Long-lasting RNAi activity in mammalian neurons☆

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Abstract The effect of RNA interference (RNAi) induced by synthetic small interfering RNAs (siRNAs) on proliferating mammalian cells appears to last for approximately 3–7 days after its induction. Here we show that the RNAi activity induced by a synthetic 21-nucleotide siRNA duplex in postmitotic neurons, mouse primary hippocampal neurons and neurons that differentiated from mouse embryonal carcinoma P19 cells persists for at least 3 weeks, suggesting long-lasting RNAi activity in mammalian neurons. In addition, we also show that an apoptotic (or antiviral) pathway triggered by long dsRNAs is generated during neuronal differentiation of P19 cells, by which the sequence-specific RNAi activity involving long dsRNA appears to be masked.

Key words: RNA interference; Mammalian neuron; Synthetic small interfering RNA; Long double-stranded RNA; Apoptosis

1. Introduction

Double-stranded RNAs (dsRNAs) induce sequence-specific posttranscriptional gene silencing or RNA interference (RNAi) in various kinds of species including flies, worms, protozoa, vertebrates and higher plants (reviewed in [1–4]). DsRNAs introduced or generated in cells are digested by an RNase III-like enzyme, Dicer, into 21–25-nucleotide (nt) RNA duplexes [5–8], and the resultant duplexes, referred to as small interfering RNA (siRNA) duplexes, function as sequence-specific mediators of RNAi in the RNA-induced silencing complexes (RISCs) [7,8]. In mammalian cells except for a part of undifferentiated cells [9–12], however, long dsRNAs (>30 bp) trigger a rapid and non-specific RNA degradation involving the sequence-non-specific RNase, RNase L [13], and a rapid translation inhibition involving the interferon-inducible, dsRNA-activated protein kinase, PKR [14], instead of induction of RNAi: these rapid responses to long dsRNAs may mask the sequence-specific RNAi activity in differentiated mammalian cells. Elbashir et al. [15] have demonstrated that synthetic 21-nt siRNA duplexes can induce sequence-specific RNAi activity in cultured mammalian cells without triggering the rapid and non-specific RNA degradation and translation inhibition. RNAi induction by synthetic siRNA duplexes has not only allowed us to analyze the molecular mechanism of mammalian RNAi, but also provided us with a powerful reverse genetic tool for suppressing the expression of a gene of interest in various mammalian cells [16].

The persistence of RNAi activity appears to be an important parameter when considering the effect of RNAi on the regulation of the expression of genes in cells, particularly in mammalian cells [17,18]. Previous studies indicated that the RNAi activity induced by synthetic siRNA duplexes persisted for approximately 3–7 days in cultured mammalian cells [12,19,20]. However, it should be noted that these observations were obtained from experiments using proliferating mammalian cells. Therefore, it is conceivable that whenever cell division occurs, the number of RISCs carrying siRNA duplexes decreases in those cells.

A neuron is known as a terminally differentiated and cell cycle-arrested cell. RNAi activity in mammalian neurons has been reported [21,22], but the duration and features of RNAi in neurons are still unknown. In this study, we investigated RNAi activity induced by synthetic siRNA duplexes in mouse primary hippocampal neurons and neurons that differentiated from mouse embryonal carcinoma P19 cells by treatment with retinoic acid. The results indicated that the RNAi activity in the neurons persisted for at least 3 weeks, whereas undifferentiated P19 cells studied as proliferating cells lost the RNAi activity by day 5 after its induction.

2. Materials and methods

2.1. Cell culture

Primary culture of dissociated hippocampal neurons was carried out as described previously [23,24] with minor modifications. Briefly, mouse E17 embryonic hippocampal tissue (ICR mouse strain) was isolated, treated with 90 U/ml papain (Worthington) at 37°C for 15 min, and gently dissociated with pipetting. The dissociated hippocampal neurons were seeded into polyethyleneimine-coated 24-well culture plates at a density of approximately $5\times10^5/cm^2$. The cells were grown at 37°C in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum (Invitrogen), and 5% heat-inactivated horse serum (Invitrogen) in a 5% CO_2-humidified chamber. Transfection as described below was carried out 4 h after seeding the neurons onto culture plates. After a 2-day incubation, 10 μM cytosine arabinoside (Ara-C) (Sigma) was added, and further incubation at 37°C was carried out.

P19 mouse embryonic carcinoma cells were grown at 37°C in α-MEM (Sigma) supplemented with 10% fetal bovine serum (Sigma), 100 U/ml penicillin (Sigma) and 100 μg/ml streptomycin (Sigma) in a

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Abbreviations: RNAi, RNA interference; siRNA, small interfering RNA; dsRNA, double-stranded RNA; PKR, interferon-inducible, dsRNA-activated protein kinase; nt, nucleotide

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5% CO_2-humidified chamber. For induction of neuronal differentiation of P19 cells, the cells were cultured in the presence of 5×10^{-7} M all-*trans*-retinoic acid (RA) (Sigma) in bacterial-grade Petri dishes for 4 days. After a 4-day incubation, aggregated cells were collected, dissociated into single cells by treatment with trypsin–EDTA and seeded into poly-L-lysine-coated 24-well culture plates at a density of 1×10^5 cells/cm^2 in the normal medium described above. After a 1-day incubation, the medium was changed to the Neurobasal (Invitrogen) medium containing B27 supplement (Invitrogen), 0.5 mM glutamine, and 10 μM Ara-C (Sigma); thereafter, the medium was changed every 2 days. Transfection was carried out over 240 h after RA treatment.

2.2. Synthetic oligonucleotides

RNA and DNA synthetic oligonucleotides were obtained from Proligo and Sigmagenosis, respectively. Preparation of RNA duplexes was performed as described previously [25]. The sequences of siRNA synthesized newly were as follows: ssRLa1: 5′-UGGCUUCCAAGGUGUACGAUU-3′, asRLa1: 5′-UCGUACACCUUGGAAGCCAUU-3′.

The GFP-22 and non-silencing siRNAs and the *silencer* GAPDH siRNA were purchased from Qiagen and Ambion, respectively.

2.3. In vitro transcription

For preparation of a 200-bp-long dsRNA against the *Photinus luciferase* gene, a plasmid containing the region from positions 434 to 633 in the pGL3-control vector (Promega) was constructed. Briefly, the region was amplified by polymerase chain reaction (PCR) with the following primers: 5′-TGGAGGTACCTTACGCTGAGTACTTCG-3′ and 5′-GCGACCGCGGCATACTGTTGAGCAATTC-3′, and the resultant PCR product was digested with *Kpn*I and *Sac*II and inserted into the pBluescript vector (Stratagene). In vitro transcription with the plasmid as a template was carried out using MEGAscript T7 and T3 kits (Ambion). Duplex formation was carried out as described previously [25].

2.4. Transfection and luciferase assay

Reporter plasmids and siRNA duplexes were cotransfected into primary hippocampal neurons and undifferentiated P19 cells using Lipofectamine 2000 (Invitrogen), and into differentiated neuronal P19 cells using NeuroPORTER transfection reagents (Gene Therapy Systems) according to the manufacturers' instructions with minor modifications. In the case of introduction of just siRNA duplexes into the neurons, the transfection was carried out using GeneSilencer siRNA transfection reagent (Gene Therapy Systems) according to the manufacturer's instructions. Before the transfection, the culture medium was replaced with 0.5 ml of the fresh medium without antibiotics, and to each well (24-well culture plates), 0.25 μg of pGL3-control plasmid (Promega), 0.05 μg phRL-TK plasmid (Promega), and 0.2 μg of siRNAs were applied. In the case of targeting the *GFP* gene, 0.25 μg of pEGFP-C1 (BD Biosciences), 0.25 μg of pDsRed2-C1 (BD Biosciences), 0.2 μg of GFP-22 siRNA (Qiagen) were applied. The cells were incubated for 4 h at 37°C. After the 4-h incubation, 0.5 ml of the fresh culture medium without antibiotics was added, and further incubation at 37°C was carried out. For medium change, a half volume of the medium was changed with the fresh medium at 10 days after transfection in primary hippocampal neurons, and every 2 days in differentiated P19 cells. When transfection efficiency was examined using the pEGFP-C1 plasmid and Cy3-labeled siRNA duplexes, it appeared that the present procedures yielded approximately 5–8% and 60–70% transfected neurons using the plasmid and siRNA duplexes, respectively (data not shown).

In order to maintain the exponential cell growth of undifferentiated P19 cells, the cells were collected 24 h after transfection, counted using a hemocytometer, and divided into two portions. One portion was subjected to preparation of cell lysate followed by luciferase expression assay, and other portion was diluted and grown in 6-well culture plates containing the medium without antibiotics. Thereafter, the number of cells was counted when the luciferase assay was carried out. The expression of luciferase was examined using a Dual-Luciferase reporter assay system (Promega) according to the directions provided by the manufacturer.

2.5. Reverse transcription (RT) PCR

Total RNA was isolated from the cultured cells using Trizol reagent (Invitrogen). RT for synthesizing the first-strand cDNAs was carried out using oligo(dT) primer and SuperScript II reverse transcriptase (Invitrogen) according to the manufacturer's instructions, and the resultant cDNAs were subjected to a real-time PCR using the LightCycler thermal cycler system (Roche) with a LightCycler FastStart DNA Master SYBR Green I kit (Roche) according to the manufacturer's instructions. PCR primers were designed so that they can span exon–intron junctions on the transcripts of genes. The sequences of the primers are available upon request. The expression levels of the genes examined were normalized to that of the control *Gapdh* gene.

2.6. TdT-mediated dUTP biotin nick end labeling (TUNEL)

Genomic DNA breaks were examined by TUNEL using an Apoptosis Screening Kit (Wako) according to the manufacturer's instructions. The assay was carried out 48 h after transfection with dsRNAs.

3. Results

3.1. Long-lasting RNAi activity in primary hippocampal neurons

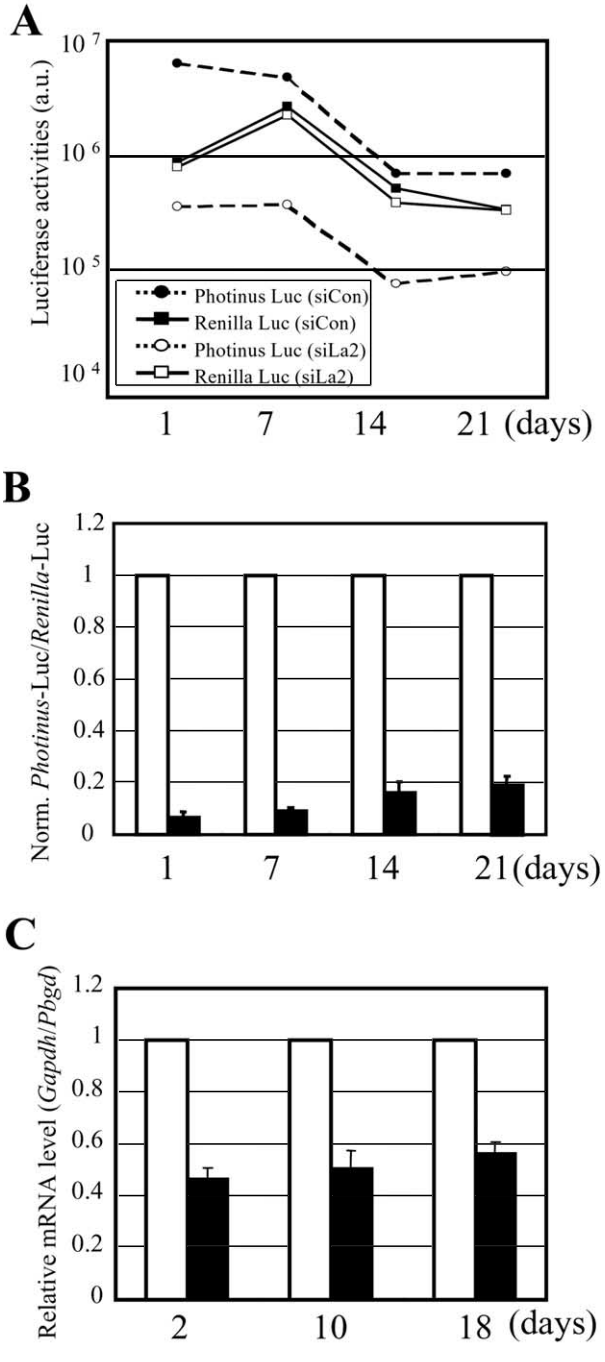
In order to induce RNAi in postmitotic neurons, we prepared primary hippocampal neurons from E17 mouse embryos, and introduced synthetic 21-nt siRNAs targeting the exogenous reporter gene, *Photinus luciferase*, together with a pGL3-control plasmid carrying the *Photinus luciferase* gene and a phRL-TK plasmid carrying the *Renilla luciferase* gene as a control into the neurons. For realizing an efficient RNAi induction, we used the La2 siRNA duplex having the potential for inducing a strong RNAi activity in cultured mammalian cells [25]. As shown in Fig. 1, the La2 siRNA duplex can specifically and strongly inhibit the expression of *Photinus luciferase* in the primary hippocampal neurons, which agrees with the previous study using cultured mammalian cells, HeLa and NTera2D1 cells [25]. The most important point to note in this experiment is that the duration of the RNAi activity in the neurons was quite a long, i.e. a strong RNAi activity lasted for at least 3 weeks after induction of RNAi. To further confirm this, we examined RNAi activities induced by the RLa1 siRNA duplex targeting the *Renilla luciferase* gene present in phRL-TK plasmid (see supplementary Fig. s1A), the GFP-22 siRNA duplex (Qiagen) targeting the *GFP* gene present in pEGFP-C1 plasmid (see supplementary Fig. s1B) and the *silencer* GAPDH siRNA duplex (Ambion) targeting the endogenous *Gapdh* gene (Fig. 1C) in the primary hippocampal neurons. Consistently, all the results demonstrate that long-lasting RNAi activity occurs in the neurons. We also add that little or no morphological change of the primary hippocampal neurons used was observed during the experiments (see supplementary Fig. s1C). To our knowledge, this is the first report on the longest duration of RNAi activity in the former RNAi activities induced by synthetic siRNA duplexes in mammalian cells.

3.2. Long-lasting RNAi activity in P19 neurons

To further confirm such a long-term effect of RNAi in other neurons and to examine the properties of RNAi during neuronal differentiation, we investigated RNAi in P19 cells, a mouse embryonal carcinoma cell line, which can be induced by RA to differentiate into neuroectodermal derivatives including neuron and glia [26], and in this study, we used P19 cells that differentiated into neurons over 240 h after RA treatment and a 3-day treatment with Ara-C as terminally differentiated and cell cycle-arrested neurons (provisionally named 'P19 neurons'). Additionally, the expression profile of genes related to neuronal differentiation in P19 neurons

appears to be similar to that in E17 hippocampal neurons (see supplementary Fig. s2).

We introduced the La2 siRNA duplex together with the pGL3-control and phRL-TK plasmids into P19 neurons and undifferentiated P19 cells as in the primary hippocampal neurons (Fig. 1). As shown in Fig. 2, the La2 siRNA duplex can specifically inhibit the expression of *Photinus luciferase* in either P19 neurons or undifferentiated P19 cells, suggesting that there is no significant difference in RNAi activity between P19 neurons and undifferentiated P19 cells. Next we examined the duration of RNAi activity in P19 neurons and undifferentiated P19 cells. The expression levels of the *luciferase* genes were examined at various hours after induction of RNAi. When undifferentiated P19 cells were examined, a strong RNAi activity was detected for 2 days after RNAi induction; thereafter, the cells gradually lost the activity and lost it completely by day 5 (120 h) after the induction (Fig. 3A). Note that the RNAi activity is being lost with an increase in the number of the cells (Fig. 3B). Therefore, these observations agree with results of previous studies using proliferating mammalian cells [12,19]. In contrast to the results of undifferentiated P19 cells, P19 neurons, like primary hippocampal neurons, revealed a long-lasting RNAi activity. When RNAi was induced in P19 neurons, a strong RNAi activity lasted for as long as 18 days after the induction (Fig. 3C). We must mention that the maintenance of P19 neurons for as long as 18 days after RNAi induction was a limit in culture under our current condition. Taken all together, our present data suggest that once RNAi is induced in mammalian neurons, a long-lasting RNAi activity occurs.



3.3. Significant difference in response to long dsRNAs between undifferentiated P19 cells and P19 neurons

Undifferentiated P19 cells appear to allow long dsRNAs to trigger sequence-specific gene silencing, i.e. RNAi [11]. In this study, we have shown that synthetic 21-nt siRNA duplexes can induce RNAi in either undifferentiated or differentiated P19 cells (Figs. 2 and 3): an authentic RNAi pathway occurs in either of the cells. These observations raise the question whether P19 neurons (differentiated P19 cells), like undifferentiated P19 cells, allow long dsRNAs to induce the sequence-specific RNAi activity. To address this, a 200-bp-long dsRNA against *Photinus luciferase* (detailed in Section 2) was prepared and cotransfected with the pGL3-control and phRL-TK plasmids into either differentiated or undifferentiated P19 cells. When the long dsRNAs were introduced into undifferentiated P19 cells, consistently, sequence-specific gene silencing of *Photinus luciferase* was observed without induction of cell death (Fig. 4A). In contrast, when P19 neurons were used, excessive cell death was observed the day after

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Fig. 1. Persistence of RNAi activity in postmitotic neurons. The La2 siRNA duplex against the *Photinus luciferase* gene [25] or a non-silencing siRNA duplex (Qiagen) together with pGL3-control and phRL-TK plasmids carrying *Photinus* and *Renilla luciferase* reporter genes, respectively, were cotransfected into mouse primary hippocampal neurons. RNAi activity was examined every week up to 3 weeks after RNAi induction. A: Absolute *Photinus* and *Renilla* luciferase expressions. The expression levels are plotted in arbitrary luminescence units (a.u.). B: Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the La2 siRNA duplex are normalized to the ratios obtained in the presence of the non-silencing siRNA duplex. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations. C: Gene silencing of the endogenous *Gapdh* gene. The *silencer* GAPDH siRNA duplex (40 nM) (Ambion) was transfected into primary hippocampal neurons using GeneSilencer siRNA transfection reagent (Gene Therapy Systems). Total RNA was extracted from the neurons at indicated days after transfection and subjected to cDNA synthesis with reverse transcriptase followed by real-time PCR. The expression level of *Gapdh* was normalized to that of the *hydroxymethylbilane synthase* (*Pbgd*) gene as a control, and the resultant expression level in the presence of the silencer GAPDH siRNA duplex was normalized to the expression level determined in the presence of non-silencing siRNA duplex (Qiagen). The resultant normalized ratios are indicated. Data are averages of three independent experiments. Error bars represent standard deviations.

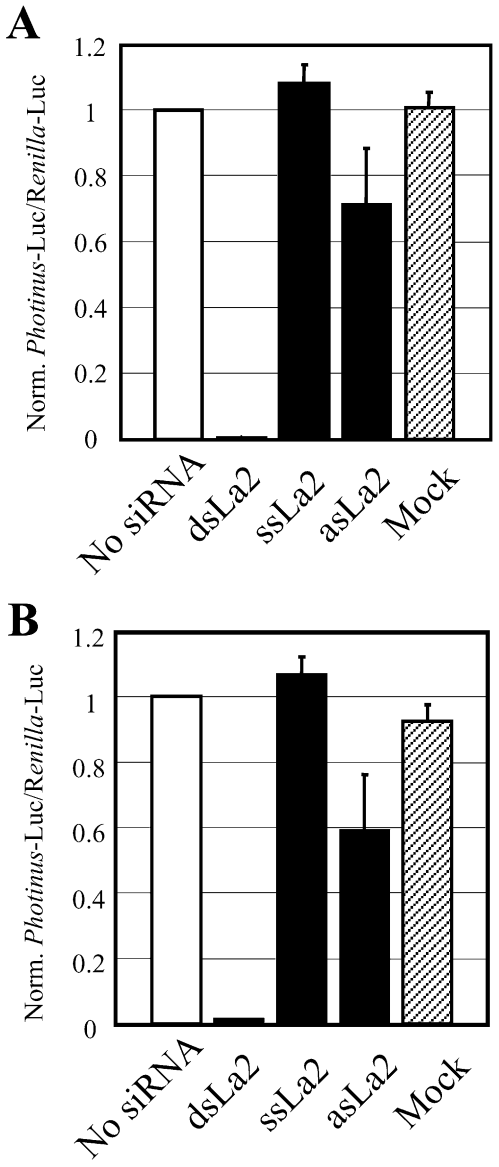

Fig. 2. RNAi activities induced by synthetic siRNAs in undifferentiated (A) and differentiated (B) P19 cells. The La2 siRNAs [25] or a non-silencing siRNA duplex (Mock) (Qiagen) together with pGL3-control and phRL-TK plasmids were cotransfected into either undifferentiated or differentiated (neurons) P19 cells. Prefixes attached to the name of La2 siRNA, i.e. ds, ss, and as, represent double-stranded (duplex), and sense- and antisense-stranded siRNAs, respectively. Twenty-four hours after transfection, cell lysate was prepared and dual luciferase assay was carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated: the ratios of luciferase activity determined in the presence of the siRNAs are normalized to the ratios obtained for a control in the absence of siRNA (No siRNA). Data are averages of at least three independent experiments. Error bars represent standard deviations.

transfection of the long dsRNAs. Note that although the observed ratio of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity was below 0.2 in the presence of the long dsRNAs in P19 neurons (Fig. 4A), the expression level of the control (*Renilla*) luciferase was significantly lower than those in the presence of synthetic 21-nt siRNA duplexes (Fig. 4B), which appeared to be due to excessive cell death triggered by the long dsRNAs. To further

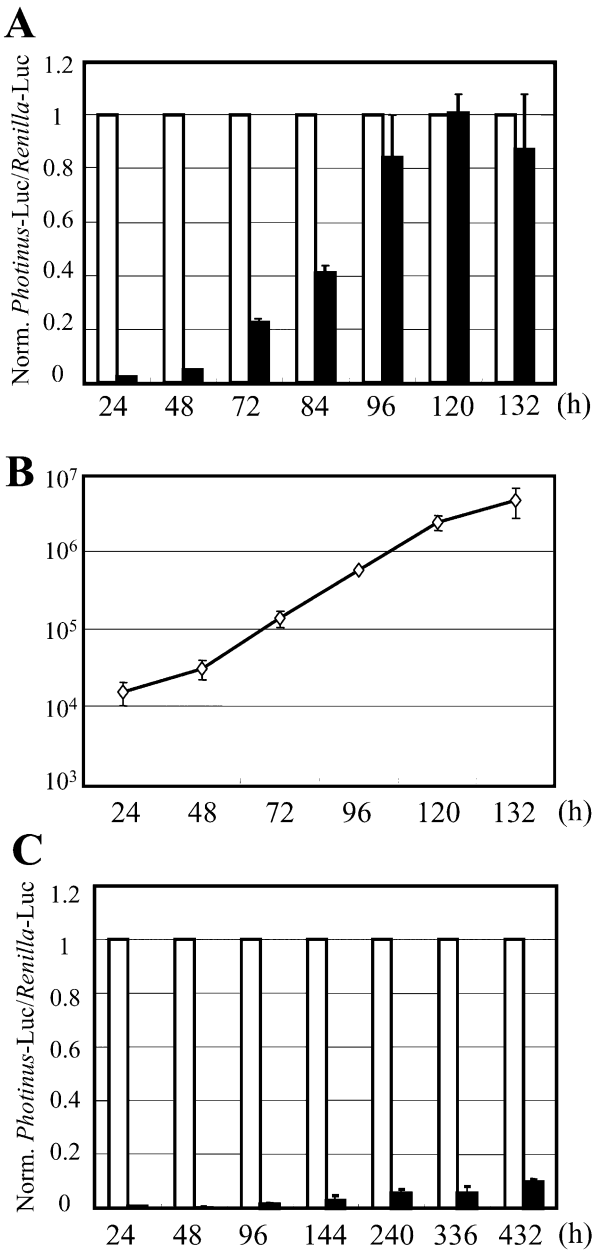

Fig. 3. Persistence of RNAi activity in undifferentiated and differentiated P19 cells. A: RNAi activity in undifferentiated P19 cells. RNAi activity was examined every 24 h up to 132 h after RNAi induction. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 1. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations. B: Growth of undifferentiated P19 cells. The dual luciferase assay was carried out as in A, and the number of cells was counted immediately before preparation of cell lysate. Data indicate the mean number of cells at each time after RNAi induction. Error bars represent standard deviations. C: RNAi activity in P19 neurons. RNAi activities were examined up to 432 h (18 days) after RNAi induction. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in A. Open and solid bars indicate the data in the presence of the non-silencing siRNA and La2 siRNA duplexes, respectively. Data are averages of at least three independent experiments. Error bars represent standard deviations.

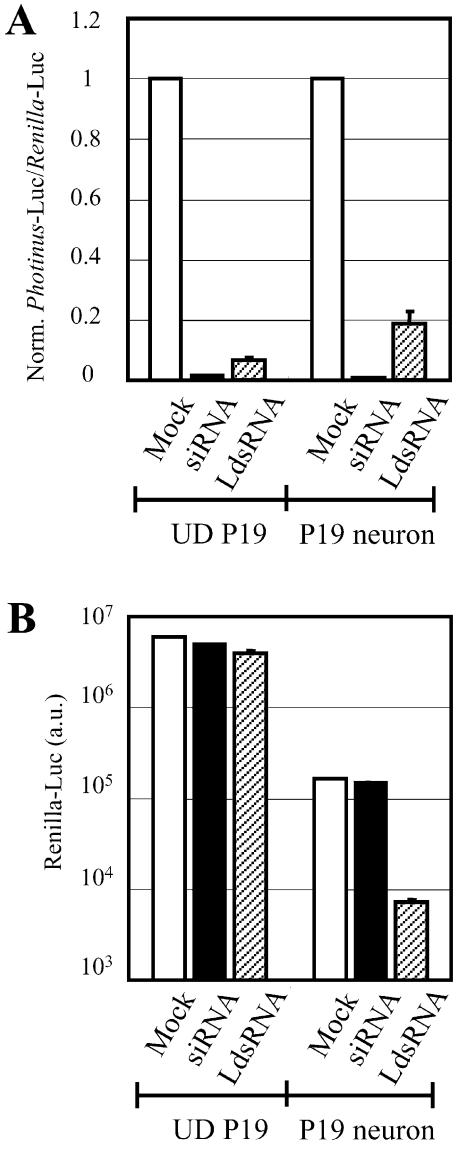

Fig. 4. Response to long dsRNAs in undifferentiated and differentiated P19 cells. A: Induction of RNAi by long dsRNAs. Two-hundred-bp-long dsRNA targeting *Photinus* (LdsRNA) and the La2 (siRNA) and non-silencing (Mock) siRNA duplex as a positive and negative control, respectively, were cotransfected with pGL3-control and phRL-TK plasmids into either undifferentiated (UD P19) or differentiated (P19 neuron) P19 cells. Twenty-four hours after transfection, dual luciferase assay was carried out. Ratios of normalized target (*Photinus*) luciferase activity to control (*Renilla*) luciferase activity are indicated as in Fig. 1. Data are averages of at least three independent experiments. Error bars represent standard deviations. Note that excessive cell death was observed in P19 neurons, but not in undifferentiated P19 cells after transfection with long dsRNAs. B: Absolute *Renilla* luciferase expression. The expression levels of *Renilla* luciferase were plotted in arbitrary luminescence units (a.u.).

confirm the induction of cell death (apoptosis) by the long dsRNAs, we examined the cells transfected with the long dsRNAs and siRNAs by using a TUNEL assay. As shown in Fig. 5, the results consistently indicate that while neither the siRNAs nor the long dsRNAs can induce apoptosis in undifferentiated P19 cells, either P19 neurons or primary hippocampal neurons undergo apoptosis in the presence of the long dsRNAs, but not in the presence of siRNA duplexes. We next examined the levels of expression of the *PKR* and *RNase L* genes involved in such an apoptotic pathway [13,14,27]. The results of a real-time RT-PCR of the genes indicate that both the genes were rapidly expressed in the course of neuronal differentiation of P19 cells (Fig. 6), and that the expression

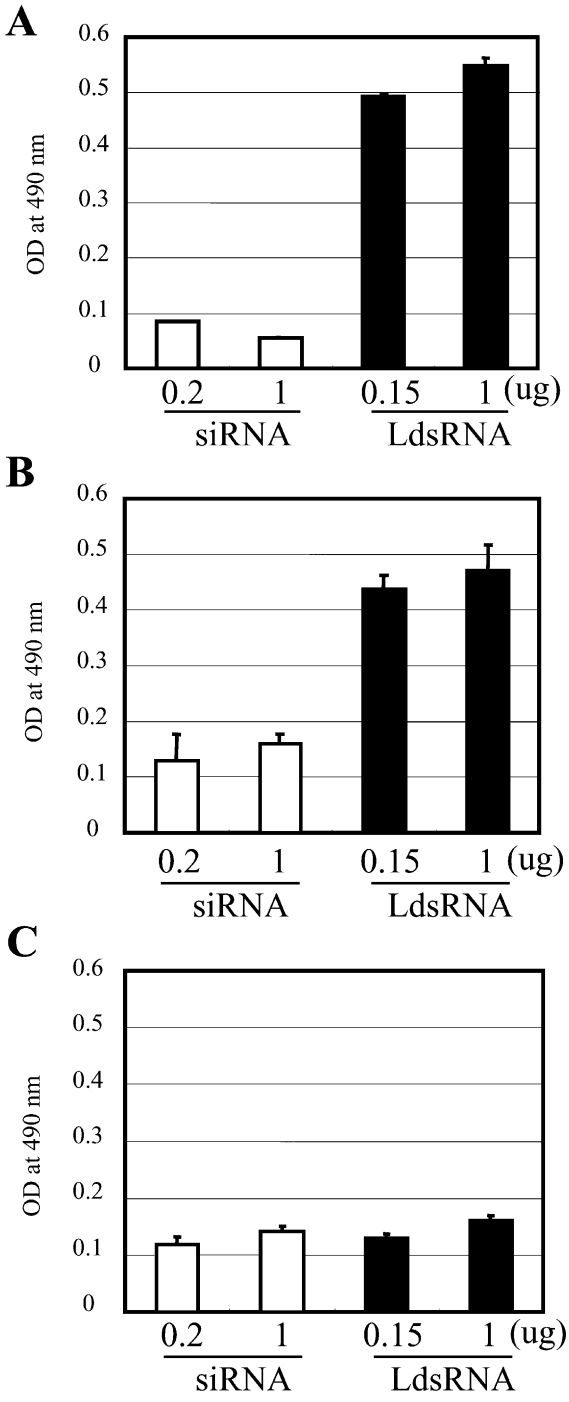

Fig. 5. Apoptosis induced by long dsRNAs in postmitotic neurons. The La2 siRNA duplex (siRNA) and 200-bp-long dsRNAs (LdsRNA) were transfected into primary hippocampal neurons (A), P19 neurons (B) and undifferentiated P19 cells (C). The amounts of dsRNA transfected are indicated. Forty-eight hours after transfection, double-stranded DNA breaks were examined by TUNEL. The absorbance was measured at 490 nm with a spectrophotometer. Data are averages of at least three independent experiments. Error bars represent standard deviations.

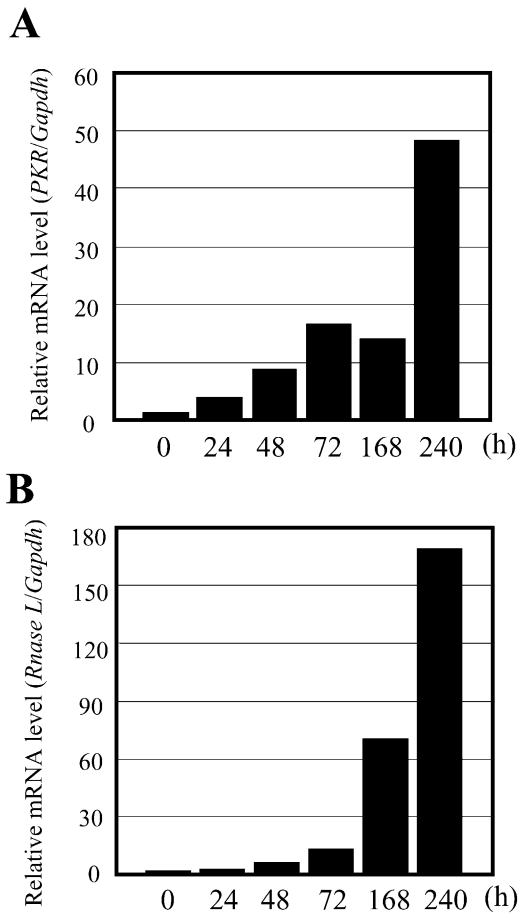

Fig. 6. Expression profiles of the *PKR* (A) and *RNase L* (B) genes during neuronal differentiation of P19 cells. The expression levels of *PKR* (A) and *RNase L* (B) were examined by real-time PCR. The expression levels of the genes are normalized to that of the *Gapdh* gene examined as a control, and plotted when the expression level of either *PKR* or *RNase L* in undifferentiated P19 cells (before treatment with RA; 0 h) is given as 1.

levels of the genes in primary hippocampal neurons were significantly higher than those in undifferentiated P19 cells (date not shown). Consequently, the data presented here indicate that primary hippocampal neurons and P19 neurons, unlike undifferentiated P19 cells, are sensitive to the long dsRNAs, which induce an apoptotic pathway and appear to mask the sequence-specific RNAi activity involving the long dsRNAs in those cells.

4. Discussion

4.1. Long-term effect of RNAi on mammalian neurons

The persistence of RNAi activity is an important parameter in the application of RNAi to mammalian cells. While the effect of RNAi on proliferating mammalian cells appears to last for approximately 3–7 days, our present study demonstrates that the RNAi activity induced by synthetic 21-nt siRNA duplexes persists for at least 3 weeks in mammalian neurons. Since a neuron does not undergo cell division, and since mammalian RNAi appears not to require a new supply of siRNAs involving RNA-dependent RNA polymerases [28,29], the present data suggest that the RISCs containing siRNA duplexes are most likely stable in mammalian neurons, and that the absence of cell division, due to which the number of RISCs in neurons appears unchanged, probably contributes to such a long-term effect of RNAi activity on neurons. Under our culture conditions, although the number of neurons became fewer as the culture duration increased, we could detect a strong RNAi activity in the cells for as long as 3 weeks; if the culture conditions were improved, or if neurons in vivo were used, a longer effect of RNAi activity, that is, more than 3 weeks, could be observed.

It is of interest to examine whether such a long-lasting RNAi activity in neurons participates in the long-term regulation of the expression of genes associated with neuronal functions, for example, long-term changes in synaptic plasticity. In order to evaluate such a possibility, more extensive studies must be conducted.

4.2. Generation of an apoptotic pathway involving long dsRNAs during neuronal differentiation

Most mammalian cells are sensitive to the long dsRNAs that are more than 30 bp; such long dsRNAs trigger rapid and non-specific RNA degradation and rapid translation inhibition, namely, the interferon-induced antiviral response, and triggers apoptosis of cells [13,14,27], whereas a part of undifferentiated mammalian cells appear to be resistant to the long dsRNAs; in such cells, the long dsRNAs can induce sequence-specific RNAi activity without inducing apoptosis [11,12]. In this study, we have shown that P19 cells change the nature of their response to the long dsRNAs, that is, from being resistant to being sensitive, during neuronal differentiation. We have further demonstrated that the *RNase L* and *PKR* genes involved in non-specific RNA degradation and translation inhibition, respectively, are rapidly expressed in the course of neuronal differentiation of P19 cells. Consistently, primary hippocampal neurons appear to possess a similar response against the long dsRNAs to P19 neurons. Therefore, these observations suggest that an antiviral response or a neuronal apoptotic pathway, which is triggered by the long dsRNAs, is generated in the course of the neuronal differentiation, and also that the loss of RNAi induction by the long dsRNAs in the neurons is probably due to the formation of such rapid responses to the long dsRNAs. Additionally, since PKR is known to control the activation of several transcription factors such as NF-κB, p53, or STATs [30], and since the activation of NF-κB occurs during the differentiation of P19 cells [31,32], it may be possible that PKR could participate in not only the antiviral response but also the regulation of the expression of genes during the neuronal differentiation. Apoptosis involving the long dsRNAs may also play a role in elimination of cells in which an aberrant RNAi could occur, other than the removal of virus-infected cells. In the brain, such an apoptotic elimination of neurons possessing an abnormal RNAi might contribute to the formation of proper neuron networks.

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References

[1] Fire, A. (1999) Trends Genet. 15, 358–363.
[2] Sharp, P.A. (1999) Genes Dev. 13, 139–141.
[3] Bosher, J.M. and Labouesse, M. (2000) Nat. Cell Biol. 2, E31–6.
[4] Vaucheret, H., Beclin, C. and Fagard, M. (2001) J. Cell Sci. 114, 3083–3091.
[5] Zamore, P.D., Tuschl, T., Sharp, P.A. and Bartel, D.P. (2000) Cell 101, 25–33.
[6] Elbashir, S.M., Lendeckel, W. and Tuschl, T. (2001) Genes Dev. 15, 188–200.
[7] Bernstein, E., Caudy, A.A., Hammond, S.M. and Hannon, G.J. (2001) Nature 409, 363–366.
[8] Hammond, S.M., Bernstein, E., Beach, D. and Hannon, G.J. (2000) Nature 404, 293–296.
[9] Svoboda, P., Stein, P., Hayashi, H. and Schultz, R.M. (2000) Development 127, 4147–4156.
[10] Wianny, F. and Zernicka-Goetz, M. (2000) Nat. Cell Biol. 2, 70–75.
[11] Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Proc. Natl. Acad. Sci. USA 98, 14428–14433.
[12] Yang, S., Tutton, S., Pierce, E. and Yoon, K. (2001) Mol. Cell. Biol. 21, 7807–7816.
[13] Player, M.R. and Torrence, P.F. (1998) Pharmacol. Ther. 78, 55–113.
[14] Gale, M.Jr. and Katze, M.G. (1998) Pharmacol. Ther. 78, 29–46.
[15] Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K. and Tuschl, T. (2001) Nature 411, 494–498.
[16] Harborth, J., Elbashir, S.M., Bechert, K., Tuschl, T. and Weber, K. (2001) J. Cell Sci. 114, 4557–4565.
[17] Jacque, J.M., Triques, K. and Stevenson, M. (2002) Nature 418, 435–438.
[18] Kapadia, S.B., Brideau-Andersen, A. and Chisari, F.V. (2003) Proc. Natl. Acad. Sci. USA 100, 2014–2018.
[19] Holen, T., Amarzguioui, M., Wiiger, M.T., Babaie, E. and Prydz, H. (2002) Nucleic Acids Res. 30, 1757–1766.
[20] Elbashir, S.M., Harborth, J., Weber, K. and Tuschl, T. (2002) Methods 26, 199–213.
[21] Krichevsky, A.M. and Kosik, K.S. (2002) Proc. Natl. Acad. Sci. USA 99, 11926–11929.
[22] Yu, J.Y., DeRuiter, S.L. and Turner, D.L. (2002) Proc. Natl. Acad. Sci. USA 99, 6047–6052.
[23] Numakawa, T. et al. (2003) J. Biol. Chem. 278, 41259–41269.
[24] Hatanaka, H., Tsukui, H. and Nihonmatsu, I. (1988) Brain Res. 467, 85–95.
[25] Hohjoh, H. (2002) FEBS Lett. 521, 195–199.
[26] Jones-Villeneuve, E.M., McBurney, M.W., Rogers, K.A. and Kalnins, V.I. (1982) J. Cell Biol. 94, 253–262.
[27] Stark, G.R., Kerr, I.M., Williams, B.R., Silverman, R.H. and Schreiber, R.D. (1998) Annu. Rev. Biochem. 67, 227–264.
[28] Schwarz, D.S., Hutvagner, G., Haley, B. and Zamore, P.D. (2002) Mol. Cell 10, 537–548.
[29] Stein, P., Svoboda, P., Anger, M. and Schultz, R.M. (2003) RNA 9, 187–192.
[30] Gil, J. and Esteban, M. (2000) Apoptosis 5, 107–114.
[31] Belhumeur, P., Lanoix, J., Blais, Y., Forget, D., Steyaert, A. and Skup, D. (1993) Mol. Cell. Biol. 13, 2846–2857.
[32] Kalvakolanu, D.V. and Sen, G.C. (1993) Proc. Natl. Acad. Sci. USA 90, 3167–3171.