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The latency-associated transcript of herpes simplex virus type 1 promotes survival and stimulates axonal regeneration in sympathetic and trigeminal neurons

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Herpes simplex virus type 1 (HSV-1) primarily infects mucoepithelial tissues of the eye, the orofacial region, and to a lesser extent the genitalia. The virus is retrogradely transported through the axons of sensory and sympathetic neurons to their cell bodies to establishe a life-long latent infection. Throughout this latency period, the viral genome is transcriptionally silent except for a single region encoding the latency-associated transcript (LAT). The function of LAT is still largely unknown. To understand how HSV-1 latency might affect neurons, the authors transfected primary cultures of sympathetic neurons and trigeminal sensory neurons obtained from rat embryos with LAT-expressing plasmids. LAT increased the survival of both sympathetic and trigeminal neurons after induction of cell death by nerve growth factor (NGF) deprivation. Because HSV-1 is transported through axons both after initial infection and during reactivation, the authors considered the possibility that LAT may affect axonal growth. They found that LAT expression increased axonal regeneration by twofold in both types of neurons. Inhibition of the mitogen-activated protein kinase (MAPK) pathway reverses stimulation of both neuronal survival and axonal regeneration, which indicates that these effects are mediated through the MAPK pathway. These data provide evidence that HSV-1 LAT promotes survival of sympathetic as well as trigeminal neurons. The authors show for the first time that LAT stimulates axonal regeneration in both sympathetic and trigeminal neurons. *Journal of NeuroVirology* (2007) **13,** 56–66.

Keywords: axon; herpes; LAT; survival; sympathetic; trigeminal

Introduction

Herpes simplex virus type 1 (HSV-1) is a member of the alpha-herpesvirus subfamily that infects approximately two thirds of the United States population (Schillinger *et al*, 2004). A common characteristic of the alpha-herpesviruses is their ability to establish lifelong latent infections in neurons (Baringer and Swoveland, 1973; Cook *et al*, 1974). HSV-1 initially infects the mucoepithelial tissues of the orofacial region and, to a much lesser extent, of the genitalia. Subsequently, the virus is retrogradely transported to the cell bodies of the sensory and sympathetic neurons in the corresponding ganglia (Bastian *et al*, 1972; Warren *et al*, 1978). The virus establishes latency in the nuclei of the infected neurons. During latent neural infection with HSV-1, significant gene expression is restricted to one mRNA, the latency-associated transcript (LAT) (Stevens *et al*, 1987; Deatly *et al*, 1987). The primary transcript of the HSV-1 LAT gene is 8.3 kb in length and this is rapidly processed to yield shorter transcripts, the most prominent of which is a stable 2.0 kb intron (Farrell *et al*, 1990). This transcript has been detected exclusively in the

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nuclei of latently infected neurons (Wagner *et al*, 1988).

LAT has been shown to regulate the establishment of viral latency and reactivation (Block *et al*, 1993; Thompson and Sawtell, 1997) and alter the properties of the host cell. Previous studies indicate that deletion of the HSV-1 LAT gene causes an increase in programmed cell death in virally infected trigeminal ganglia *in vivo* when compared with those infected with the wild type virus (Perng *et al*, 2000; Branco and Fraser, 2005). However, because several HSV-1 gene products can alter host cell survival, it was not known whether LAT directly affects programmed cell death in neurons or acts indirectly by altering either viral replication or the expression of other viral proteins. A recent study by Gupta *et al* (2006) has addressed this question and shown that a microRNA encoded in the 3′ region of the LAT exon 1 exhibits antiapoptotic activity in the continuous Hela and SY5Ycell lines. It has also been reported that transfection of the LAT intron reduces cell death in actively proliferating cell lines (Perng *et al*, 2000; Ahmed *et al*, 2002), but the relevance of data obtained from lytically infected and mitotically active cells to latent infection of postmitotic neurons is uncertain. Previous studies have shown that latent HSV-1 infection of mouse trigeminal ganglia can alter the expression of neuronal genes including those involved in immune response, signaling, gene expression, and axonal remodeling (Higaki *et al*, 2002; Kramer *et al*, 2003; Kent and Fraser, 2005), which suggests that LAT may alter other properties of neuronal cells in addition to those listed.

In our studies we transfected plasmids expressing the 2.0-kb LAT intron into primary rat sympathetic and trigeminal neurons to examine the effects of LAT on neuronal survival and on axonal growth. Our data indicate that expression of this region of HSV-1 LAT increases survival of both cultured sympathetic and trigeminal primary neurons by inhibition of the apoptotic pathway. These data are in agreement with those obtained in continuous cell lines and *in vivo* mouse and rabbit models (Perng *et al*, 2000; Inman *et al*., 2001; Ahmed *et al*, 2002; Jin *et al*, 2003; Branco and Fraser, 2005) and suggest that the antiapoptotic effect of LAT applies to all host neuronal cell types. We show here for the first time that HSV-1 LAT stimulates axonal growth in cultured neurons, thus indicating a novel role for LAT in the biogenesis of these processes. We provide evidence that the mitogen-activated protein kinase (MAPK) pathway plays a role in effects of LAT on survival and axonal growth of neurons.

Results

Expression of LAT in sensory and motor neurons

During latent infection with HSV-1 *in vivo*, LAT is the only abundantly expressed RNA. We wished to examine the effects of LAT on primary cultures of sympathetic motor neurons as well as trigeminal sensory neurons. Both types of cultures were transfected with a plasmid containing a 2.2-kb LAT insert whose expression was driven by the cytomegalovirus (CMV) promoter ensuring high levels of expression. The LAT does not normally give rise to a protein product (Doerig *et al*, 1991; Lagunoff and Roizman, 1994; Drolet *et al*, 1998; Thomas *et al*, 2002). LAT expression was examined by *in situ* hybridization. LAT expression was confined to the nucleus in primary cultures of trigeminal and sympathetic neurons (Figure 1), and therefore would not be available to the cellular translation apparatus. Thus, the distribution of LAT in primary sensory and sympathetic motor neurons in our in vitro system is similar to that observed *in vivo* (Wagner *et al*, 1988).

Because the conditions required for in situ hybridization interfere with most cytochemical reactions, we used enhanced green fluorescent protein (EGFP) expression as a surrogate marker to identify neurons transfected with LAT. We previously found that in sympathetic neurons 85% to 90% of neurons expressing EGFP were also LAT positive, and we did not detect LAT-positive cells that were EGFP negative (Hamza *et al*, 2006). Similarly, we found that trigeminal neurons transfected with LAT-containing plasmids express LAT exclusively in their nuclei (Figure 1B and C). Thus, EGFP could be reliably used as a surrogate marker for LAT expression in primary cultures of sympathetic and sensory neurons. In our experiments, we studied transfected neurons by examining only those neurons expressing EGFP.

Effects of LAT expression on neuronal survival

Expression of LAT increases the survival of trigeminal neurons infected with HSV-1 *in vivo* in a rabbit model and continuous cell lines transfected with plasmids expressing LAT sequences (Perng *et al*, 2000; Gupta *et al*, 2006). We wished to determine if LAT also promotes the survival of other host cell populations and whether its effects on survival are direct or indirect. We therefore established primary cultures of sympathetic and trigeminal neurons obtained from rat embryos and transfected them with plasmids expressing LAT constructs. The cultures were treated for 2 days with an antimitotic agent to kill dividing non-neuronal cells prior to transfection. We initially focused on sympathetic neurons because they are the most readily transfected (~25% efficiency versus 1% to 5% in trigeminal neurons), and the most thoroughly characterized in terms of cell death (Glebova and Ginty, 2005).

Embryonic rat sympathetic neurons undergo apoptosis when they are deprived of Nerve groath factor (NGF) and this cellular model has been widely used to study programmed cell death (Glebova and Ginty, 2005). We therefore used these cells to study the antiapoptotic effects of LAT. Expression of LAT increased the percentage of viable cells, i.e., percentage of neurons that did not exhibit DNA condensation after NGF deprivation (73.6% ± 6.1% in

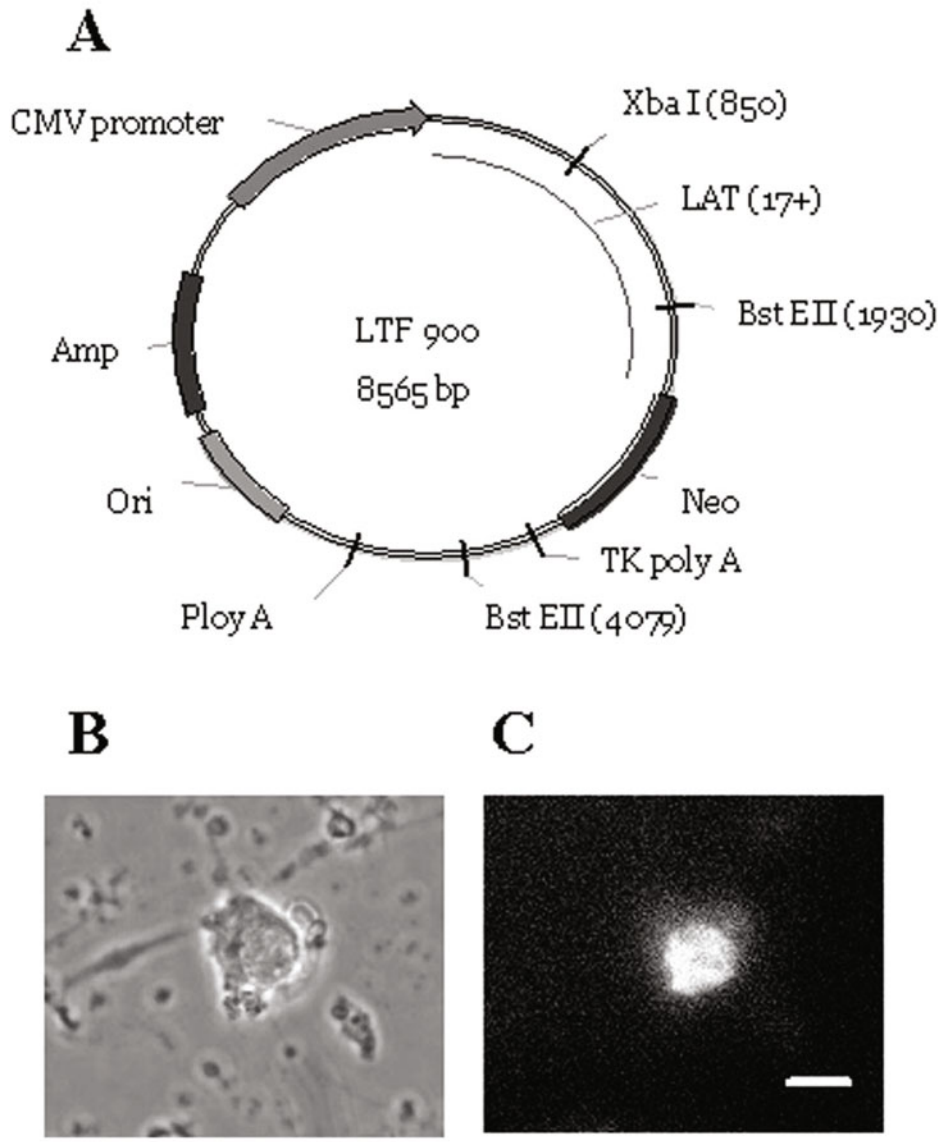

Figure 1 Expression of LAT in sympathetic and trigeminal neurons. (**A**) A map showing the LAT containing plasmid (LTF 900) that was used in the experiments is presented. The LAT sequences expressed include bp 119311 to 121571 of the HSV-1 strain 17+ sequence. (**B**) and (**C**) Phase- contrast (**B**) and fluorescence (**C**) micrographs of a trigeminal neuron transfected with the plasmid expressing the 2.0-kb intron. LAT was detected in the nucleus of the neuron by *in situ* hybridization (**C**). Scale bar is 20 μm.

comparison to 52.6% ± 8.2% in pCDNA3.1- transfected neurons) (Figure 2G). Using the same assay, we also observed increased cell survival in cultures of trigeminal neurons that were deprived of NGF (80.4% ± 5.4% in comparison to 45.9% ± 5.8% in pCDNA3.1-transfected neurons) (Figure 2H). Thus LAT appears to prevent or retard cell death in both neuronal cell populations that can serve as hosts for HSV-1.

The finding that expression of LAT reduced the number of cells with nuclear condensations suggested that it can block apoptosis as it has been previously shown. Consistent with this, we found that expression of LAT reduced by ~50% the number of tunel-positive nuclei in primary sympathetic neurons (29.3% ± 8.2% in comparison to 54.1% ± 5.8% in pCDNA3.1 transfected neurons) (Figure 3) and trigeminal neurons (not shown) deprived of NGF. Additionally, LAT inhibited the activation of caspase 3 in sympathetic neurons deprived of NGF (10.3% ± 3.8% in comparison to 23.8% ± 4.8% in pCDNA3.-transfected neurons) (Figure 3), indicating that LAT interferes with the apoptosis pathway in sympathetic as well as sensory neurons.

To identify the region(s) of the LAT sequences used here that are responsible for increasing sympathetic neuronal survival, 5′ deletion mutants of the LAT insert were used. The deleted segments ranged from

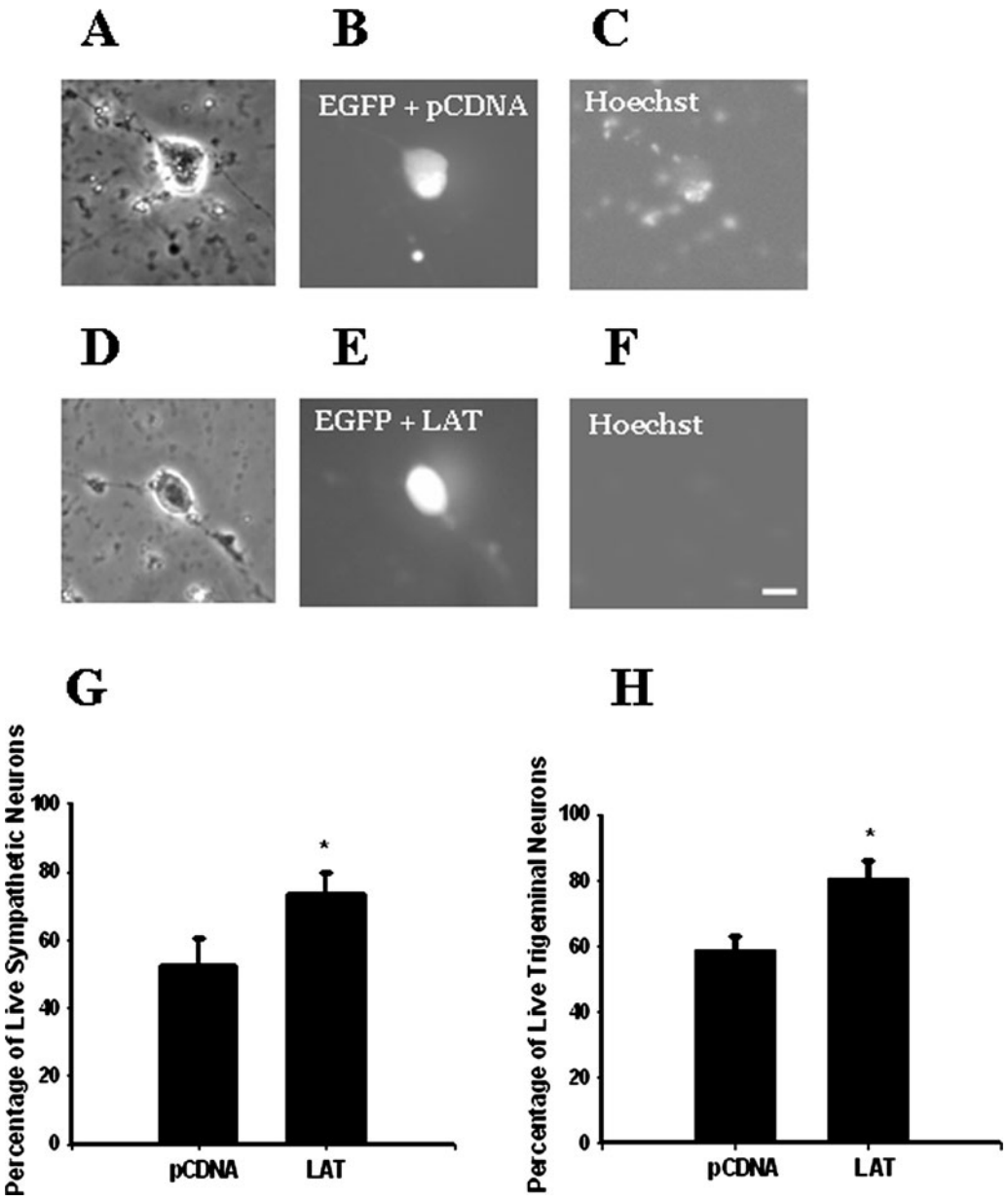

Figure 2 LAT increases survival of sympathetic and trigeminal neurons. Sympathetic neurons were cotransfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector. Two days later, neuronal death was induced by replacing NGF-containing medium with DMEM. Fourteen hours later, the cultures were fixed, and then stained with Hoechst to detect dead neurons. The same procedure was done to cultures of trigeminal sensory neurons. Phase-contrast (**A**, **D**), green fluorescence (**B**, **E**), and Hoechst stain (**C**, **F**) micrographs of sympathetic neurons. Cultures were cotransfected with plasmids containing EGFP and the pCDNA3.1 vector (**A**, **B**, **C**) or LAT (**D**, **E**, **F**). The neuron transfected with LAT did not show nuclear staining with Hoechst (**F**), whereas the one transfected with pCDNA3.1 showed nuclear staining with Hoechst (**C**). LAT increased survival of sympathetic ($n = 53$ neurons, $P < .05$) (**G**) and sensory ($n = 56$ neurons, $P < .001$) (**H**) neurons by 40% to 50%. Scale bar is 20 μm.

~800 b to ~1.85 kb of the LAT insert (~600 b to ~1.65 kb of the LAT intron) (Figure 4A). Mutants 901 and 902 retained some ability to enhance cell survival (percentages of live neurons were 79.4 ± 7 and 78.8 ± 7.2, respectively; as compared to 48 ± 5 in untransfected neurons). The mutant 904 had no significant effect on survival (percentage of live neurons was 58.8 ± 8.6) (Figure 4B). Mutant 903 increased neuronal survival (72% ± 9.7%) more than mutant 904, but the effect was not statistically significant. These results suggest that the first ~800 bases from the 5′ end of the LAT construct used in this study are primarily responsible for the observed effects on neuronal survival.

We next investigated whether the observed LAT effects involve the MAPK pathway in order to gain insight into the mechanism of LAT action. This was based on the fact that NGF activation of the MAPK

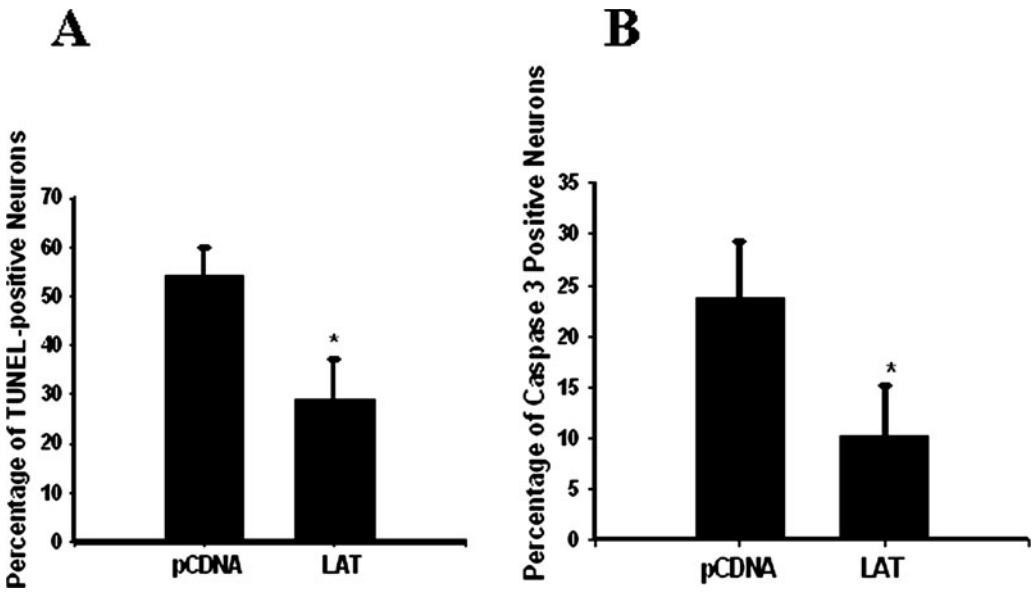

Figure 3 LAT inhibits apoptosis and decreases the activation of caspase 3. Neuronal cultures were cotransfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector. Two days later, neuronal death was induced by replacing the NGF-containing medium with DMEM. Fourteen hours later, the cultures were fixed, and the cells stained with TUNEL for detection of apoptotic cells, or with caspase 3 active. **A,** LAT decreased the percentage of apoptotic sympathetic neurons ($n = 41$). **B**, LAT decreased the percentage of sympathetic neurons positive for activated caspase 3 by ~50% ($n = 50$) ($^*P < .05$).

pathway results in both neuronal survival and process growth (Mazzoni *et al*, 1999). We treated neurons with PD98059, a MEK1 inhibitor, which results in inhibition of the MAPK pathway. Treatment of sympathetic neurons with PD98059 reversed the effects of LAT on neuronal survival following NGF withdrawal (the percentage of live neurons was 50.6 ± 5.5 with PD98059 as compared to73.6 ± 7.7 without PD98059) (Figure 5A). To verify the involvement of the MAPK pathway, we cotransfected sympathetic neurons with LAT expressing plasmids and plasmids expressing a dominant negative MEK (DnMEK). DnMEK also reversed the effects of LAT on neuronal survival (60.9% ± 8.6% with DnMEK in comparison to 95.5% ± 3.6% without DnMEK) (Figure 5B). These data indicate that activation of the MAPK pathway plays a role in the antiapoptotic effect of LAT on neurons.

Effects of LAT expression on axonal regeneration

In primary neuronal cultures, both sympathetic and sensory neurons begin to extend axons within hours after plating, and these axons continue to grow at a high rate during the first few days of culture. We wished to study the effects of LAT on axonal growth. However, in the standard protocol, cultures are transfected 5 days after plating the cells, and the neurons

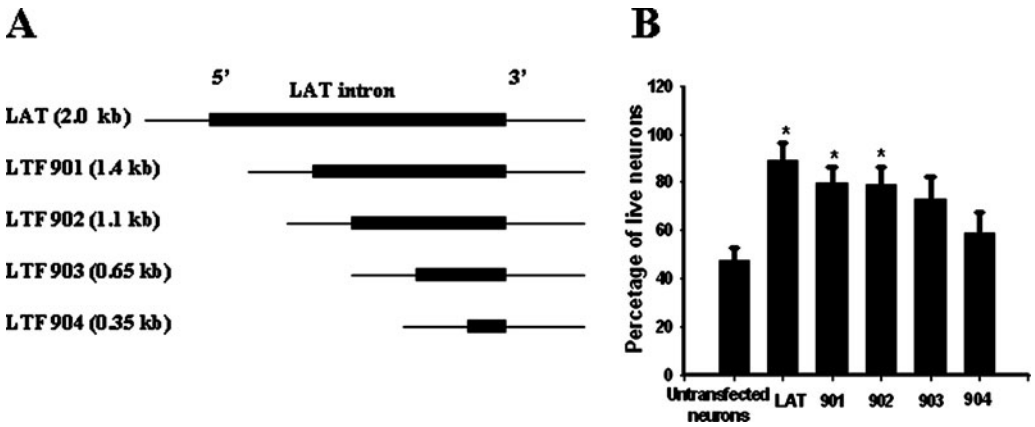

Figure 4 Effects of LAT deletion mutants on neuronal survival.**A,** Schematic representation of plasmids containing LAT (wild type) and the deletion mutants (901–904). **B,** Mutants 901($n = 40$) and 902 ($n = 33$) retained the ability to increase survival of sympathetic neurons ($^*P < .05$), whereas mutant 904 ($n = 34$) had no significant effect. Mutant 903 ($n = 44$) increased neuronal survival more than mutant 904, but the effect was not statistically significant.

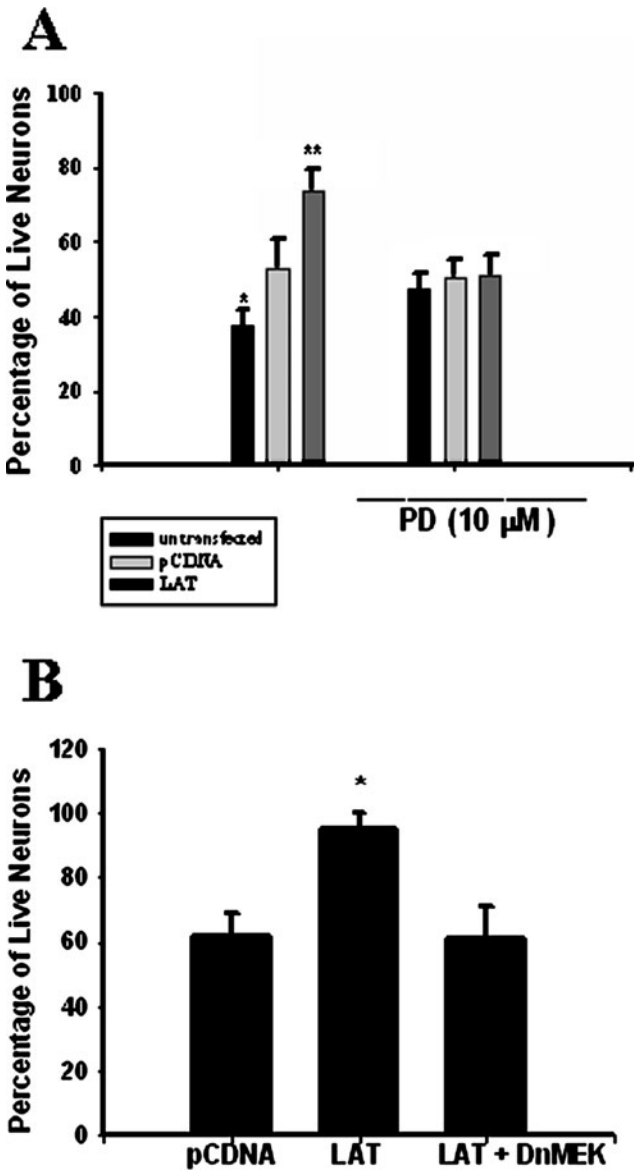

Figure 5 LAT promotion of neuronal survival is reversed by both MEK inhibitor PD98059 and dominant-negative MEK.**A**, Sympathetic neuronal cultures were cotransfected with plasmids containing EGFP and plasmids containing pCDNA3.1 or LAT, then neuronal death was induced as described in Figure 2 and the cells were fixed and stained with Hoechst. Treatment of neurons with PD98059 (10 μM) for 3 days before induction of neuronal death reversed the antiapoptotic effect of LAT ($n = 74$, $P < .01$). **B**, Sympathetic neurons were cotransfected with plasmids containing EGFP and plasmids containing pCDNA3.1, or pCDNA3.1 and LAT, or LAT and dominant-negative MEK (DnMEK). Equal amounts of plasmid DNA (2 μg/well) were used for transfection. Neuronal death was induced, and the cells were stained with Hoechst. DnMEK reversed the positive effect of LAT on neuronal survival ($n = 34$, $P < .001$).

have already extended long axons. Thus measurement of axonal length would represent initial axonal growth rather than growth after transfection.

We therefore utilized an alternative method to study the effect of LAT on axonal growth. Explants were cultured from the superior cervical ganglia as well as from the trigeminal ganglia of the rat embryos. Non-neuronal cells were eliminated, and by this time (6 days) the explants extended long processes. The axons were then cut, and the neurons were dissociated and plated on coverslips. Sixteen hours later, the neurons were cotransfected with plasmids containing EGFP and either the LAT insert or the empty pCDNA3.1 vector.

This protocol took advantage of our finding that the axons of these neurons do not begin to regenerate until 24 to 48 h after plating. However, these neurons can be transfected after 16 h of dissociation and plating, which allows the expression from the transfected constructs to take place before the beginning of axonal growth. After allowing time for the axons to regenerate, we found that LAT stimulated axonal regeneration in both sympathetic neurons (406.9 $\pm$ 63 μm as compared to 184.9 $\pm$ 34 μm in pCDNA3.1-transfected neurons) (Figure 6) and trigeminal sensory neurons (839.2 $\pm$ 133.6 μm as compared to 365 $\pm$ 80 μm in pCDNA3.1-transfected neurons) (Figure 7). LAT increased the average axonal length of neurons by twofold when compared to neurons transfected with the control plasmid.

To examine whether the MAPK pathway plays a role in LAT stimulation of axonal regeneration, we treated sympathetic neurons with PD98059 to inhibit the MAPK pathway. Treatment with PD98059 reversed LAT stimulation of axonal growth (200.5 $\pm$ 80 with PD98059 treatment in comparison to 413.4 $\pm$ 61.3 without PD98059 treatment) (Figure 8), indicating that activation of the MAPK pathway plays a role in LAT stimulation of axonal regeneration as well as promotion of neuronal survival.

Discussion

HSV-1 infects 67.7% of the population aged 12 years and older in the United States, and the prevalence increases with age from 44% among people aged 12 to 19 years to 90% among those aged $\geq$70 years (Schillinger *et al*, 2004). The widespread nature of HSV-1 infection with a life-long latency, which is interrupted by frequent reactivations is a peculiar characteristic of this virus. Although the virus infects the mucoepethelial tissue of the face and genitalia, the latency of HSV-1 specifically takes place in neurons (Bastian *et al*, 1972; Warren *et al*, 1978). The LAT is the only viral transcript consistently found in latently infected neurons as compared to transcripts for all of the over 70 open reading frames encoded by the viral DNA of HSV-1during lytic infection of other cell types (Stevens *et al*, 1987; Mitchell *et al*, 2003).

Previous studies have shown that latent HSV-1 infection may increase survival and inhibit apoptosis of trigeminal sensory neurons in rabbits (Perng *et al*, 2000; Thompson and Sawtell, 2001). In other work, latent wild-type HSV-1 infection of mouse trigeminal ganglia *in vivo* reduced the number of apoptotic neurons in comparison with latent infection with a

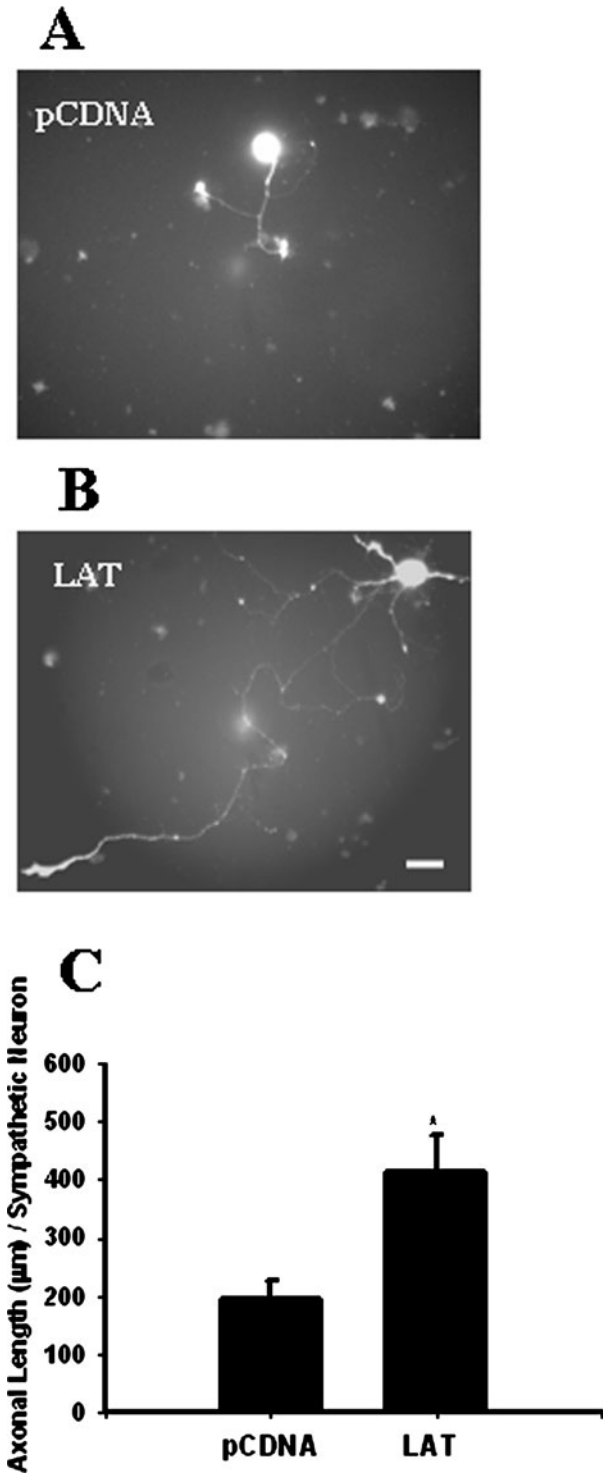

Figure 6 LAT increases axonal regeneration in sympathetic neurons. Superior cervical ganglia were explanted in 35-mm plastic dishes. Non-neuronal cells were eliminated and 2 days later the axons were cut and the neurons were dissociated and plated on 18-mm coverslips. Sixteen hours later, the neurons were cotransfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector. Five days later, the cells were fixed and neurons expressing EGFP are photographed and the total axonal length for each neuron is measured. Green fluorescence micrographs of sympathetic neurons cotransfected with plasmids containing EGFP and the pCDNA3.1 vector (**A**) or LAT (**B**). LAT ($n = 50$) caused a twofold increase in axonal length in comparison with neurons transfected with pCDNA3.1 ($n = 61$) (**C**) ($^*P < .001$). Scale bar is 20 μm.

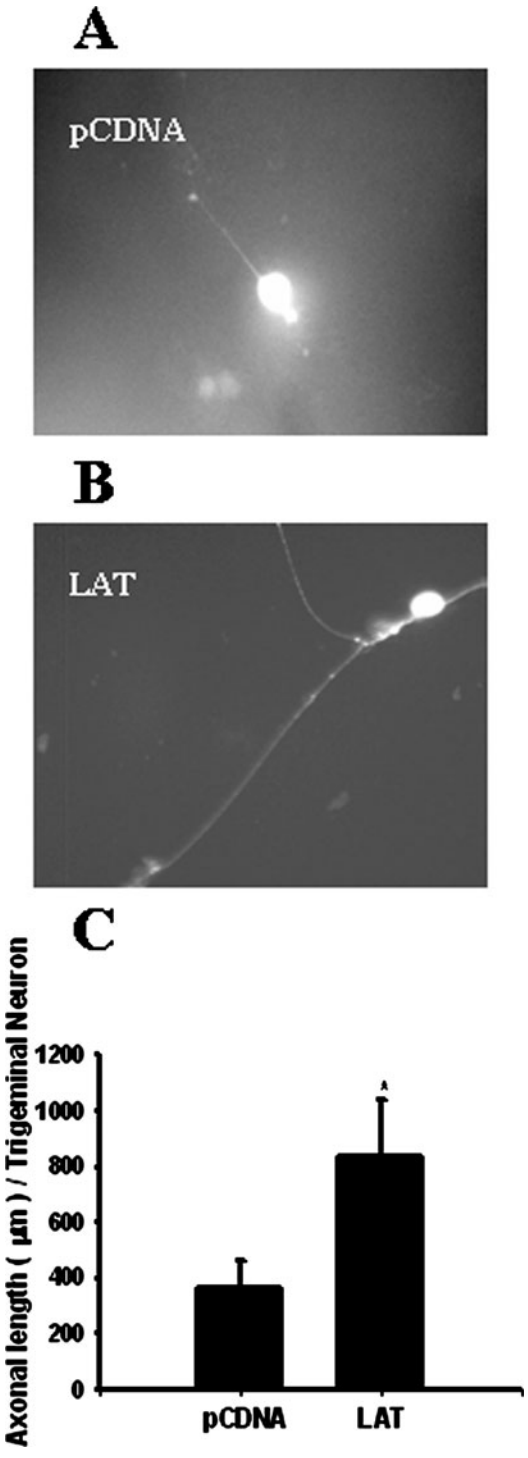

Figure 7 LAT increases axonal regeneration in trigeminal sensory neurons. Trigeminal ganglia were explanted in 35-mm plastic dishes. Non-neuronal cells were eliminated and 2 days later the axons were cut and the neurons were dissociated and plated on 18-mm coverslips. Sixteen hours later, the neurons were cotransfected with plasmids containing EGFP and LAT or the pCDNA3.1 vector and treated with medium containing NT3 (10 ng/ml). Five days later, the cells are fixed and neurons expressing EGFP are photographed and the total axonal length for each neuron is measured. Green fluorescence micrographs of trigeminal neurons cotransfected with plasmids containing EGFP and the pCDNA3.1 vector (**A**) or LAT (**B**). LAT ($n = 48$) caused a twofold increase in axonal length in comparison with neurons transfected with pCDNA3.1 ($n = 66$) (**C**) ($^*P < .005$). Scale bar is 20 μm.

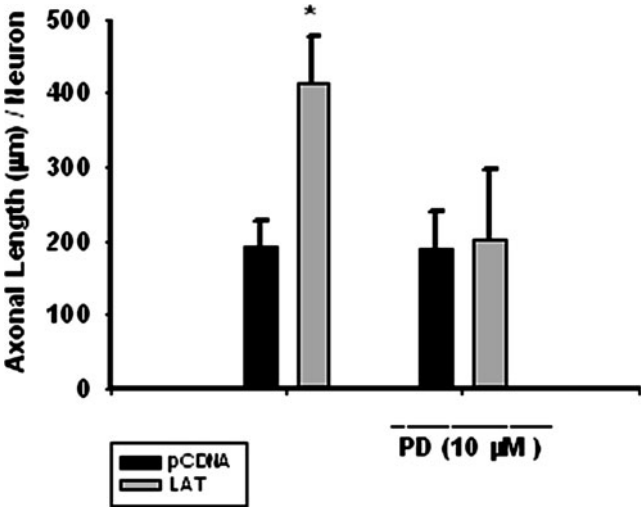

Figure 8 MEK inhibitor PD98059 reverses LAT stimulation of axonal regeneration. Superior cervical ganglia were explanted, dissociated and transfected as mentioned in Figure 6. After transfection, the cells were treated with medium containing PD98059 (10 μM) and axons were allowed to grow for 5 days, then the cultures were fixed and the total axonal length for each neuron were measured. Treatment with PD98059 reversed the stimulating Effect of LAT on axonal regeneration ($n = 33$) in comparison to untreated neurons ($n = 35$) ($^*P < .001$).

LAT-negative deletion mutant HSV-1 strain (Branco and Fraser, 2005). In a number of other studies, expression of LAT in neuronal cell lines resulted in a reduction in the percentage of cells that became apoptotic (Perng *et al*, 2000; Moxley *et al*, 2002). Although these studies provided evidence that LAT has an antiapoptotic effect, there was no evidence in the literature that LAT was able to inhibit apoptosis in primary sympathetic neurons, which is another, albeit infrequent, host for the latency of HSV-1. Also, the induction of apoptosis (at least in the majority of these studies) was achieved through methods including virus-induced apoptosis and proapoptotic drugs such as etoposide and camptothecin (Perng *et al*, 2000; Ahmed *et al*, 2002; Gupta *et al*, 2006). These methods do not represent the pathophysiological factors that induce apoptosis in neurons. Our results provide direct evidence that the expression of LAT increases survival of primary neurons by inhibiting apoptosis induced by NGF withdrawal. The induction of apoptosis by withdrawal of NGF from culture media has been widely used and accepted as the most likely mechanism behind apoptosis of primary neurons (Glebova and Ginty, 2005).

Our data expand the information regarding the anti-apoptotic effect of LAT on sensory neurons and extend the spectrum of the antiapoptotic effect to include sympathetic neurons. In agreement with previous studies (Inman *et al*, 2001; Ahmed *et al.*, 2002; Branco and Fraser, 2005; Gupta *et al*, 2006), our findings show that ectopic expression of LAT sequences results in increasing neuronal survival. We have shown this to be true for both primary sympathetic as well as sensory neurons, indicating that this effect of LAT may be universal for all types of host neurons. Deletion of sequences comprising the 3′ 200 bp of the LAT exon 1 and first 600 bp of the LAT intron resulted in a partial positive effect on neuronal survival as compared to expression of the full-length insert. The recent work by Gupta *et al.* (2006) indicates that a microRNA encoded in the 3′ region of exon 1 of LAT is in part responsible for this effect. The LAT construct used in the present study begins 36 bp downstream of the sequence encoding the microRNA. Although the LAT construct used in our study did not contain this sequence, as it begins 36 bp downstream of the sequence encoding the microRNA, our study shows that the ~800 bp from the 5′ end of our LAT construct also has an antiapoptotic effect, and the ~300 bp downstream to the ~800 bp still exert a partial effect on neuronal survival. These results are in agreement with Gupta *et al* (2006), who showed that deletion of the sequences encoding the microRNA did not completely ablate the antiapoptotic activity of LAT. Enhancing the survival of the neuron is an effect that is of crucial importance to the biology of infection of the virus. Long-term survival of neurons containing the latent viral genome results in a stable reservoir of latent virus until the occurrence of the as yet unknown molecular events that result in reactivation.

The effect of LAT on axonal regeneration in this study represents a novel finding. Our data show that LAT is capable of stimulating axonal regeneration in both types of neurons used in this study. Because initial HSV-1 infection of neurons may be associated with axonal injury, regeneration of axons may be important for a number of reasons. First, neurotrophins, such as NGF, which are important for the survival of the neurons, are delivered by retrograde transport from the target organs to the neuronal cell bodies through the axons (Ure and Campenot, 1997; Riccio *et al*, 1997), which is important for the survival of the neurons. Second, because HSV-1 reactivation results in anterograde transport of the virus to the target tissues (Miranda-Saksena *et al*, 2000), axonal regeneration may be necessary to maintain this pathway to the peripheral tissues. The effect on axonal regeneration was not limited to a single type of neuron, and whether or not this effect extends to other types of neurons that are not the natural hosts for HSV-1 latency needs to be investigated.

In this study we explored whether LAT affects one of the key signaling pathways involved in neuronal survival and axonal growth. The MAPK pathway is activated by binding of NGF to TrkA receptors, which activates the small GTP-binding protein Ras. Ras activates a number of downstream effectors including Raf, MEK, and ERK, and this signaling cascade decreases neuronal apoptosis by suppressing the expression or the function of a number of key elements in the apoptotic pathway including Bax, c-jun, and p53 (Mazzoni *et al*, 1999; Anderson and Tolkovsky

et al, 1999). In addition, through activation of the MAPK pathway, NGF stimulates axonal growth (Doherty *et al*, 2000; Goold and Gordon-Weeks, 2004). Our data show that inhibition of MEK reversed the LAT stimulation of survival and axonal growth, and this provides the first evidence that LAT may exert its effects through activation of the MAPK pathway.

In conclusion, our results clearly indicate that HSV-1 latency not only affects the survival of the host neurons, but also alters the growth of its processes. We suggest that latently infected neurons will be more able to survive and regenerate their damaged axons. The question of whether these effects have pathophysiological consequences on the infected neurons needs to be addressed in further studies. Our data increase our understanding of the virus-neuron interaction during latency, which might help in establishing new therapeutic strategies for latent and productive viral infections.

Materials and methods

Materials
Human recombinant bone morphogenetic protein-7 (BMP7) was generously provided by Curis (Cambridge, MA). PD98059 was purchased from Biomol (Plymouth Meeting, PA).

Plasmids
A plasmid coding enhanced green fluorescent protein (pEGFP-C1) was purchased from Clontech (Palo Alto, CA). Plasmids expressing the full-length HSV-1 strain 17+ 2.0-kb LAT intron and truncations thereof were generated as follows. A fragment from the HSV-1 LAT gene (bp 119311 to 121571 of HSV-1 strain 17+) (McGeoch *et al*, 1988), which included ~200 bp of the 3′ end of LAT exon 1 and the full-length 2.0-kb LAT intron, was cloned into a plasmid containing the CMV IE enhancer and a polyadenylation signal. For the 5′ deletion series, the CMV enhancer was followed by the splice donor site and the restriction sites Asp718, Bgl II, and XbaI. For plasmid 901, a partial digest with Bst EII was used, filled in, and an XbaI linker inserted. This plasmid was digested with Xba I and closed to generate a plasmid with the CMV enhancer, the LAT splice donor, and the remaining 1.4 kb of the LAT intron. The other plasmids were generated in a similar manner, with 902 cut at Hpa I to generate a 1.1-kb 3 end, 903 cut at Sal I to generate a 0.65-kb fragment, and 904 as another partial with BstEII to generate a 0.35-kb DNA fragment.

The dominant-negative MEK1 plasmid carrying M substitution at K97 was a generous gift from Dr. Natalie Ahn (University of Colorado, Boulder, CO).

Cell cultures
Superior cervical ganglia were dissected from embryos (E20) of Holtzman rats (Harlan Sprague-Dawley, Rockford, IL). The cells were dissociated after treatment with trypsin (2.5 mg/ml) and collagenase (1 mg/ml) for 35 min. Cells were plated on poly-D-lysine–coated (200 μg/ml) coverslips and maintained on serum-free medium containing β-nerve growth factor (NGF; 100 ng/ml) (Higgins *et al*, 1991). One day later, the cultures were treated with medium containing the antimitotic drug cytosine-β-D-arabinofuranoside (ara-c) (1 μM) for 2 days to eliminate non-neuronal cells, and then the culture were allowed to recover for 2 days before transfection. Trigeminal ganglia were dissected from embryos (E19) of Holtzman rats (Harlan Sprague-Dawley), and then dissociated and plated as described above. Subsequently, the cultures were treated for 2 days with ara-c (1 μM) to reduce the number of non-neuronal cells.

For the axonal regeneration experiments, superior cervical ganglia or trigeminal ganglia were dissected and explanted on a poly-D-lysine–coated (200 μg/ml) 35-mm plastic dishes, and maintained on serum-free medium containing NGF (100 ng/ml) or NGF (100 ng/ml) and NT-3 (10 ng/ml) for trigeminal ganglia. Two days later, the explants were treated with ara-c for 2 days, and then allowed to recover for 2 days. Subsequently, the ganglia were axotomized and dissociated, then the cells were plated on poly-D-lysine–coated coverslips. Sixteen hours later, the cultures were transfected and then maintained on serum-free medium plus NGF (100 ng/ml) or NGF (100 ng/ml) and NT-3 (10 ng/ml) for trigeminal cultures.

Transfection
Cells were cotransfected with a plasmid encoding the enhanced green fluorescence protein (pEGFP-C1; Clontech) and the expression vector LTF900 containing the LAT insert, or one of a set of four expression vectors (LTF901, LTF902, LTF903, and LTF904) containing different deletion mutants of LAT. In control experiments, cells were cotransfected with pEGFP-C1 and the pCDNA3.1 vector (Invitrogen). Transfections were performed using Lipofectamine 2000 (Invitrogen). Briefly, cells were treated with 150 μl of Dulbecco's modified Eagle's medium (DMEM) containing 1.6 μg of plasmid DNA and 6 μg of lipofectamine for 6 h. Subsequently, cells were washed and allowed to recover for 2 days.

In situ *hybridization*
Antisense and sense riboprobes for LAT were prepared as follows: a fragment of LAT (bp 119615 to 120426 of HSV-1 strain 17+) was amplified by polymerase chain reaction (PCR), and then subcloned into the PCR II-TOPO vector (Invitrogen). Digoxigenin-labeled riboprobes were generated by *in vitro* transcription according to the manufacturer's instructions (Roche, Indianapolis, ID). Cultures were fixed with 4% formaldehyde for 1 h and washed twice with 2× SSC (300 mM NaCl, 30 mM sodium citrate, pH 7.0). Cultures were treated with proteinase K (5 μg/ml) for 10 min then washed twice with 2× SSC. Cells were incubated at 50°C for 3 h with a

prehybridization solution that contained 50% formamide, 4× SSC, 1 mM EDTA, 1× Denhardt's solution, 10% dextran sulfate, yeast tRNA (250 μg/ml), and denatured salmon sperm DNA (250 μg/ml). Cells were hybridized by incubation at 50°C for 16 h with 2 μg/ml of probe in hybridization solution. The LAT signal was detected using an anti-digoxigenin Fab fragment conjugated to rhodamine (Roche) and visualized by fluorescence microscopy.

Assessment of cell survival

Neuronal death was induced by withdrawal of NGF from the medium through washing the cultures twice with DMEM containing bovine serum albumin (500 μg/ml) and then maintaining the cells on DMEM for 14 to 16 h. Dead cells were detected through the recognition of nuclear morphology by incubating the cells with Hoechst stain (bisbenzimide; Sigma, St. Louis, MO) for 20 min. Apoptotic cells were detected by TUNEL assay according to the manufacturer's instructions (Roche). Activated capase 3 was detected in cells by immunostaining with antihuman/mouse caspase 3 active rabbit immunoglobulin (IgG) (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions.

Statistical analysis

All of the experiments reported were repeated at least three times. Data are presented as mean ± SEM. Statistical significance was determined by a one-way analysis of variance (ANOVA) followed by Tukey's *post hoc* test, or a two-tailed unpaired *t* test.

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