Gene expression, Neurite extension, Neurotrophic factor

Introduction

Cell fates, such as proliferation and differentiation, are determined by the cell response to extracellular signals and require a series of functionally distinct processes, including signal transduction, transcription, translation and function of de novo proteins. Accordingly, these sequential steps can be regarded as preparation for the future phenotype. Each preparation step corresponds to a latent process in cell differentiation in PC12 cells (Chung et al., 2010) and to the G1 phase in the cell cycle (Jones and Kazlauskas, 2001). Conceivably, the future phenotype is predetermined during the latent process.

PC12 cells, an adrenal chromaffin cell line, are a well-characterized model of nerve cells, which respond to nerve growth factor (NGF) by differentiating into a sympathetic neuron-like phenotype (Burstein et al., 1982; Greene and Tischler, 1976). 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). We previously found that NGF-dependent differentiation of PC12 cells can be divided into two processes: a latent process and a subsequent extension process (supplementary material Fig. S1) (Chung et al., 2010). 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 hour was sufficient to trigger the process. The latent process activity for the subsequent neurite extension was sustained for 12 hours after stimulation and eventually decreased. The extension process showed rapid neurite extension and required ERK and phosphoinositide 3-kinase (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.

In the present study, we identified three genes, *Metrnl*, *Dclk1* and *Serpinb1a*, as the latent process (LP) genes that induce expression during the latent process and are required for subsequent neurite extension. 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. Furthermore, overexpression of all LP genes

together, but not that of each LP gene separately, cooperatively enhanced NGF-induced neurite extension. We also found that *Metrnl* and *Dclk1* were required for neurite extension in rat hippocampal neurons. Given that expression of the LP genes occurred during the latent period (0–12 hours) and before the extension period (12–24 hours), our findings demonstrate that the LP genes are common decoders of neurite extension length regardless of neurotrophic factors and cooperative enhancers of neurite extension.

Results

Identification of the LP genes expressed during the latent process and required for subsequent neurite extension

We 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 (supplementary material Table S1) and the expression of these genes was examined by using quantitative real-time polymerase chain reaction (qRT-PCR). Twenty-two of the 30 genes were induced during the latent process in response to NGF stimulation (supplementary material Fig. S2A–C). Temporal expression patterns of these genes fell into three groups: (1) transient expression with a peak at 3 hours after the stimulation (four genes); (2) transient and sustained expression with a peak at 3 hours and a duration of 12 hours, respectively (12 genes); and (3) sustained expression with a duration of 24 hours (six genes).

We examined whether these 22 genes were essential for the neurite extension. We tested the effect of specific knockdown of the genes on NGF-induced neurite extension (Fig. 1A). The siRNAs targeting 3 of 22 genes significantly inhibited neurite extension; these genes were *Metrnl*, *Dclk1* and *Serpinb1a*. We confirmed that expression of the three genes was suppressed by the addition of the siRNAs (supplementary material Fig. S2D). 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* or *Dclk1* [*Carp* (*Ania-4*), see below] or *Serpinb1a* (supplementary material Fig. S2E). *Metrnl* and *Dclk1* rescue constructs partially rescued the inhibited neurite extension caused by the siRNA, whereas the *Serpinb1a* rescue construct did not show any statistically significant effect. The partial and non-significant effect of LP gene rescue constructs might have occurred because of the distinct expression profiles between endogenous and the expressed genes. Taken together, we identified the three genes as the LP genes because they were induced during the latent process and were essential for neurite extension. The gene product of *Metrnl* is a protein akin to meteorin, a secreted protein expressed in undifferentiated neural progenitors (Nishino et al., 2004). *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*) and *Dclk1-short* (Schenk et al., 2007; Vreugdenhil et al., 1999). In this study, we used the common siRNA and primers for both variants of *Dclk1*, except that cDNA of *Carp* (*Ania-4*) and *Dclk1-short*-specific siRNA were used for rescue and overexpression experiments and knockdown in hippocampal neurons, respectively. *Serpinb1a* is a member of a subgroup of the serine protease inhibitors (serpins) superfamily

that is important in regulating several biological processes, including neurogenesis (Parmar et al., 2002; Silverman et al., 2001). The time courses of *Metrnl*, *Dclk1* and *Serpinb1a* expression were classified into temporal expression pattern groups 1, 2 and 3, as described at the start of this section, respectively (Fig. 2B–D and supplementary material Fig. S2A–C). However, because the expression of some of the other 19 genes was not suppressed by siRNA (supplementary material Fig. S2D), we cannot exclude the possibility that some of them are also LP genes.

We examined whether the LP genes are also required for the 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 hours to induce the latent process and then with NGF for an additional 12 hours to induce neurite extension. The siRNAs targeting the LP genes significantly suppressed the expression of the LP genes (supplementary material Fig. S2F,G) 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. 1H,I). These results indicate that the LP genes are commonly essential for the NGF-, PACAP- and forskolin-induced latent process.

Expression of LP genes in response to pulse or step stimulation with NGF

Because pulse stimulation with NGF induces the latent process similar to step stimulation with NGF (Chung et al., 2010), we examined whether pulse stimulation with NGF can also induce expression of the LP genes (Fig. 2A–D) and found that it could (Fig. 2B–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 only in the initial 3 hours after stimulation (Chung et al., 2010). Consistent with this notion, expression of all LP genes increased within 3 hours, 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 hour negatively regulates the expression of *Metrnl* (Fig. 2B).

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, we examined ERK activation by step and pulse stimulations with NGF and found that activation of ERK was similarly sustained for at least 12 hours by both types of stimulation (supplementary material Fig. S3A,B).

We next examined whether ERK activity is required for expression of LP genes. Prior addition of U0126, a MEK inhibitor, almost completely inhibited ERK activation (supplementary material Fig. S3C) and concomitantly suppressed LP gene expression (Fig. 2E–H). We investigated whether sustained ERK activation is needed for the expression of LP genes by the addition of U0126 at 1 or 3 hours after NGF
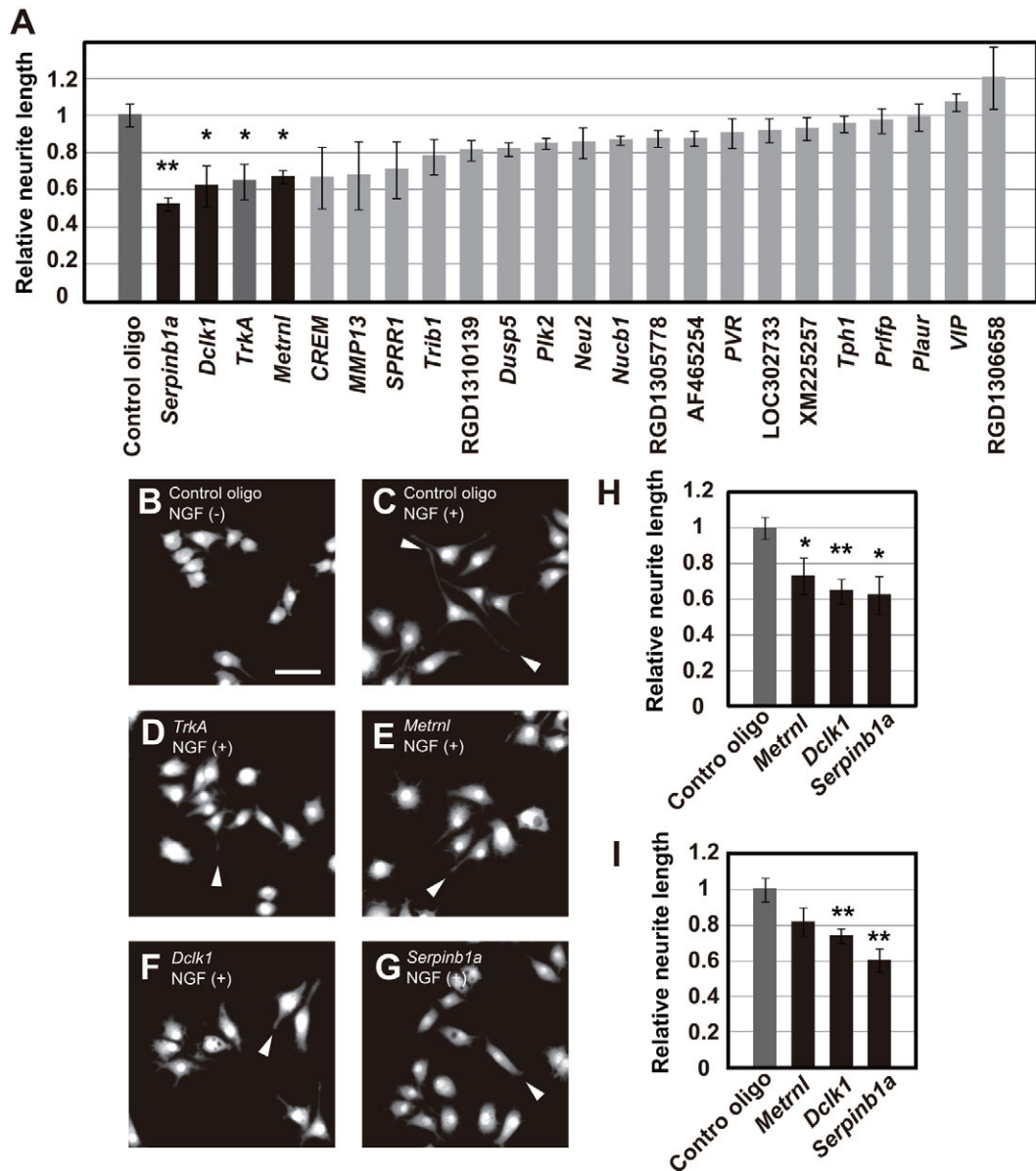
Fig. 1. Effects of siRNA-mediated knockdown of LP gene candidates on neurite extension. (**A**) PC12 cells were treated with the indicated siRNA for 12 hours and then subjected to differentiation by NGF (50 ng/ml) for 24 hours. The relative neurite lengths of the cells transfected with the indicated siRNA compared with that of cells transfected with negative control siRNA are shown. As a positive control, siRNA 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's *t*-test was used to evaluate the statistical significance of differences (**P<0.01, *P<0.05). See also supplementary material Fig. S2D and Table S1. (**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 hours. The termini of neurites are highlighted with arrowheads. (**H,I**) PC12 cells were treated with the indicated siRNAs for 12 hours followed by PACAP (100 nM; H) or forskolin (10 nM; I) treatment for 12 hours and then treated with NGF for additional 12 hours. The neurite lengths were quantified and statistically analyzed as described in A. See also supplementary material Fig. S2F,G. Scale bar: 50 μm.

stimulation. The expression of *Metrnl* was not suppressed (Fig. 2F); however, when ERK activity was inhibited after 1 hour, the peak level of *Metrnl* at 3 hours was significantly higher than that in the absence of U0126. This result suggests that the expression of *Metrnl* after 1 hour is negatively regulated by sustained ERK activity. The expression of *Dclk1* was suppressed when ERK activity was inhibited after 1 hour, whereas it was not suppressed when ERK activity was inhibited after 3 hours (Fig. 2G). The expression of *Serpinb1a* was suppressed when

ERK activity was inhibited after 1 hour, whereas it was suppressed only at 24 hours after the NGF stimulation when ERK activity was inhibited after 3 hours (Fig. 2H). We confirmed the inhibition of ERK activity by the addition of U0126 at 1 hour after the NGF stimulation (supplementary material Fig. S3C). Taken together, expression of all LP genes showed distinct dependency on the duration of ERK activity. ERK activation during the initial 1 hour was sufficient to induce *Metrnl* expression. By contrast, the expression of *Dclk1* required ERK activation for 3 hours and
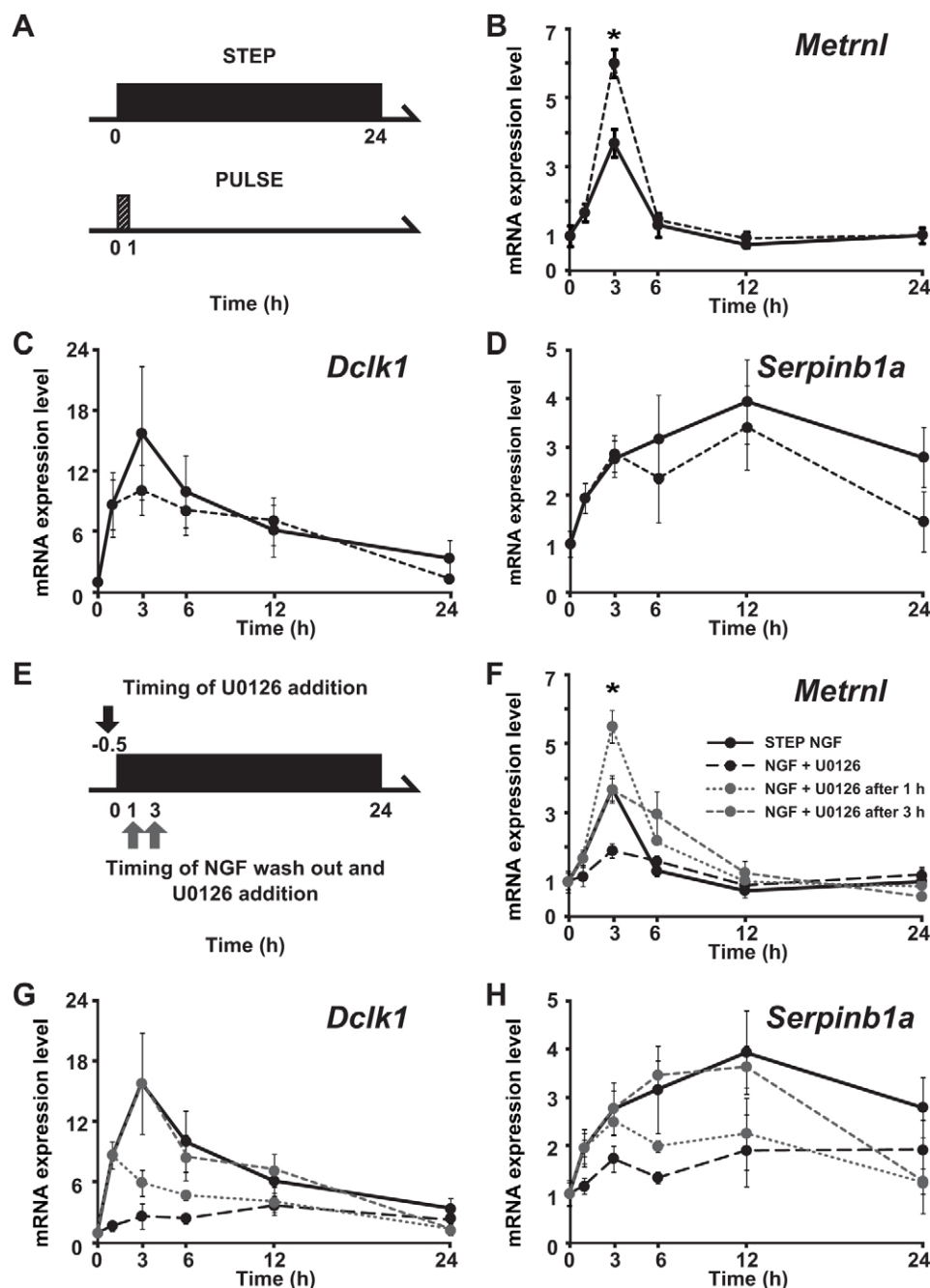
Fig. 2. **Temporal expression patterns of the LP genes in response to pulse or step stimulation with NGF and in the presence of U0126.** (**A**) Stimulation scheme: step (upper) and pulse (lower) stimulation by NGF (50 ng/ml). For step stimulation, PC12 cells were continuously exposed to NGF. For pulse stimulation, cells were exposed to NGF for 1 hour, then NGF-containing medium was washed out and replaced with NGF-free medium. Time courses of expression of *Metrnl* (**B**), *Dclk1* (**C**) and *Serpinb1a* (**D**) were determined by qRT-PCR at 1, 3, 6, 12 and 24 hours of pulse (dashed lines) and step (solid lines) stimulation with NGF. (**E**) Stimulation scheme: arrows represent the timing of the addition of U0126 and that of NGF withdraw accompanied by the addition of U0126. (**F,G**) PC12 cells were treated in the absence (black solid lines) or the presence of U0126 (50 μM) at 30 minutes before stimulation with NGF (50 ng/ml; black dashed lines) or stimulated with NGF followed by withdrawal of NGF and the addition of U0126 at 1 hour (gray dotted lines) or 3 hours (gray dashed lines) after stimulation. Time courses of expression of *Metrnl* (F), *Dclk1* (G) and *Serpinb1a* (**H**) were determined by qRT-PCR. The values are shown as the means ± s.e.m. The Student's *t*-test was used to evaluate the statistical significance of differences in expression levels at 3 hours after pulse and step stimulation (*$P < 0.05$ in B) and at 3 hours in the absence of U0126 and when U0126 was added after 1 hour (*$P < 0.05$ in F).

expression of *Serpinb1a* required ERK activation for longer than 3 hours. Sustained ERK activation is required not only for induction but also for maintenance of the expression of *Dclk1* and *Serpinb1a*. We also examined the expression of the LP genes by adding K252a, an inhibitor of the neurotrophin receptor Trk, and found that, similar to U0126, K252a completely suppressed LP gene expression (supplementary material Fig. S3D–F) and ERK activation by NGF (supplementary material Fig. S3C), indicating that NGF- and TrkA-mediated expression of LP genes mainly depends on ERK activity. By 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 (supplementary material Fig. S3C,G–I), 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 *Gap43* (Ginty et al., 1991) or neurite extension (Yao et al., 1998), whereas ERK is necessary for both (Cowley et al., 1994; Marshall, 1995). We also confirmed that U0126 inhibited NGF-induced neurite extension and H-89 did not (supplementary material Fig. S5A).

We next investigated how LP genes are transcriptionally regulated. We examined the effect of siRNAs-mediated knockdown of the immediate early genes, such as *Fos* and *Jun* (Eriksson et al., 2007), on NGF-induced expression of the LP genes (supplementary material Fig. S4A–E). The expression of the LP genes was not affected under the conditions in which the expression of *Fos* and *Jun* were suppressed by 64% at 1 hour

(*Fos*) and 51% at 1 hour (*Jun*), respectively. In addition, we examined the 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 (supplementary material Fig. S4F-K). Under the conditions in which the expression of *Egr1*, *Fra1* and *JunB* were suppressed by 53% at 1 hour (*Egr1*), 67% at 3 hours (*Fra1*), and 72% at 1 hour (*JunB*), respectively, *Dclk1* expressions were inhibited (supplementary material Fig. S4J). Moreover, when the expression of *Fra1* was suppressed, *Serpinb1a* expression was also inhibited (supplementary material Fig. S4K). These results indicate that *Egr1*, *Fra1* and *JunB* are required for *Dclk1* expression and that *Fra1* is required for *Serpinb1a* expression. This also highlights the fact that the expression of LP genes is differently regulated by the immediate early genes.

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

We measured the LP gene expression during the latent period (0–12 hours) and neurite extension length during the extension period (12–24 hours) in response to other neurotrophic factors, including PACAP, forskolin, epidermal growth factor (EGF) and insulin (Fig. 3 and supplementary material Fig. S5B,C). We 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 (Fig. 3A,B), 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. 3B). This result indicates that PACAP and forskolin can induce the latent process.

We 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. 3C). Likewise for neurite extension length, PACAP and forskolin were more potent for induction of LP gene expression than NGF. By 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. 3C).

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 might trigger the PACAP- and forskolin-dependent latent process that is required for neurite extension.

Expression of the LP genes, but not ERK phosphorylation, correlates with neurite extension length

We examined the quantitative relationship between the LP genes and neurite extension length. We 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. 4). Higher concentrations of NGF induced LP gene expression, whereas lower concentrations did not (Fig. 4B). Although ERK phosphorylation showed a graded response, LP gene expression showed a switch-like response (Fig. 4A,B,D). By contrast, PACAP induced the expression of LP genes in a dose-dependent manner, which appeared as a graded response

(Fig. 4B,D). Similarly, forskolin induced the LP gene expression in a dose-dependent manner, which appeared as a graded response (Fig. 4B,D). 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 did (Fig. 4A,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. 2E–H), whereas U0126 inhibited only the expression of *Metrnl* but not that of *Dclk1* or *Serpinb1a* induced by PACAP and forskolin (supplementary material Fig. S5D–I). 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. 4), suggesting that *Metrnl* expression is regulated not only by ERK, but also by other signaling pathways at least in response to PACAP and forskolin. We also examined the effect of H-89 on the PACAP- or forskolin-induced expression of the LP genes (supplementary material Fig. S5J–O). The expression of *Metrnl* was partially suppressed, whereas that of *Dclk* was not affected and that of *Serpinb1a* was enhanced. Taken together with the effect of H-89 on NGF-induced LP gene expression (supplementary material Fig. S3C,G–I), the expression of the LP genes shows 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 (supplementary material Fig. S5A). 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 below). 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 (supplementary material Fig. S6A,B). We found that neurite extension was observed only during the late phase (18–24 hours) but not during the early phase (12–18 hours) of the extension period (supplementary material Fig. S6E,F), indicating that the effect of the LP genes for neurite extension was evoked with a 6-hour 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. 4C,D and supplementary material Fig. S6C–F). 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,
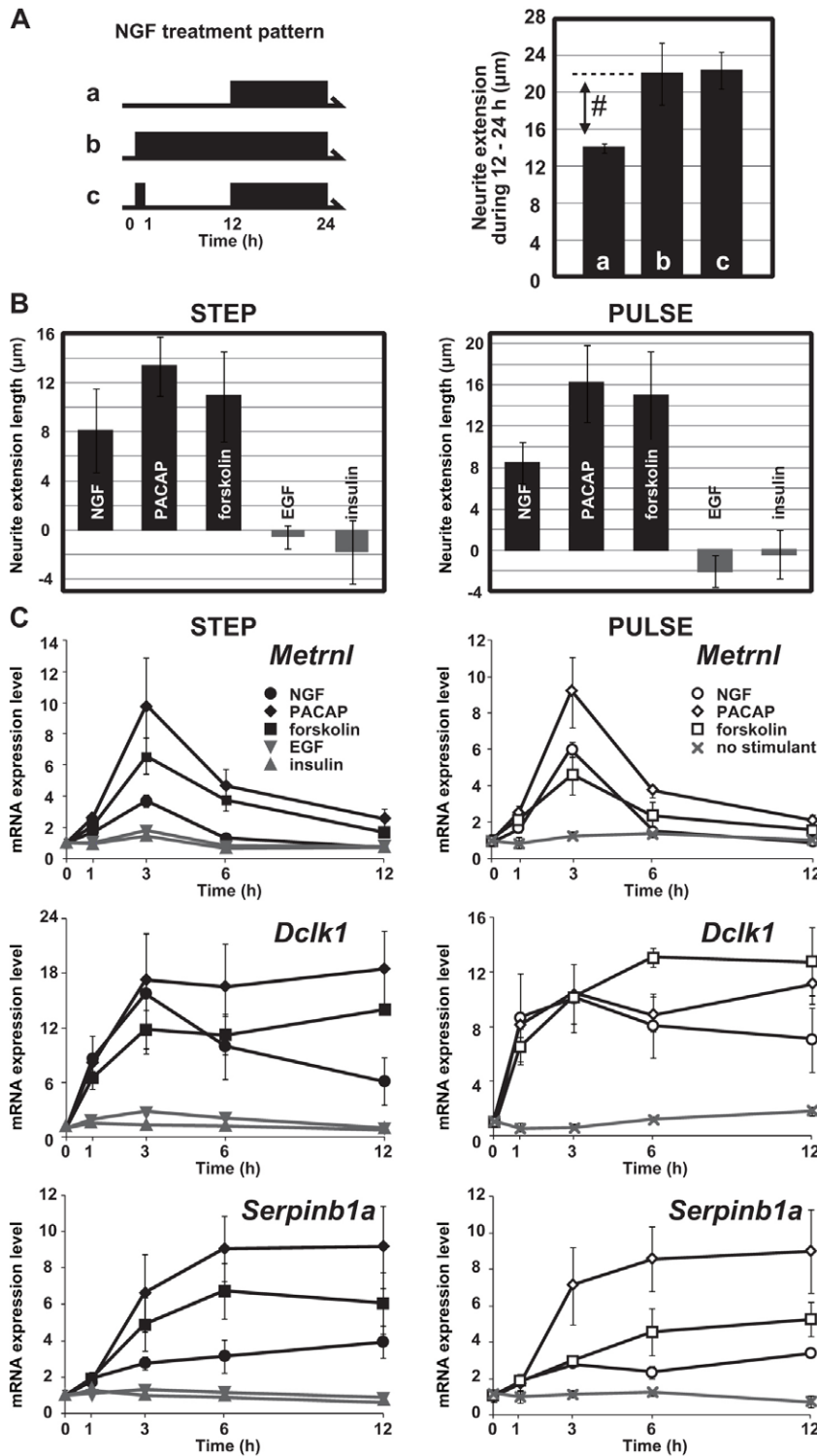
Fig. 3. **Induction of the LP gene expression during the latent process and neurite extension during the extension period.** (**A**) Scheme of NGF treatment pattern (left) and neurite length extended during the extension period (12–24 hours; right). Neurite extension length (#) was measured by subtraction of neurite length extended during the extension process (12–24 hours) of untreated cells by the first stimulation from which the neurite length extended during the extension process (12–24 hours) 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.

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. 5B–D). By contrast, the integrated phosphorylation levels of ERK during the latent process were not correlated with the neurite extension lengths ($r=0.20$; Fig. 5A). When the peak amplitudes of the LP genes and ERK phosphorylation were used instead of their integrated levels,

similar results were obtained (supplementary material Fig. S7). 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 hours) and neurite extension was particularly induced during the late extension period (18–24 hours), information of neurite extension length is
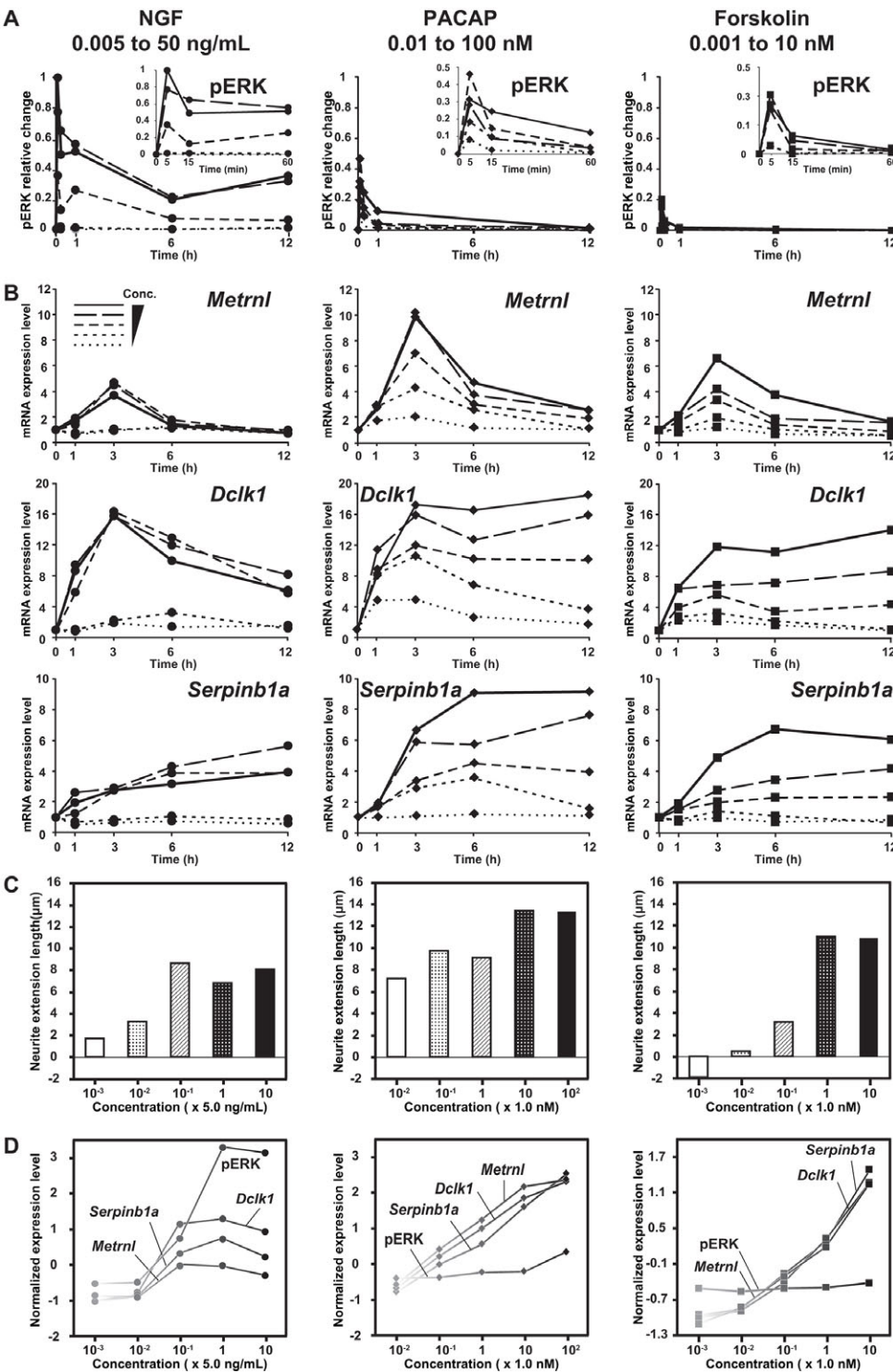
Fig. 4. Temporal expression pattern of LP genes and phosphorylated ERK during the latent period and neurite extension length during extension period. (**A**) The time course of ERK phosphorylation (pERK) in response to step stimulation with NGF (0.005, 0.05, 0.5, 5, 50 ng/mL), PACAP (0.01, 0.1, 1, 10, 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 minutes. (**B**) The time course of expression of the LP genes in response to step stimulation with NGF, PACAP and forskolin. Their expression was quantified by qRT-PCR. The values represent means of triplicate samples. (**C**) Step stimulation with NGF, PACAP and forskolin dose response of the neurite extension lengths. The values represent means of triplicate samples. (**D**) Step stimulation with NGF, PACAP and forskolin dose response of the integrated levels of ERK phosphorylation (0–12 hours), and those of the expression of the LP genes (0–12 hours). The values represent the normalized integrated levels (mean 0 and variance 1) of ERK phosphorylation and those of the expression of the LP genes.
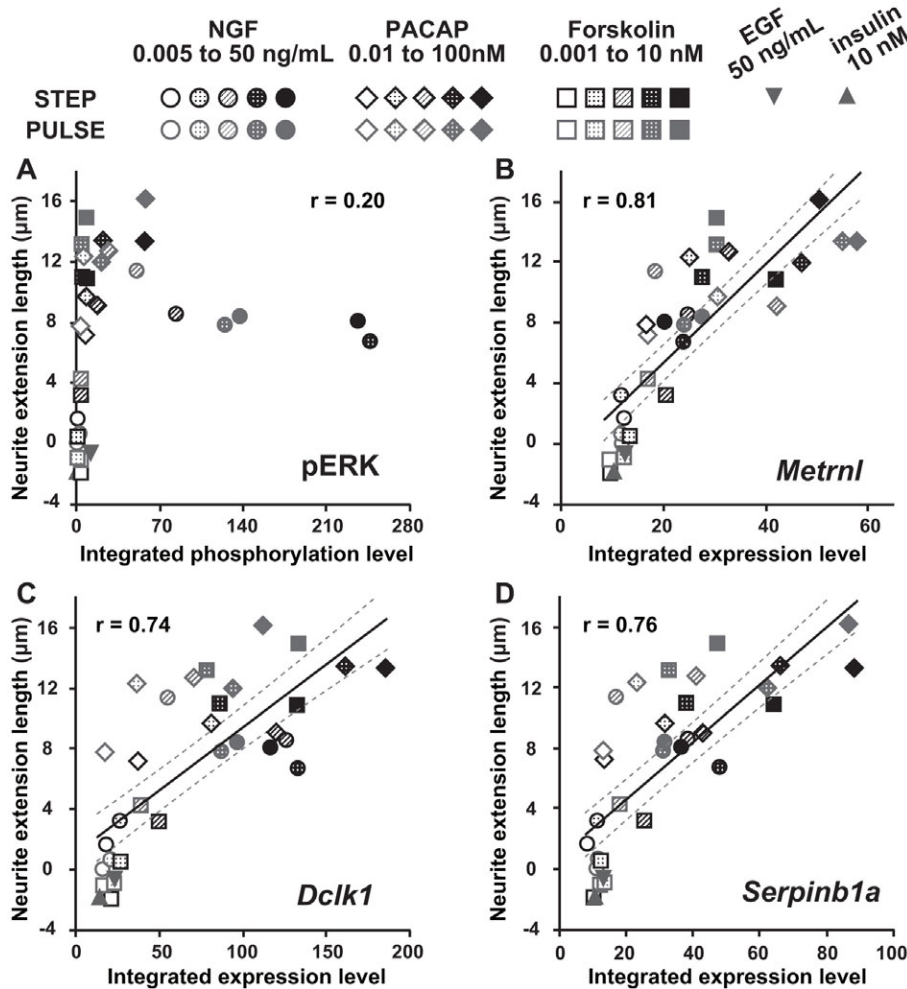
Fig. 5. Correlation of the integrated levels of ERK phosphorylation and LP genes with neurite extension length. A scatter plot of the neurite extension length (12–24 hours) versus ERK phosphorylation (**A**), *Metrnl* (**B**), *Dclk1* (**C**) and *Serpinb1a* (**D**) expression (0–12 hours). PC12 cells were 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 (pERK, 0.20; *Metrnl*, 0.81; *Dclk1*, 0.74; *Serpinb1a*, 0.76). Regression lines (lines) and 95% confidence limits (dashed lines) for *Metrnl*, *Dclk1* and *Serpinb1a* are depicted.

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.

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

We examined whether overexpression of the LP genes enhance neurite extension (Fig. 6A). We 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 (supplementary material Fig. S8). 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. Co-overexpression of all LP genes showed a significant enhancement of NGF-induced neurite extension compared with the control, whereas 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 hours than the latter, even in cells transfected with the control plasmid (Fig. 6A). Therefore, we cannot conclude that the co-overexpression of the LP genes is sufficient to bypass the latent process. We 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. 6B). We found that siRNA targeting each LP gene significantly inhibited the cooperative neurite extension, but the effects of the genes 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.

The role of the LP genes in rat hippocampal neurons

We further examined the role of the LP genes in neurite extension in rat hippocampal neurons. We tested the effect of the siRNA-mediated knockdown of the LP genes on neurite extension of primary dissociated hippocampal neurons (Fig. 6C). The siRNAs targeting *Dclk1-short* and *Metrnl* significantly inhibited neurite extension, but siRNA targeting *Serpinb1a* did not show an inhibitory effect. This result indicated that *Metrnl* or *Dclk1* are required for neurite extension in hippocampal neurons, although whether both genes are involved in the latent process in the neurons remains to be explored. We did not exclude the
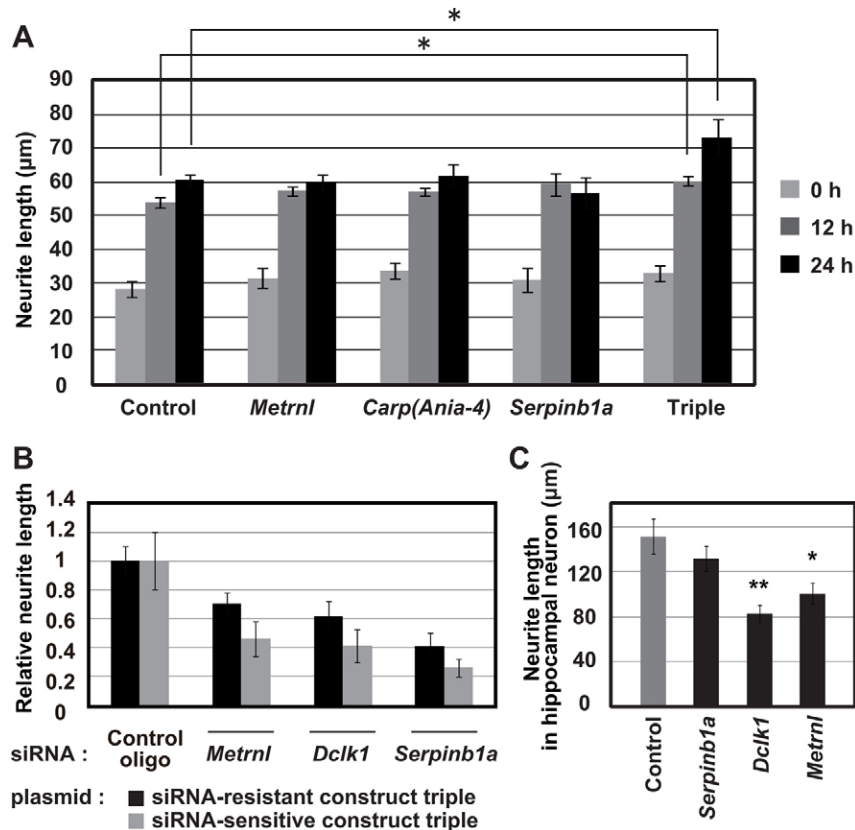
Fig. 6. The role of the LP genes in neurite extension in PC12 cells and hippocampal neurons. (**A**) The effect of overexpression of the LP genes on the neurite extension. *Metrnl*, *Carp* (*Ania-4*), and *Serpinb1a* and all LP genes (triple) were expressed in PC12 cells for 48 hours after transfection, 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 hours) and at 12 hours and 24 hours after NGF. Overexpression of all LP genes together showed a significant enhancement of NGF-induced neurite extension compared with the control. The values are shown as means ± s.e.m. The Student's *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 hours and assayed for neurite extension. (**C**) The role of the LP genes in rat hippocampal neurons. The effect of siRNA-mediated knockdown of the LP genes on neurite length in rat hippocampal neurons. siRNA oligo sequences are shown in supplementary material Table S1 except that of *Dclk1-short* specific siRNA (forward, 5′-UUAAAAGAAAAAAAAUCGCAA-3′; reverse, 5′-GCGAUUUUUUUUCUUUUAACA-3′). The values are shown as the means ± s.e.m. The Student's *t*-test was used to evaluate the statistical significance of differences (**P<0.01, *P<0.05).

possibility of involvement of *Serpinb1a* in the neurite extension in hippocampal neurons because it is possible that other members of the serpin superfamily compensate for the loss of *Serpinb1a* function.
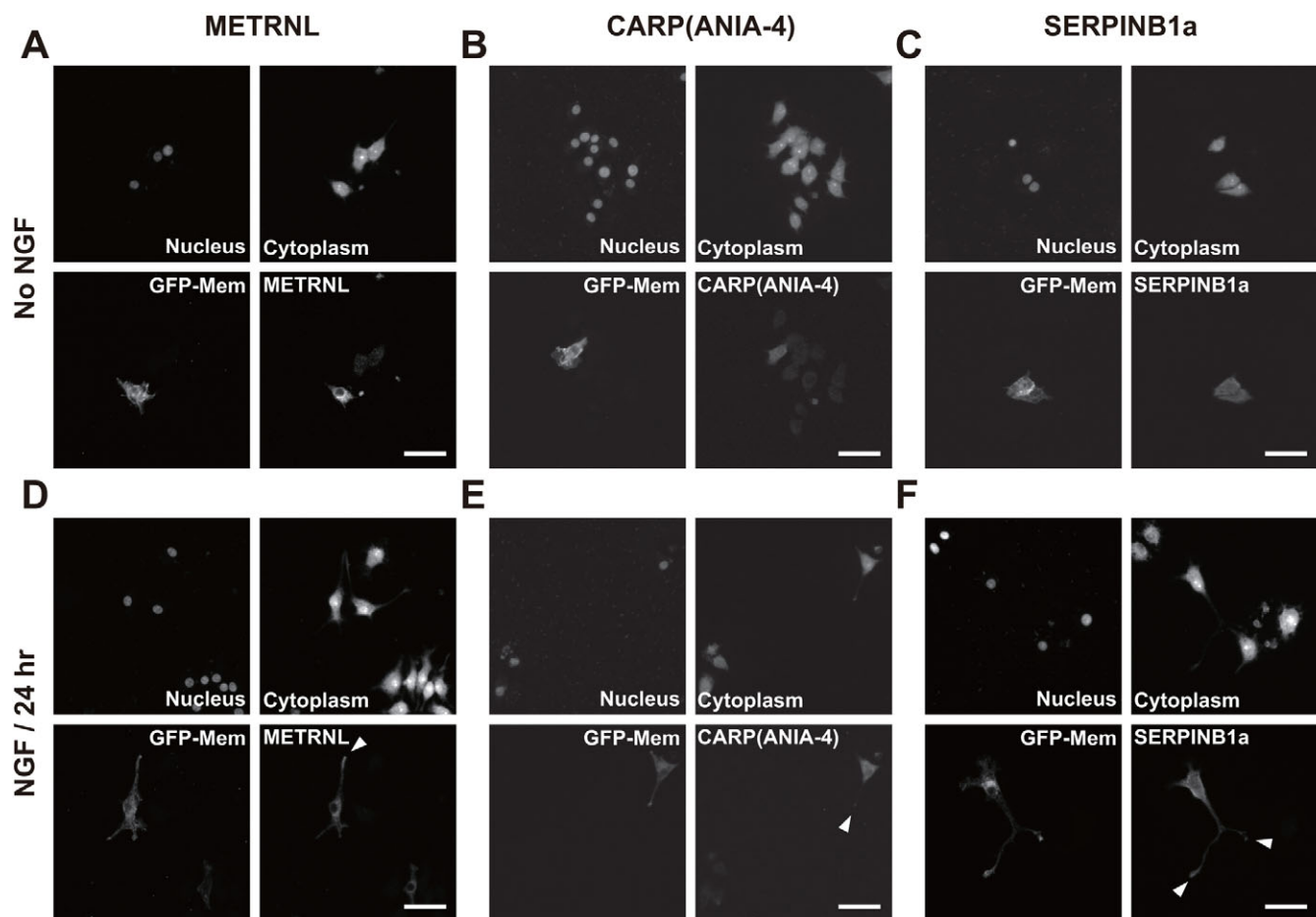
Subcellular localization of the LP gene products

To investigate the potential functions of the LP genes, we analyzed the localization of the LP gene products, METRNL, CARP (ANIA-4), and SERPINB1a, in PC12 cells. We raised rabbit polyclonal antibodies against the LP gene products, but the antibodies did not specifically detect the endogenous proteins (data not shown). Therefore, we transfected a plasmid bearing hemagglutinin (HA)-tagged LP genes and analyzed the localization of the LP gene products (Fig. 7). METRNL showed diffusible distribution throughout the cell body except for the nucleus before NGF stimulation (Fig. 7A) and localized at the terminuses of neurites after NGF stimulation (Fig. 7D, arrowheads). CARP (ANIA-4) showed diffusible localization throughout the cell body including the nucleus before NGF

stimulation (Fig. 7B), but was not markedly localized at the termini of neurites after NGF stimulation (Fig. 7E, arrowheads). SERPINB1a showed diffusible localization throughout the cell body including the nucleus before NGF stimulation (Fig. 7C) and localized at the termini of neurites after NGF stimulation (Fig. 7F, arrowheads). The distinct localization of METRNL, CARP (ANIA-4), 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 might need the latent period to be completed. In addition, the localization of METRNL and SERPINB1a at the termini of neurites after the NGF stimulation suggests that the LP gene products are also involved in the neurite extension process.

Discussion

We identified *Metrnl*, *Dclk1* and *Serpinb1a* as LP genes. The expression levels of the LP genes during the latent process were positively correlated with neurite extension length during the

Fig. 7. The subcellular localization of HA-tagged LP gene products in transformed PC12 cells. (A–F) PC12 cells were transfected with a construct of the indicated HA-tagged LP gene for 24 hours, and incubated with no stimulant (A–C) or treated with NGF (50 ng/ml) for 24 hours (D–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 33342 and CellMask, respectively. The termini of neurites are highlighted with arrowheads. Scale bar: 50 µm.

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. 8). Co-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.

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
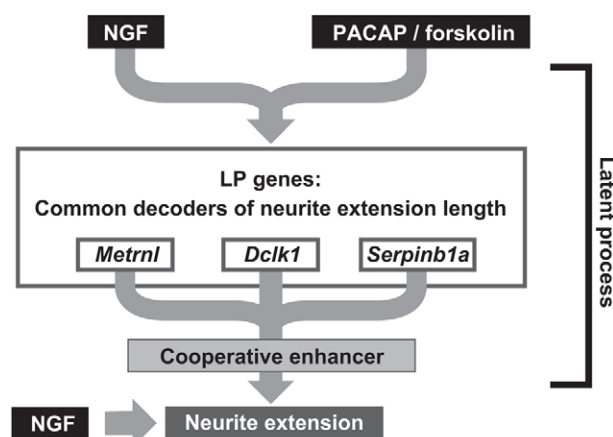
Fig. 8. The role of LP genes as common decoders of neurite extension length. The expression of the LP genes, *Metrnl*, *Dclk1* and *Serpinb1a*, during the latent process decodes information for neurite extension length before neurite extension starts. The LP genes cooperatively enhance NGF-induced neurite extension. Thus, the LP genes are common decoders of neurite extension length and cooperative enhancers of neurite extension.

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., 2011; 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 to be 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 biological 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, which belongs to another branch of the family, 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, we 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 different temporal expression patterns of the LP genes, 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.

We found that *Metrnl* and *Dclk1* are required for neurite extension in rat hippocampal neurons. 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). By contrast, the expression of *Carp* (*Ania-4*) is highly upregulated 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 that 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 a common upstream molecule that might be an initial decoder for neurite extension length. One candidate for the common upstream regulator of the LP genes is a protein of immediate early genes (Hazzalin and Mahadevan, 2002). Although it has been reported that *Fos* and *Jun* expression are required for NGF-induced neurite extension (Eriksson et al., 2007), it was 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, we 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 common upstream regulators 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.

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 hours) 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. 1). However, we cannot exclude the possibility that *Plaur* is an LP gene because we did not confirm the suppression of mRNA of *Plaur* by RNAi (supplementary material Fig. S2D).

The 22 genes that we selected as candidate LP genes were classified into three groups in terms of a temporal pattern (supplementary material Fig. S2A–C). 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 might 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. Because 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.

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.

Materials and Methods
Cell culture
PC12 cells (provided by Masato Nakafuku, Cincinnati Children's Hospital Medical Center, Cincinnati, OH) were cultured in a humidified 5% CO_2 atmosphere at 37℃ in complete medium, Dulbecco's modified Eagle's 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 cells/well) in the complete medium for 24 hours 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 hours or 1 hour followed by an 11-hour interval, were uniformly switched to complete medium containing 50 ng/ml of NGF. Inhibitors for TrkA [200 nM K252a (Sigma)], MEK [50 μM U0126 (Promega, Madison, WI)], or PKA [50 μM H-89 (Sigma)] were added 30 minutes before the first stimulation or 1 hour or 3 hours after first stimulation as indicated in the respective figure legends for (Fig. 2 and supplementary material Figs S3,S5). To wash out stimulants or inhibitors, the culture medium was replaced with an equal volume of complete medium four times.

Quantitative analysis of neurite length
PC12 cells (0.5×10^4 cells/well) were fixed with 10% formalin solution (Wako, Osaka, Japan) for 10 minutes. Cells were washed with phosphate-buffered saline (PBS) and incubated with 1 μg/ml Hoechst 33342 solution (Life Technologies, Carlsbad, CA) and 1 μg/ml CellMask (Life Technologies) in PBS for 1 hour 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.

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 qRT-PCR. qRT-PCR was performed with Power SYBR Green PCR Master Mix (Applied Biosystems); the primers are shown in supplementary material Table S1. As an internal control for normalization, the β-actin transcript was similarly amplified using the primers (supplementary material Table S1). 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.

siRNAs and transfections
Twenty-four hours after plating, the cells were transfected with siRNAs by using Lipofectamine 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 hours. 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 supplementary material Table S1. The siRNA duplexes were chemically synthesized (Sigma).

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 against phosphorylated ERK1/2 (1:2000 dilution; Cell Signaling Technology, Danvers, MA, #9101) or phosphorylated CREB (1:2000 dilution; Cell Signaling Technology, #9198) or pan ERK1/2 (1:2000 dilution; Cell Signaling Technology, #9102) followed by horseradish peroxidase-conjugated rabbit 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).

Rescue and overexpression experiment
The coding regions of rat *Metrnl*, *Carp* (Ania-4), and *Serpinb1a* cDNA, which we cloned from total RNA, were cloned into the vector pIRES-AcGFP1 (Clontech, Mountain View, CA) at the *Nhe*I and *Xho*I sites. We 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) to obtain high resolution of the 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′-GCTCAA-CAAAAAGAATACCAA-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 Lipofectamine 2000 reagent, according to the manufacturer's instructions (Life Technologies). The cells were fixed and stained as described above, except that the transfected cells were stained with anti-GFP antibody (Roche, Cat. No. 11814460001) in Can Get Signal (Toyobo) for 1 hour at room temperature and stained with Alexa-Fluor-488-conjugated goat anti-mouse IgG (Life Technologies) in PBS for 1 hour 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.

Analysis of effects of siRNA on neurite extension in rat hippocampal neurons
Dissociated hippocampal cells were prepared from E18 rats. Cells were incubated for 2 hours and transfected with both siRNA and pCAGGS-myc-GST by the calcium phosphate method. After incubation for an additional 72 hours, the transfected cells were fixed and stained as previously described (Kawano et al., 2005). We used anti-Myc antibody (Santa Cruz, Santa Cruz, CA, sc-789) for detecting Myc-positive cells and anti-Tau antibody (Chemico, Temecula, CA; MAB3420) for visualizing neurites. The stained images of the Myc-positive cells were captured on a confocal laser-scanning microscope LSM 510 (Carl Zeiss, Oberkochen, Germany). The longest neurite length was quantified using LSM Image Browser Rel. 4.2 (Carl Zeiss).

Immunofluorescence staining for analysis of localization

To determine the subcellular localization of LP gene products, two tandem copies of the hemagglutinin (HA) epitope tag sequence was engineered at the C-terminus of METRNL, the N-terminus of SERPINB1a, and the N- and C-termini of CARP (ANIA-4) using a mutagenesis kit for immunofluorescent detection using antibodies against the HA tag. A glycine–glycine linker was inserted between the HA tag and LP gene sequences. The cells were transfected as described above. For immunofluorescence staining, cells were fixed as described above, permeabilized by treatment with methanol for 30 minutes at 4℃. The cells were stained as described above, except that anti-HA antibody (CST, #3724) and Alexa-Fluor-546-conjugated goat anti-rabbit IgG (Life Technologies) were used as primary and fluorescent secondary antibody, respectively.

Acknowledgements

Funding

Supplementary material available online at
http://jcs.biologists.org/lookup/suppl/doi:10.1242/jcs.097709/-/DC1

References

Benarafa, C., Cooley, J., Zeng, W., Bird, P. I. and Remold-O'Donnell, E. (2002). Characterization of four murine homologs of the human ov-serpin monocyte neutrophil elastase inhibitor MNEI (SERPINB1). *J. Biol. Chem.* **277**, 42028-42033.

Burgess, H. A. and Reiner, O. (2000). Doublecortin-like kinase is associated with microtubules in neuronal growth cones. *Mol. Cell. Neurosci.* **16**, 529-541.

Burstein, D. E. and Greene, L. A. (1978). Evidence for RNA synthesis-dependent and -independent pathways in stimulation of neurite outgrowth by nerve growth factor. *Proc. Natl. Acad. Sci. USA* **75**, 6059-6063.

Burstein, D. E., Blumberg, P. M. and Greene, L. A. (1982). Nerve growth factor-induced neuronal differentiation of PC12 pheochromocytoma cells: lack of inhibition by a tumor promoter. *Brain Res.* **247**, 115-119.

Chen, L., Maures, T. J., Jin, H., Huo, J. S., Rabbani, S. A., Schwartz, J. and Carter-Su, C. (2008). SH2B1beta (SH2-Bbeta) enhances expression of a subset of nerve growth factor-regulated genes important for neuronal differentiation including genes encoding urokinase plasminogen activator receptor and matrix metalloproteinase 3/10. *Mol. Endocrinol.* **22**, 454-476.

Chin, M. H., Geng, A. B., Khan, A. H., Qian, W. J., Petyuk, V. A., Boline, J., Levy, S., Toga, A. W., Smith, R. D., Leahy, R. M. et al. (2007). A genome-scale map of expression for a mouse brain section obtained using voxelation. *Physiol. Genomics* **30**, 313-321.

Chung, J., Kubota, H., Ozaki, Y., Uda, S. and Kuroda, S. (2010). Timing-dependent actions of NGF required for cell differentiation. *PLoS ONE* **5**, e9011.

Cowley, S., Paterson, H., Kemp, P. and Marshall, C. J. (1994). Activation of MAP kinase kinase is necessary and sufficient for PC12 differentiation and for transformation of NIH 3T3 cells. *Cell* **77**, 841-852.

Deutsch, P. J. and Sun, Y. (1992). The 38-amino acid form of pituitary adenylate cyclase-activating polypeptide stimulates dual signaling cascades in PC12 cells and promotes neurite outgrowth. *J. Biol. Chem.* **267**, 5108-5113.

Dijkmans, T. F., van Hooijdonk, L. W., Schouten, T. G., Kamphorst, J. T., Vellinga, A. C., Meerman, J. H., Fitzsimons, C. P., de Kloet, E. R. and Vreugdenhil, E. (2008). Temporal and functional dynamics of the transcriptome during nerve growth factor-induced differentiation. *J. Neurochem.* **105**, 2388-2403.

Dijkmans, T. F., van Hooijdonk, L. W., Schouten, T. G., Kamphorst, J. T., Fitzsimons, C. P. and Vreugdenhil, E. (2009). Identification of new Nerve Growth Factor-responsive immediate-early genes. *Brain Res.* **1249**, 19-33.

Ebisuya, M., Yamamoto, T., Nakajima, M. and Nishida, E. (2008). Ripples from neighbouring transcription. *Nat. Cell Biol.* **10**, 1106-1113.

Eriksson, M., Taskinen, M. and Leppä, S. (2007). Mitogen activated protein kinase-dependent activation of c-Jun and c-Fos is required for neuronal differentiation but not for growth and stress response in PC12 cells. *J. Cell. Physiol.* **210**, 538-548.

Farias-Eisner, R., Vician, L., Silver, A., Reddy, S., Rabbani, S. A. and Herschman, H. R. (2000). The urokinase plasminogen activator receptor (UPAR) is preferentially induced by nerve growth factor in PC12 pheochromocytoma cells and is required for NGF-driven differentiation. *J. Neurosci.* **20**, 230-239.

Farias-Eisner, R., Vician, L., Reddy, S., Basconcillo, R., Rabbani, S. A., Wu, Y. Y., Bradshaw, R. A. and Herschman, H. R. (2001). Expression of the urokinase plasminogen activator receptor is transiently required during "priming" of PC12 cells in nerve growth factor-directed cellular differentiation. *J. Neurosci. Res.* **63**, 341-346.

Ginty, D. D., Glowacka, D., DeFranco, C. and Wagner, J. A. (1991). Nerve growth factor-induced neuronal differentiation after dominant repression of both type I and type II cAMP-dependent protein kinase activities. *J. Biol. Chem.* **266**, 15325-15333.

Greene, L. A. and Tischler, A. S. (1976). Establishment of a noradrenergic clonal line of rat adrenal pheochromocytoma cells which respond to nerve growth factor. *Proc. Natl. Acad. Sci. USA* **73**, 2424-2428.

Greene, L. A., Burstein, D. E. and Black, M. M. (1982). The role of transcription-dependent priming in nerve growth factor promoted neurite outgrowth. *Dev. Biol.* **91**, 305-316.

Hansen, T. O., Rehfeld, J. F. and Nielsen, F. C. (2003). KCl potentiates forskolin-induced PC12 cell neurite outgrowth via protein kinase A and extracellular signal-regulated kinase signaling pathways. *Neurosci. Lett.* **347**, 57-61.

Harada, T., Morooka, T., Ogawa, S. and Nishida, E. (2001). ERK induces p35, a neuron-specific activator of Cdk5, through induction of Egr1. *Nat. Cell Biol.* **3**, 453-459.

Hazzalin, C. A. and Mahadevan, L. C. (2002). MAPK-regulated transcription: a continuously variable gene switch? *Nat. Rev. Mol. Cell Biol.* **3**, 30-40.

Irving, J. A., Pike, R. N., Lesk, A. M. and Whisstock, J. C. (2000). Phylogeny of the serpin superfamily: implications of patterns of amino acid conservation for structure and function. *Genome Res.* **10**, 1845-1864.

Jones, S. M. and Kazlauskas, A. (2001). Growth-factor-dependent mitogenesis requires two distinct phases of signalling. *Nat. Cell Biol.* **3**, 165-172.

Kawaai, K., Tominaga-Yoshino, K., Urakubo, T., Taniguchi, N., Kondoh, Y., Tashiro, H., Ogura, A. and Tashiro, T. (2010). Analysis of gene expression changes associated with long-lasting synaptic enhancement in hippocampal slice cultures after repetitive exposures to glutamate. *J. Neurosci. Res.* **88**, 2911-2922.

Kawano, Y., Yoshimura, T., Tsuboi, D., Kawabata, S., Kaneko-Kawano, T., Shirataki, H., Takenawa, T. and Kaibuchi, K. (2005). CRMP-2 is involved in kinesin-1-dependent transport of the Sra-1/WAVE1 complex and axon formation. *Mol. Cell. Biol.* **25**, 9920-9935.

Marshall, C. J. (1995). Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* **80**, 179-185.

Mullenbrock, S., Shah, J. and Cooper, G. M. (2011). Global expression analysis identified a preferentially NGF-induced transcriptional program regulated by sustained MEK/ERK and AP-1 activation during PC12 differentiation. *J. Biol. Chem.* **286**, 45131-45145.

Murphy, L. O., Smith, S., Chen, R. H., Fingar, D. C. and Blenis, J. (2002). Molecular interpretation of ERK signal duration by immediate early gene products. *Nat. Cell Biol.* **4**, 556-564.

Naito, Y., Yoshimura, J., Morishita, S. and Ui-Tei, K. (2009). siDirect 2.0: updated software for designing functional siRNA with reduced seed-dependent off-target effect. *BMC Bioinformatics* **10**, 392.

Ng, Y. P., Wu, Z., Wise, H., Tsim, K. W., Wong, Y. H. and Ip, N. Y. (2009). Differential and synergistic effect of nerve growth factor and cAMP on the regulation of early response genes during neuronal differentiation. *Neurosignals* **17**, 111-120.

Nishino, J., Yamashita, K., Hashiguchi, H., Fujii, H., Shimazaki, T. and Hamada, H. (2004). Meteorin: a secreted protein that regulates glial cell differentiation and promotes axonal extension. *EMBO J.* **23**, 1998-2008.

Parmar, P. K., Coates, L. C., Pearson, J. F., Hill, R. M. and Birch, N. P. (2002). Neuroserpin regulates neurite outgrowth in nerve growth factor-treated PC12 cells. *J. Neurochem.* **82**, 1406-1415.

Pool, M., Thiemann, J., Bar-Or, A. and Fournier, A. E. (2008). NeuriteTracer: a novel ImageJ plugin for automated quantification of neurite outgrowth. *J. Neurosci. Methods* **168**, 134-139.

Ravni, A., Vaudry, D., Gerdin, M. J., Eiden, M. V., Falluel-Morel, A., Gonzalez, B. J., Vaudry, H. and Eiden, L. E. (2008). A cAMP-dependent, protein kinase A-independent signaling pathway mediating neuritogenesis through Egr1 in PC12 cells. *Mol. Pharmacol.* **73**, 1688-1708.

Remold-O'Donnell, E., Chin, J. and Alberts, M. (1992). Sequence and molecular characterization of human monocyte/neutrophil elastase inhibitor. *Proc. Natl. Acad. Sci. USA* **89**, 5635-5639.

Richter-Landsberg, C. and Jastorff, B. (1986). The role of cAMP in nerve growth factor-promoted neurite outgrowth in PC12 cells. *J. Cell Biol.* **102**, 821-829.

Sasagawa, S., Ozaki, Y., Fujita, K. and Kuroda, S. (2005). Prediction and validation of the distinct dynamics of transient and sustained ERK activation. *Nat. Cell Biol.* **7**, 365-373.

Schenk, G. J., Engels, B., Zhang, Y. P., Fitzsimons, C. P., Schouten, T., Kruidering, M., de Kloet, E. R. and Vreugdenhil, E. (2007). A potential role for calcium/calmodulin-dependent protein kinase-related peptide in neuronal apoptosis: in vivo and in vitro evidence. *Eur. J. Neurosci.* **26**, 3411-3420.

Schenk, G. J., Vreugdenhil, E., Hubens, C. J., Veldhuisen, B., de Kloet, E. R. and Oitzl, M. S. (2011). Hippocampal CARP over-expression solidifies consolidation of contextual fear memories. *Physiol. Behav.* **102**, 323-331.

Shu, T., Tseng, H. C., Sapir, T., Stern, P., Zhou, Y., Sanada, K., Fischer, A., Coquelle, F. M., Reiner, O. and Tsai, L. H. (2006). Doublecortin-like kinase controls neurogenesis by regulating mitotic spindles and M phase progression. *Neuron* **49**, 25-39.

Silverman, G. A., Bird, P. I., Carrell, R. W., Church, F. C., Coughlin, P. B., Gettins, P. G., Irving, J. A., Lomas, D. A., Luke, C. J., Moyer, R. W. et al. (2001). The

serpins are an expanding superfamily of structurally similar but functionally diverse proteins. Evolution, mechanism of inhibition, novel functions, and a revised nomenclature. *J. Biol. Chem.* **276**, 33293-33296.

Surace, C., Piazzolla, S., Sirleto, P., Digilio, M. C., Roberti, M. C., Lombardo, A., D'Elia, G., Tomaiuolo, A. C., Petrocchi, S., Capolino, R. et al. (2009). Mild ring 17 syndrome shares common phenotypic features irrespective of the chromosomal breakpoints location. *Clin. Genet.* **76**, 256-262.

Tseng, M. Y., Liu, S. Y., Chen, H. R., Wu, Y. J., Chiu, C. C., Chan, P. T., Chiang, W. F., Liu, Y. C., Lu, C. Y., Jou, Y. S. et al. (2009). Serine protease inhibitor (SERPIN) B1 promotes oral cancer cell motility and is over-expressed in invasive oral squamous cell carcinoma. *Oral Oncol.* **45**, 771-776.

Vaudry, D., Chen, Y., Ravni, A., Hamelink, C., Elkahloun, A. G. and Eiden, L. E. (2002a). Analysis of the PC12 cell transcriptome after differentiation with pituitary adenylate cyclase-activating polypeptide (PACAP). *J. Neurochem.* **83**, 1272-1284.

Vaudry, D., Stork, P. J., Lazarovici, P. and Eiden, L. E. (2002b). Signaling pathways for PC12 cell differentiation: making the right connections. *Science* **296**, 1648-1649.

von Kriegsheim, A., Baiocchi, D., Birtwistle, M., Sumpton, D., Bienvenut, W., Morrice, N., Yamada, K., Lamond, A., Kalna, G., Orton, R. et al. (2009). Cell fate decisions are specified by the dynamic ERK interactome. *Nat. Cell Biol.* **11**, 1458-1464.

Vreugdenhil, E., Datson, N., Engels, B., de Jong, J., van Koningsbruggen, S., Schaaf, M. and de Kloet, E. R. (1999). Kainate-elicited seizures induce mRNA encoding a CaMK-related peptide: a putative modulator of kinase activity in rat hippocampus. *J. Neurobiol.* **39**, 41-50.

Vreugdenhil, E., Kolk, S. M., Boekhoorn, K., Fitzsimons, C. P., Schaaf, M., Schouten, T., Sarabdjitsingh, A., Sibug, R. and Lucassen, P. J. (2007). Doublecortin-like, a microtubule-associated protein expressed in radial glia, is crucial for neuronal precursor division and radial process stability. *Eur. J. Neurosci.* **25**, 635-648.

Wibrand, K., Messaoudi, E., Håvik, B., Steenslid, V., Løvlie, R., Steen, V. M. and Bramham, C. R. (2006). Identification of genes co-upregulated with Arc during BDNF-induced long-term potentiation in adult rat dentate gyrus in vivo. *Eur. J. Neurosci.* **23**, 1501-1511.

Yao, H., York, R. D., Misra-Press, A., Carr, D. W. and Stork, P. J. (1998). The cyclic adenosine monophosphate-dependent protein kinase (PKA) is required for the sustained activation of mitogen-activated kinases and gene expression by nerve growth factor. *J. Biol. Chem.* **273**, 8240-8247.
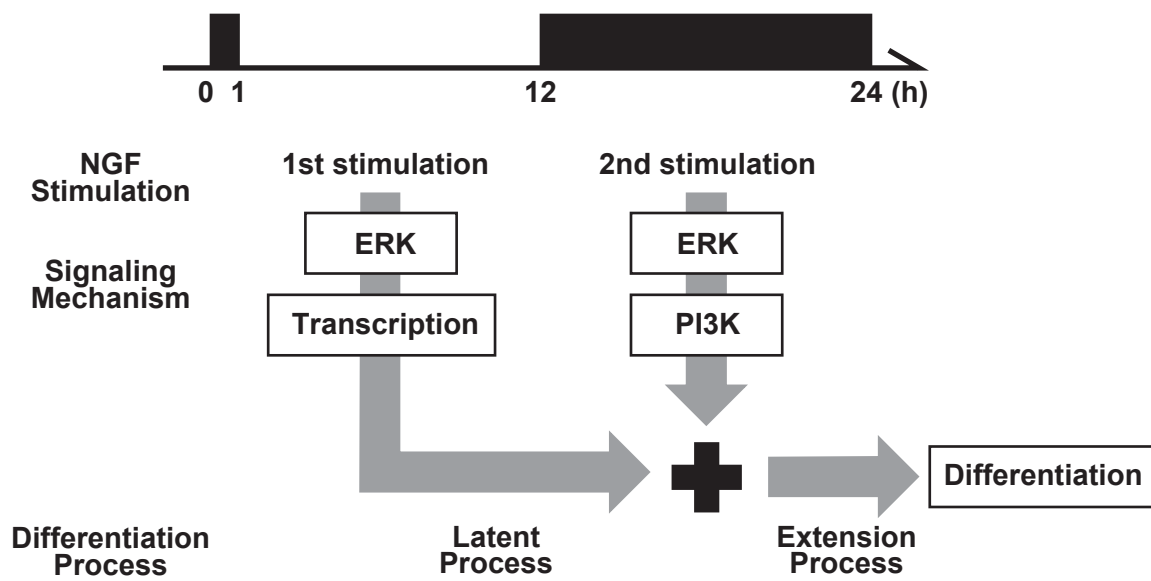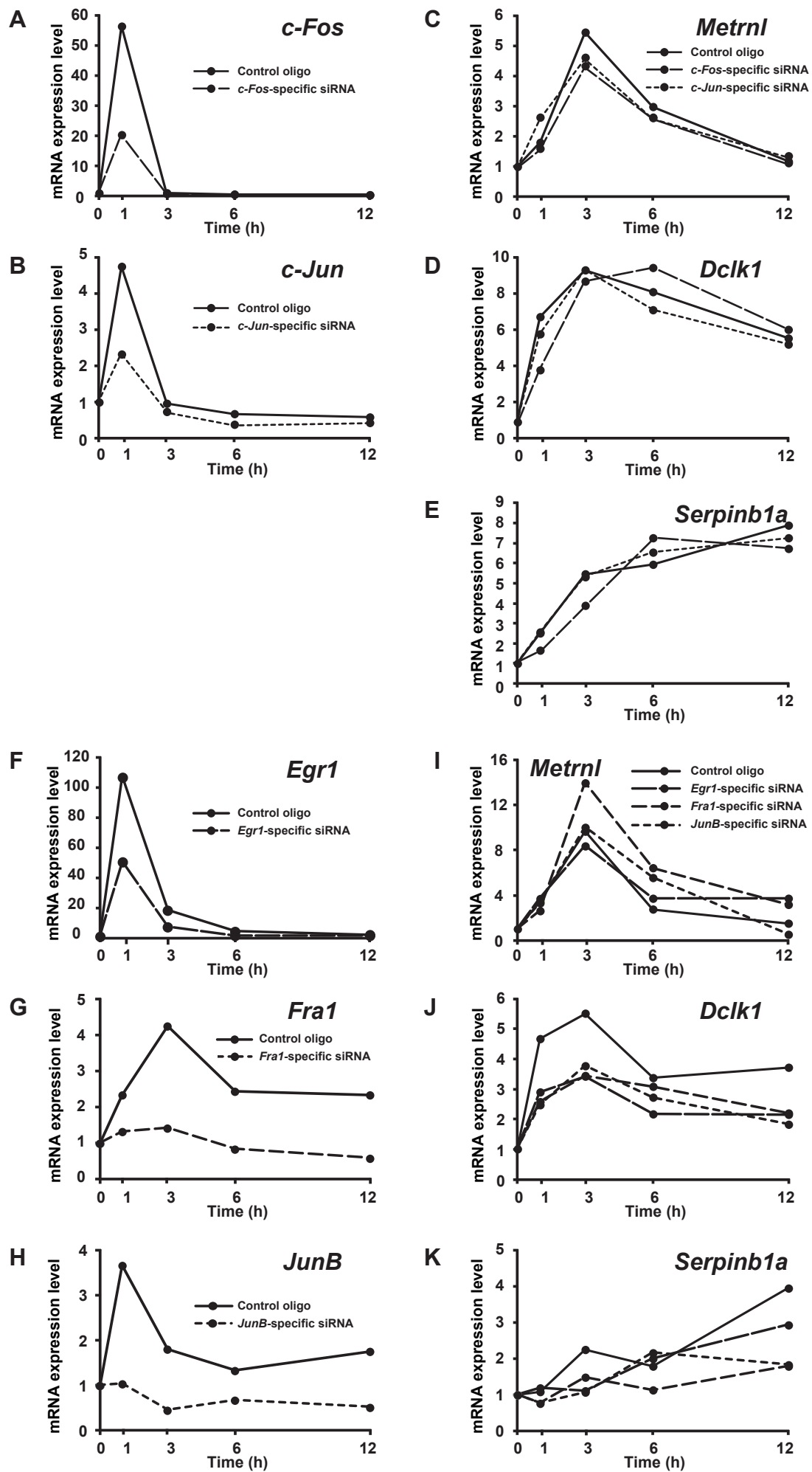
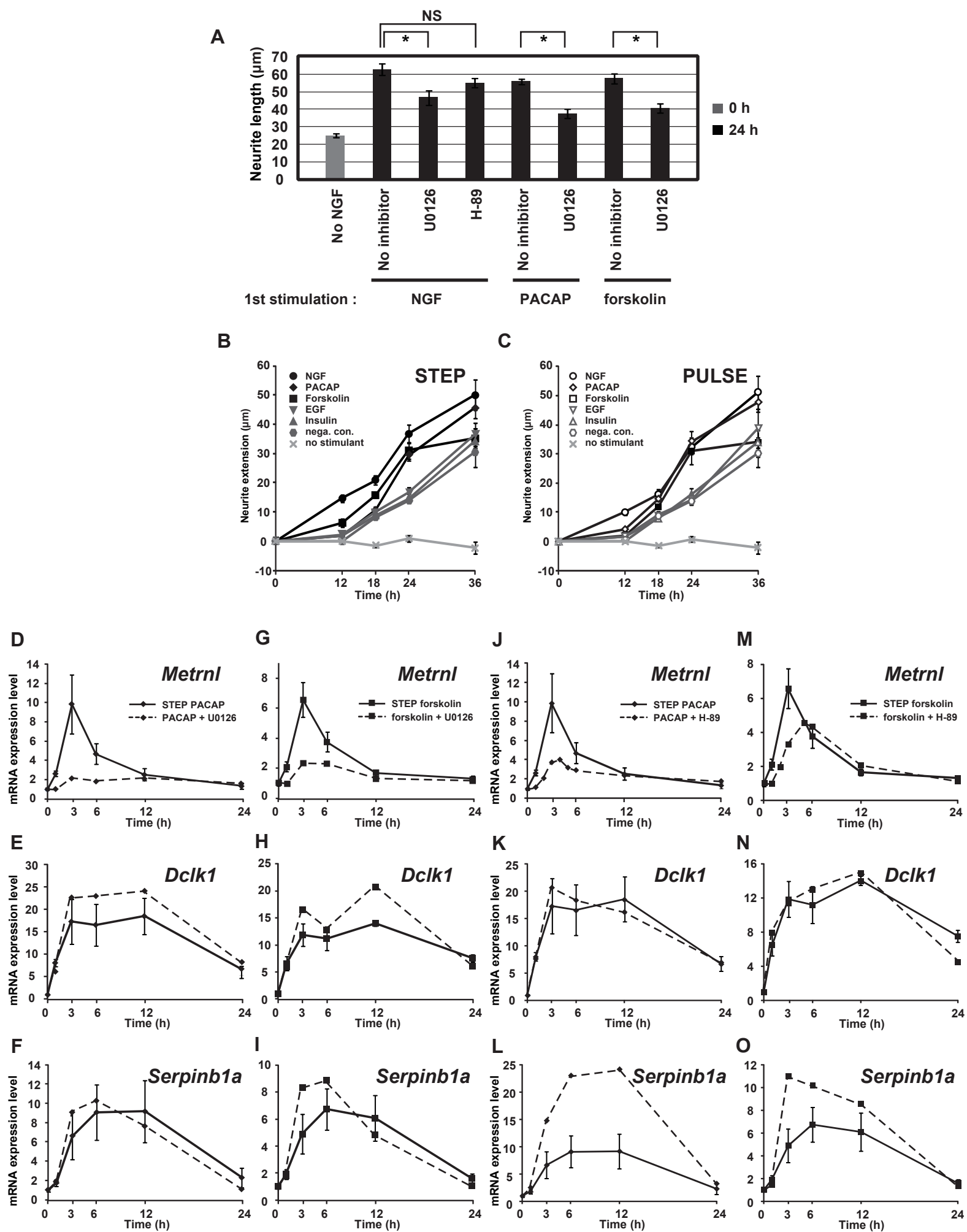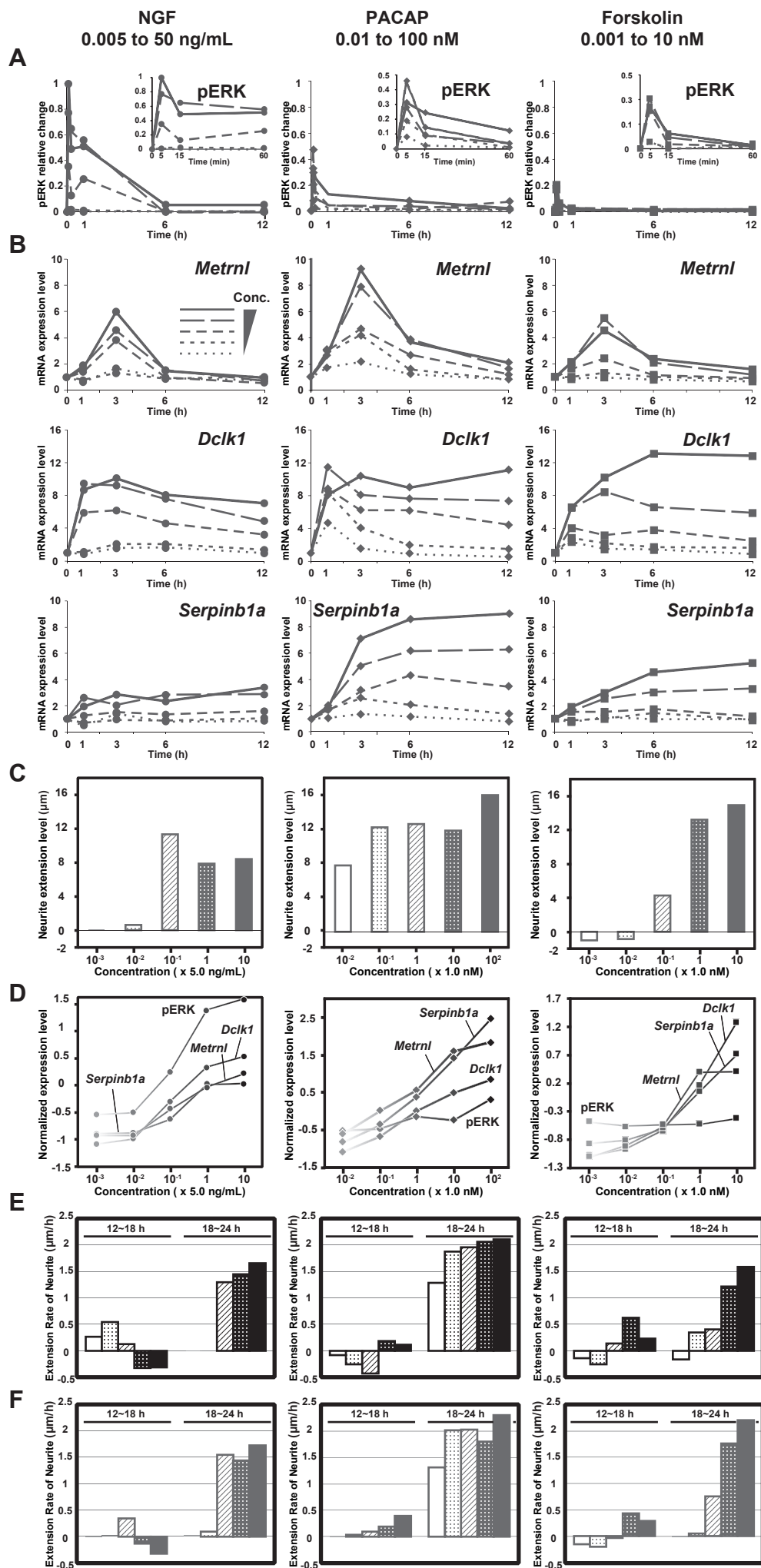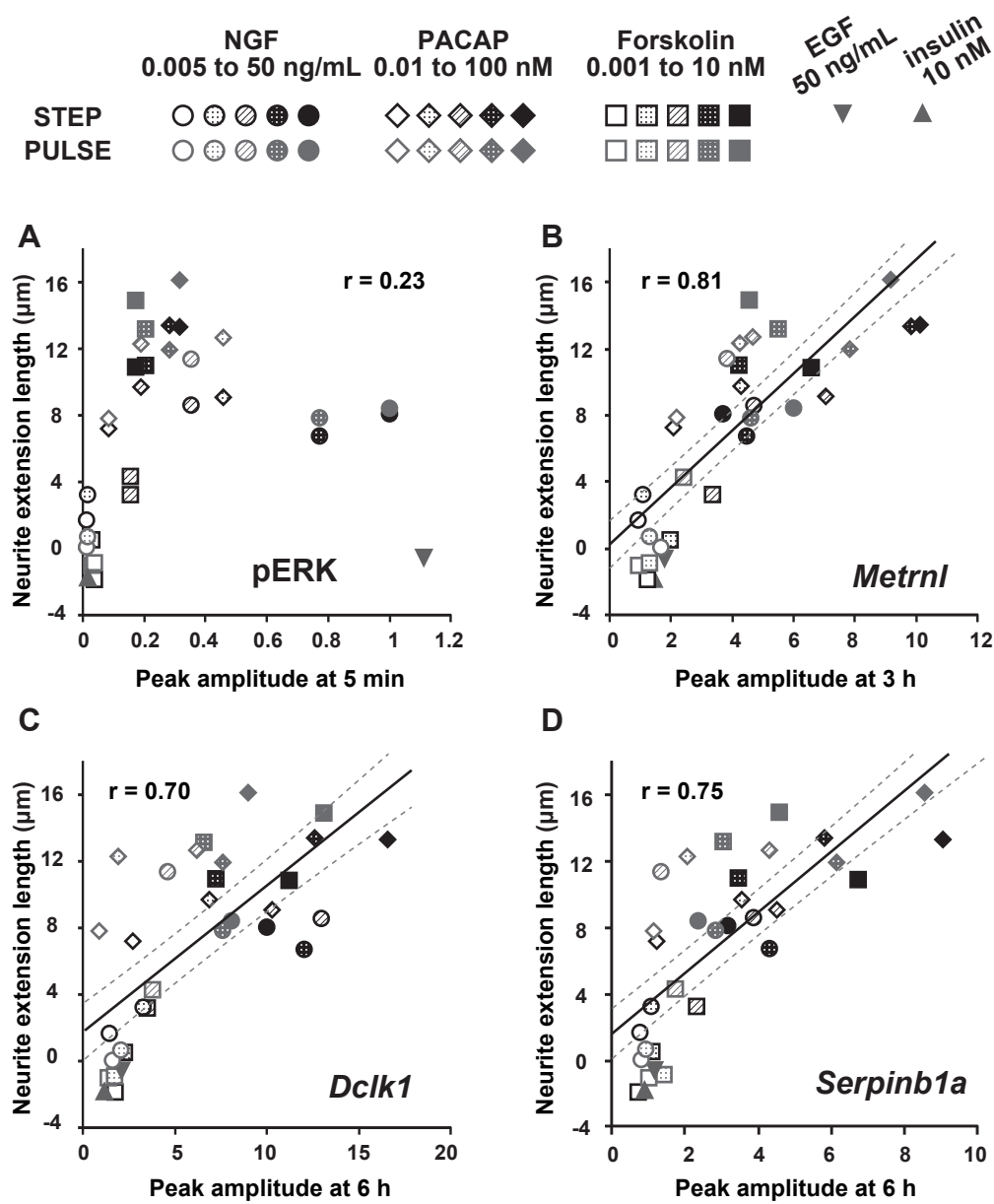**Fig. S1**
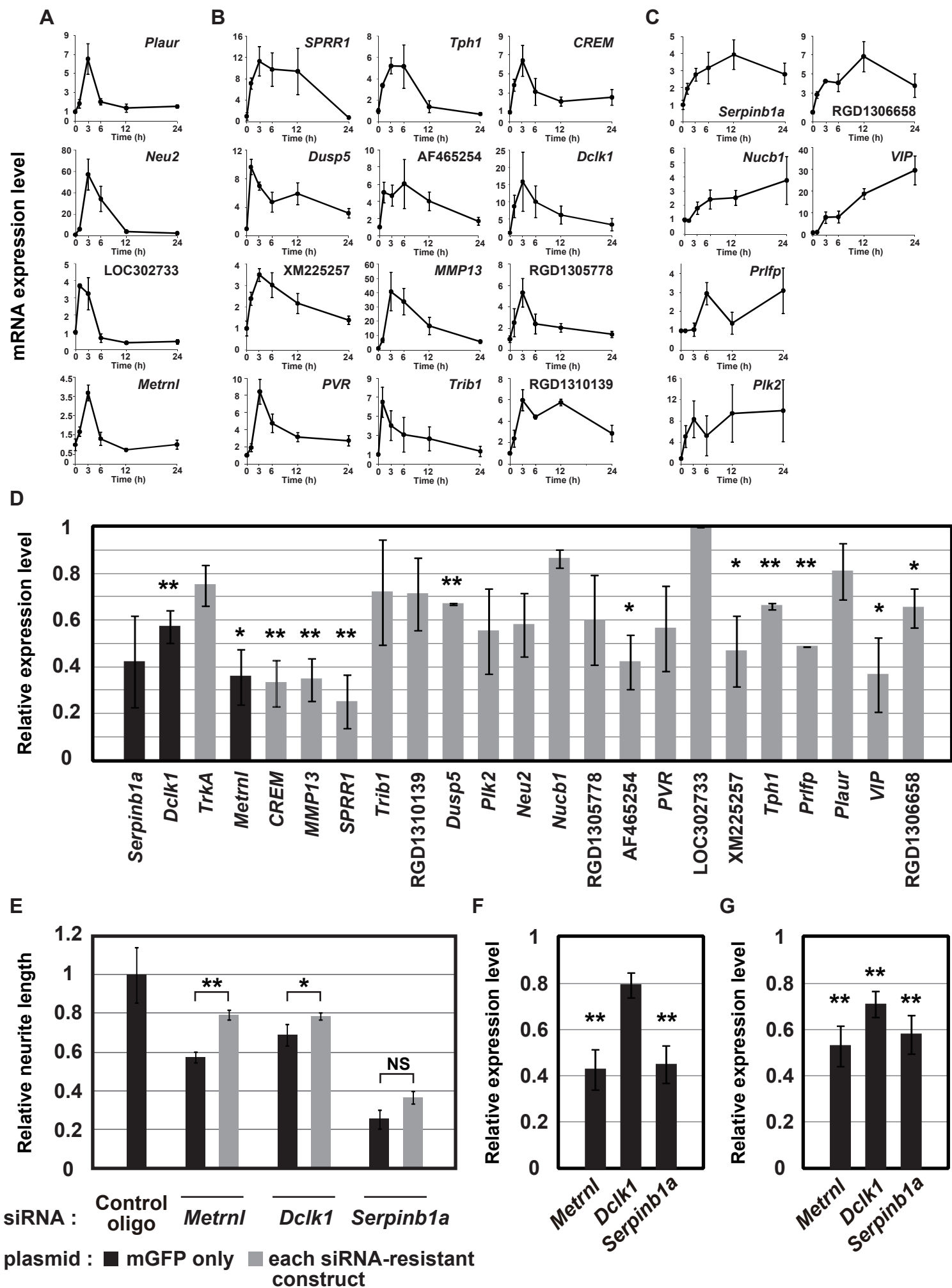
Fig. S2
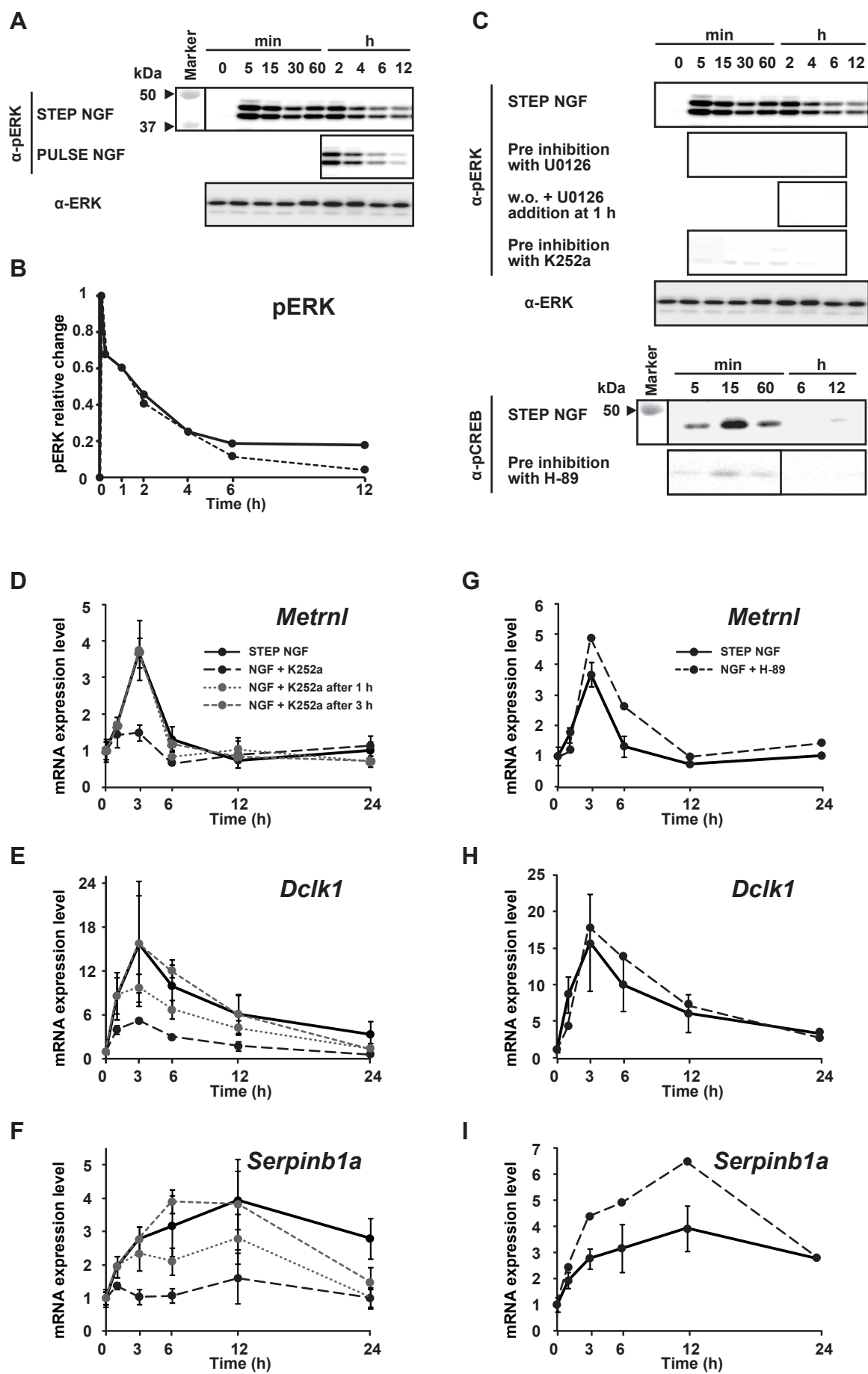
Fig. S3

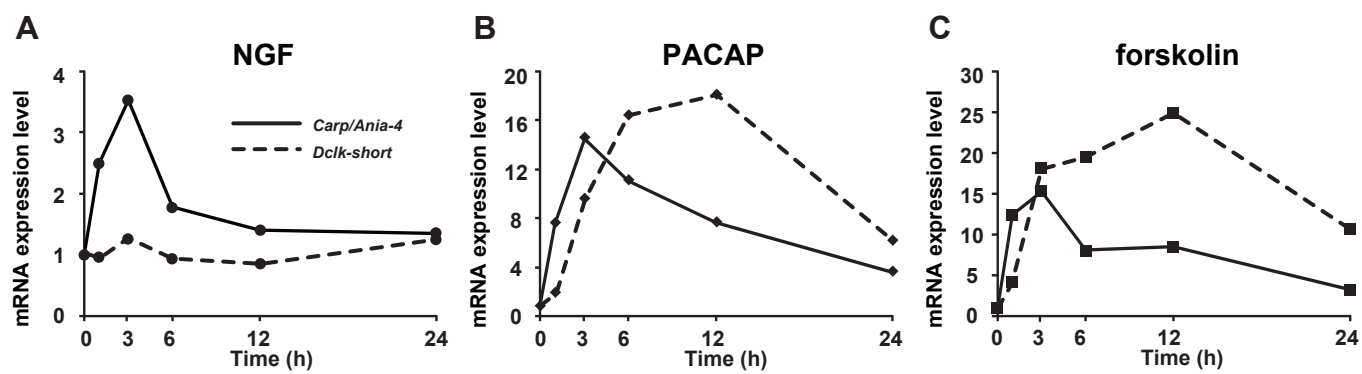**Fig. S4**

Fig. S5

Fig. S6

Fig. S7

Fig. S8

Table S1. The primer sequences used for qRT-PCR and the siRNA oligo sequences used for siRNA-mediated knockdown

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	UUCCAUUAACUUCUAAUGAGUU
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	AUGUGGACAGAGGAGCAAAUU
	R	ATTGGAGGAGAGATTCAGGTGACT	P	UUUGCUCCUCUGUCCACAUU
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 (-actin)	F	CCCGCGAGTACAACCTTCT	G	No oligo
	R	CGTCATCCATGGCGAACT	P	No oligo