

Damage-Induced Neuronal Endopeptidase (DINE/ECEL) Expression Is Regulated by Leukemia Inhibitory Factor and Deprivation of Nerve Growth Factor in Rat Sensory Ganglia after Nerve Injury

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Damage-induced neuronal endopeptidase (DINE) is a novel metalloproteinase and is expressed in response to various neuronal injuries. The expression regulation of DINE mRNA in the dorsal root ganglia (DRGs) after sciatic nerve injury is examined. A substantial increase of DINE mRNA expression was observed in relatively small-sized DRG neurons after nerve injury. The expression was observed in isolectin B4-negative and partly TrkA-positive neurons, and the expression profile was fairly similar to that of the neuropeptide galanin. More than 80% of DINE mRNA-positive neurons simultaneously demonstrated galanin immunoreactivity after nerve injury. In cultured DRG, DINE mRNA expression was enhanced by leukemia inhibitory factor (LIF) but not by other growth factors and cytokines. LIF treatment to rat sciatic nerve induced DINE mRNA expression in DRG without nerve injury, and, conversely, the intraneuronal

injection of anti-gp130 antibody after sciatic nerve injury significantly inhibited the upregulation of DINE mRNA in DRG. Furthermore, nerve growth factor (NGF) deprivation, which can induce galanin expression, also enhanced DINE mRNA expression *in vitro* and *in vivo*. Both LIF application and NGF deprivation additively enhanced DINE expression *in vitro*. These results suggest that DINE gene expression is regulated separately by both LIF and NGF deprivation, and this regulation pattern is similar to that of galanin gene expression. Because both DINE and galanin have a neuroprotective function, their simultaneous induction may provide more successful protection for injured sensory neurons.

Key words: *peptidase; gene expression; dorsal root ganglion; nerve injury; galanin; LIF; NGF deprivation*

Damage-induced neuronal endopeptidase (DINE in rats or ECEL in humans) is a newly identified membrane-bound metalloproteinase, specifically expressed in neurons of the CNS and peripheral nervous system and induced in response to nerve damage (Kiryu-Seo et al., 2000). DINE might be important for neuropeptide processing, which in turn may have functional consequences for adaptive response of neuronal injury. DINE enhances the expression and the activity of antioxidant enzymes such as Cu/Zn-superoxide dismutase (SOD), Mn-SOD, and glutathione peroxidase under oxidative stress by unknown mechanisms (Kiryu-Seo et al., 2000). The enhanced expression of antioxidant enzymes could contribute to preventing the neuronal death caused by nerve damage. After nerve injury, various molecules, such as neurotrophic factors, growth factors, cytokines, neuropeptides, and their receptors, are induced in injured neurons and/or non-neuronal cells and serve in neuroprotective and trophic mechanisms (Acheson et al., 1995; Matheson et al., 1997; Bennett et al., 1998; Groves et al., 1999; Murphy et al., 1999a;

Terenghi, 1999). Many of these molecules require processing to be activated or to mature, and thus the involvement of protease in their processing is very likely.

Although understanding the functional significance of DINE is crucial and is being studied, another intriguing characteristic of DINE is its expression regulation. The induction of DINE expression is seen in response to a wide range of nerve injuries, such as motor and sensory nerve injuries, brain and spinal cord trauma, and cerebral ischemia. The enhanced expression of DINE is strikingly restricted to neuronal cells and is not seen in glial cells. This intriguing promoter activity would be useful to achieve neuron-specific and neural damage-specific expression of various molecules in transgenic animals and gene therapy for neuronal degenerative diseases. In this study, we therefore focused on the mechanism of DINE expression regulation. In our previous study, we found that DINE mRNA is upregulated in the dorsal root ganglion (DRG) neurons by sciatic nerve injury (Kiryu-Seo et al., 2000). Using this rat sciatic nerve injury model, we characterized cell types of DINE mRNA expressing neurons first and further examined the influence of neurotrophic factors on DINE mRNA expression both *in vitro* and *in vivo*. Because nerve injury promotes a number of neurotrophic factors, such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), glial cell line-derived neurotrophic factor (GDNF), neurotrophin-3 (NT-3), insulin-like growth factor I (IGF-I), leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF), and interleukin 6 (IL-6) (Johnson et al., 1988; Murphy et al., 1993, 1999b; Acheson et al., 1995; Matheson et al., 1997; Groves et al., 1999; de Pablo et al., 2000; Shuto et al., 2001), we

Received June 10, 2002; revised Aug. 22, 2002; accepted Aug. 23, 2002.

This study was supported in part by grants from the Ministry of Health, Labor and Welfare of Japan, the Ministry of Education, Science, Technology, Sports and Culture, and the Japan Science and Technology Corporation. We are grateful to C. Kadono and T. Ogawa for technical assistance and to E. Fukui for secretarial assistance. We are also grateful to Prof. T. Tsukamoto (Sapporo Medical University) for his kind encouragement and Prof. K. Noguchi (Hyogo Medical University, Hyogo, Japan) for his critical discussion.

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assumed that these might be the possible factors. Accordingly, we addressed the consequences of those molecules on the regulation of DINE expression both *in vitro* and *in vivo*.

MATERIALS AND METHODS

Animals. Male Wistar rats, weighing ~200–300 gm, were anesthetized with pentobarbital (45 mg/kg). Their left sciatic nerves were cut with scissors at the midhigh level. For reverse transcription (RT)-PCR, 5 d after the operation, the fourth and fifth lumbar DRGs (10 DRGs from each lumbar) were quickly dissected out and frozen in liquid nitrogen. DRGs from embryonic day 15 (E15), E17, E20, postnatal day 1 (P1), P7, P15, P20, and adult were also dissected for RT-PCR studies. For *in situ* hybridization and immunohistochemistry, after a postoperative survival time of 1, 3, 5, 7, 35, and 60 d (6 DRGs each point), animals were deeply anesthetized and killed by perfusion with 200 ml of ice-cold saline, followed by 250 ml of 4% paraformaldehyde containing 0.21% picric acid in 0.1 M phosphate buffer (PB). The fourth and the fifth lumbar DRGs were quickly dissected out, postfixed, and immersed in 0.1 M PB containing 25% sucrose. Serial transverse sections were cut at 14 μ m on a cryostat and thaw mounted onto 3-aminopropyltriethoxysilane-coated slides. To reduce variability, injured and contralateral DRGs were mounted on the same slide glass.

For the LIF treatment, left sciatic nerve was exposed at the midhigh level, and the epineuriums were partly excised. The nerves were treated with 20 μ l of LIF [125 μ g/ml with 50 μ g of bovine serum albumin (BSA) in PBS; Alomone Labs, Jerusalem, Israel] or 20 μ l of PBS as a control using a Spongel (Yamanouchi, Tokyo, Japan) (five rats each). After 3 d, the fourth and fifth lumbar DRGs were dissected out for either RT-PCR or *in situ* hybridization as described above.

For the gp130 antibody treatment, left sciatic nerve was cut at the midhigh level, and 5 μ l of anti-gp130 antibody (1.0 mg/ml in PBS; R & D Systems, Minneapolis, MN) or 5 μ l of PBS as a control was injected into the nerves from the proximal stump using a Hamilton syringe (five rats each). After 24 hr, the fourth and fifth lumbar DRGs were dissected out for either RT-PCR or *in situ* hybridization as described above.

For *in vivo* NGF deprivation, male Wistar rats weighing ~200–300 gm were used, and 0.5 ml of sheep anti-NGF- β antibody (1.0 mg/ml in PBS; Chemicon, Temecula, CA) or 0.5 ml of sheep IgG (1.0 mg/ml in PBS; Cappel, Aurora, OH), as a control, was injected daily intraperitoneally (three rats each). Animals were treated for 2 d, and the fourth and the fifth lumbar DRGs were dissected out 24 hr after the final injection, for either RT-PCR or *in situ* hybridization as described above.

In situ hybridization. For *in situ* hybridization, sections were rinsed in PB, treated with 10 μ g/ml proteinase-K in 50 mM Tris-HCl and 5 mM EDTA for 4 min, and then fixed again. After rinsing in distilled water, sections were acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine, rinsed in PB, dehydrated in ascending ethanol series (70, 95, and 100%), defatted in chloroform, rinsed in ethanol, and air dried. All prehybridization procedures were performed RNase free. 35 S-Labeled RNA probes were prepared by *in vitro* transcription of DINE cDNA (accession number AB026293, nucleotides 1287–1761) fragment or galanin cDNA (accession number M18102, nucleotides 125–499) fragment in linearized pGEM-T Easy vector (Promega, Madison, WI) by using T7 RNA polymerase (Promega) and 35 S-UTP (DuPont NEN, Natick, MA). The labeled probes (5×10^6 cpm/ml per slide, minimum) in hybridization buffer (50% deionized formamide, 0.3 M NaCl, 20 mM Tris-HCl, 5 mM EDTA, 10 mM PBS, 10% dextran sulfate, 1 \times Denhardt's solution, 0.2% sarcosyl, 500 μ g/ml yeast transfer RNA, and 200 μ g/ml salmon sperm DNA) was denatured for 2 min at 80°C, quenched on ice, and placed on the sections. Hybridization was performed in a humid chamber overnight at 55°C. Hybridized sections were rinsed briefly in 5 \times SSC and 1% 2-mercaptoethanol at 55°C and washed in 50% deionized formamide, 2 \times SSC, and 10% 2-mercaptoethanol (high-stringency buffer) for 30 min at 65°C. After rinsing the sections in RNase buffer (0.5 M NaCl, 10 mM Tris-HCl, and 1 mM EDTA), they were treated with 1.0 μ g/ml RNase-A in RNase buffer for 30 min at 37°C and washed in RNase buffer. Sections were then incubated in high-stringency buffer as described above, rinsed in 2 and 0.1 \times SSC for 10 min each at room temperature, dehydrated in an ascending ethanol series, and air dried. Sections were then dipped in Kodak autoradiography emulsion (6:4; Eastman Kodak, Rochester, NY) diluted in water. Sections were then exposed for 3–14 d at 4°C, developed in a Kodak D19 developer, dehydrated in a graded series of ethanol to xylene, and coverslipped before microscopic observation.

Immunohistochemistry and in situ hybridization (double-labeling stud-

ies). For double-labeling studies, the sections were air dried, rinsed in 0.1 M PBS, and then preincubated in 0.1 M PBS containing 1% BSA for 10 min. The sections were then incubated with primary antibodies diluted with 0.1 M PBS containing 1% BSA and 0.1% Triton X-100 for 12–24 hr in a humid chamber at 4°C. The following primary antibodies were used for double-labeling studies: rabbit anti-TrkA (1:1000; Upstate Biotechnology, Lake Placid, NY), IB4 from *Griffonia simplicifolia* (biotin labeled, 1:500; Sigma, St. Louis, MO), rabbit anti-galanin (1:8000; Peninsula Laboratories, Belmont, CA). After rinsing in 0.1 M PBS, the sections were incubated with biotinylated anti-rabbit IgG (1:500; Vector Laboratories, Burlingame, CA) diluted in 0.1 M PBS containing 1% BSA and 0.1% Triton X-100 for 12–24 hr in a humid chamber at 4°C, except the sections that had been incubated with IB4. After rinsing in 0.1 M PBS, the sections were incubated with the avidin–biotin complex (1:100; Vector Laboratories) diluted in 0.1 M PBS for 3 hr at room temperature. The positive antigens were then visualized by incubation with 0.02% 3,3'-diaminobenzidine tetrahydrochloride and 0.003% hydrogen peroxide in 50 mM Tris-HCl, pH 7.6, for 5–60 min at room temperature. After identifying positive immunoreactions by light microscopy, the sections were rinsed in 50 mM Tris-HCl and fixed in 4% paraformaldehyde in 0.1 M PB for 20 min and then transferred into the prehybridization step.

Imaging and quantification. Sections were observed under dark- and bright-field illuminations. Images were directly captured from the microscope using a black-and-white film or a digital camera and composed using Adobe Photoshop 5.0J (Adobe Systems, San Jose, CA). For *in situ* hybridization, cells were identified as positively labeled when bipolarized illumination revealed silver grains clustered over the cell body. Only those DRG neurons with a nucleus and silver grain densities five times over background were measured. Cells were outlined manually using a computer mouse to measure the cell size. The proportions of DINE mRNA-positive DRG cells were determined by counting the number of all of the positively labeled neuronal profiles in five sections from ipsilateral DRGs of three animals and also the total number of neuronal profiles in the same visible sections.

RT-PCR. Total RNA was extracted from DRGs of 10 animals. Total RNA (each 5 μ g) was converted to cDNA with Superscript reverse transcriptase (Invitrogen, Gaithersburg, MD) and nucleotide oligo-dT. Aliquots from the RT reaction were used for PCR amplification using primer pairs ubiquitously expressing glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a control. The specific primers were used in PCR analysis for the detection of DINE and galanin mRNAs. The reaction products were electrophoretically separated on 1.2% agarose gel and visualized by staining with ethidium bromide. Data were also expressed as the mean density of the band in question relative to the density of the GAPDH band in the same sample. The density was measured at five points for each band. The means were compared by an unpaired Student's *t* test.

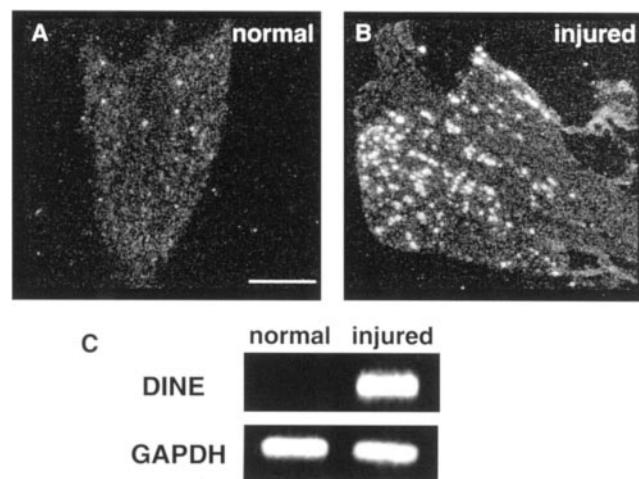


Figure 1. Expression profile of DINE mRNA in DRG 5 d after sciatic nerve injury. Less than 1% of DRG neurons expressed DINE mRNA in non-injured DRG (*A*). Sciatic nerve injury induced dramatic increase of DINE mRNA expression (*B*). RT-PCR confirmed the increase of DINE mRNA expression after nerve injury (*C*). GAPDH mRNA expression was examined as an internal control. Scale bar, 500 μ m.

Table 1. Population of DINE mRNA-expressing neurons in DRG after nerve injury

	1 d	3 d	5 d	7 d	35 d	60 d
Injured DRGs (%)	3.3	7.5	18.5	16.8	16.3	17.0
Contralateral DRGs (%)	0.2	0.3	0.4	0.4	1.0	2.0

DRG organ culture. For the primary culture, DRGs were isolated from 1-d-old Wistar rats, collected into ice-cold L-15 (Invitrogen) medium, and then seeded on 24-well plates (six DRGs per each well), which had been coated with polyethyleneimine. The feeding medium was a DMEM-F12 (Invitrogen), to which 0.5% penicillin–streptomycin (Sigma), 5 μ M uridine (Sigma), 5 μ M fluorodeoxyuridine (Sigma), and 50 ng/ml NGF (Promega) were added. DRGs were cultured in the presence of NGF at 37°C in a 5% CO₂ atmosphere. After 40 hr, the medium in each well was exchanged, and the neurotrophic factors, growth factors, and cytokines were applied to each well to investigate regulating factors of DINE mRNA induction. The following reagents were applied: 50 ng/ml BDNF (Alomone Labs), 10 ng/ml NT-3 (Calbiochem, La Jolla, CA), 50 ng/ml GDNF (Alomone Labs), 50 ng/ml IGF (Promega), 10 ng/ml LIF (Alomone Labs), 10 ng/ml CNTF (Alomone Labs), and 10 ng/ml IL-6 (Alomone Labs). Anti-hgp130 antibody (1.0 μ g/ml; R & D Systems) was also used to confirm the inductive pathway of DINE mRNA expression. Cultures with excessive NGF (500 ng/ml), cultures with NGF deprivation, and control cultures with 50 ng/ml NGF were also studied. After 96 hr, total RNA from every four wells was extracted, and RT-PCR study was performed as described above.

RESULTS

The expression profile of DINE mRNA in DRGs

In sections of normal DRG of L4–L5, DINE mRNA expression was found in a very small number of neurons (<1% of the total number of DRG neurons) (Fig. 1*A*). Sciatic nerve injury markedly induced DINE mRNA expression in injured DRG (Fig. 1*B*). RT-PCR study confirmed the increase of DINE mRNA expression after nerve injury (Fig. 1*C*). The number of DRG neurons expressing DINE mRNA increased to 18% at 5 d after nerve injury and maintained this level up to 60 d after the nerve injury (Table 1). DINE mRNA was mainly expressed in small (diameter <30 μ m) DRG neurons and hardly observed in large-diameter neurons (Fig. 2). Because Tandrup et al. (2000) reported that the loss of small DRG neurons became significant from 8 weeks after axotomy, the data at 60 d after axotomy may be influenced by the neuronal death. Small DRG neurons were classified into two major groups, TrkA-positive and IB4-positive neurons (Snider and McMahon, 1998). The double-labeling study for immunohistochemistry and *in situ* hybridization showed that 41% of TrkA-positive neurons expressed DINE mRNA, and 43% of DINE mRNA-expressing neurons were TrkA positive in the injured DRGs (Fig. 3*A*). On the other hand, DINE mRNA expressions were hardly seen in IB4-positive neurons in DRGs (Fig. 3*B*). These colocalization

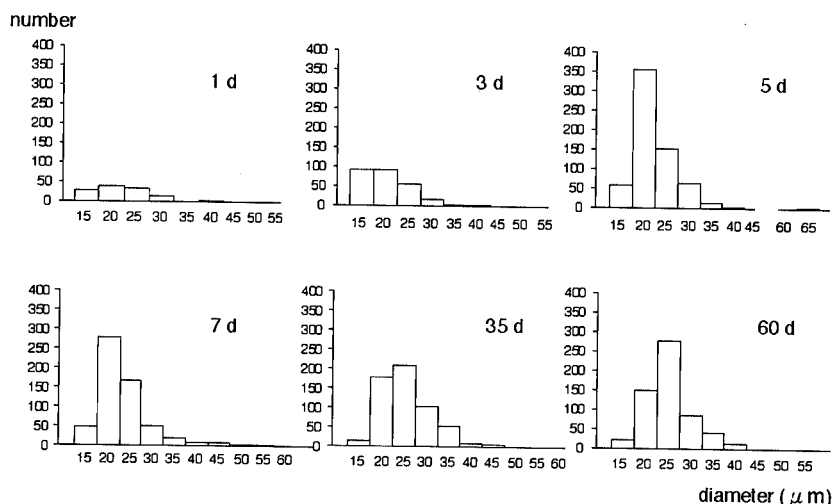


Figure 2. Size of DINE mRNA-positive neurons in DRGs. Most DINE mRNA-positive neurons in DRG were small or medium sized.

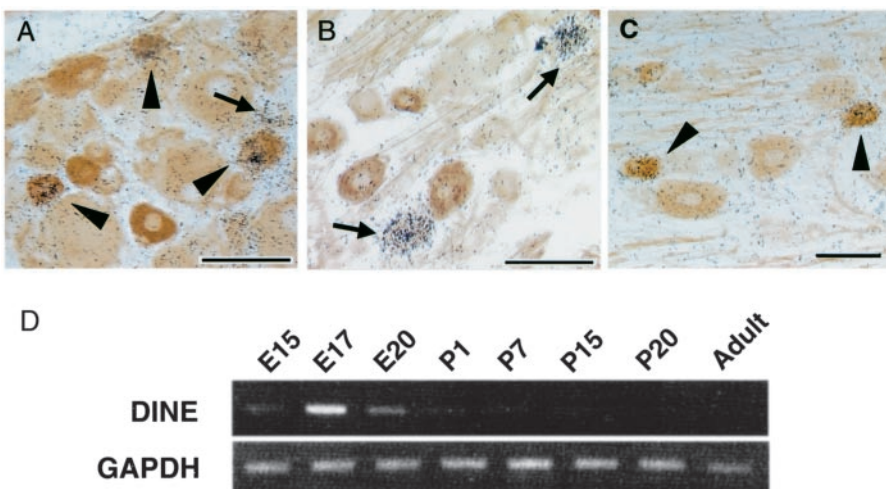


Figure 3. Characterization of DINE mRNA-positive neurons in DRGs. DINE mRNA (silver grain accumulations) was expressed partly in TrkA-positive neurons (*A*) but was hardly expressed in IB4-positive neurons (*B*). Frequently, DINE mRNA signal and galanin immunoreactivity were colocalized in the same DRG neurons (*C*). Arrowheads indicate double-labeled neurons. Arrows indicate DINE mRNA only positive neurons. Scale bars, 50 μ m. *D*, The developmental expression profile of DINE mRNA was examined by RT-PCR.

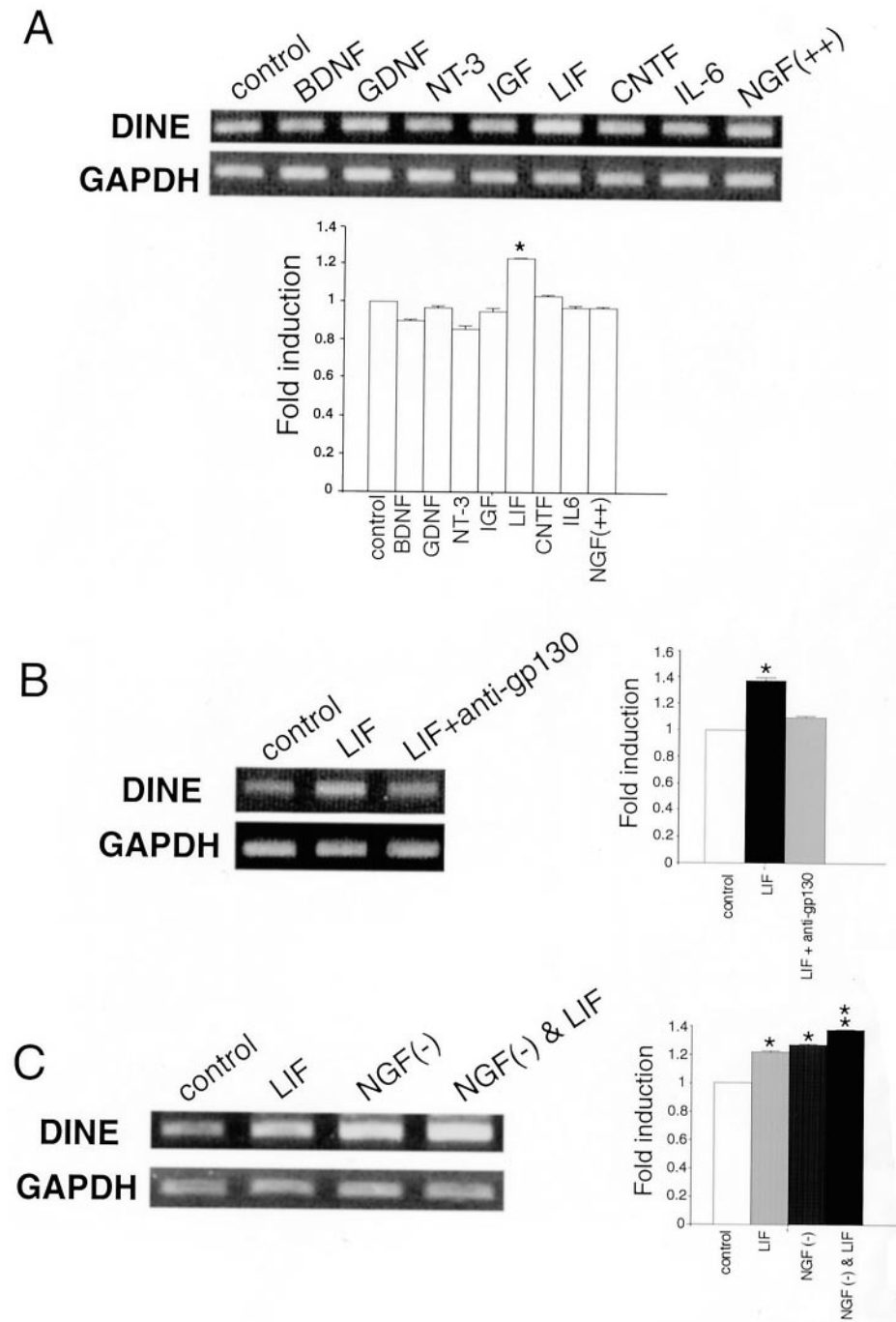


Figure 4. DINE mRNA expression in organ cultured DRGs after stimulus by various growth factors. *A*, Expression of DINE mRNA was enhanced by LIF but not growth factors. *NGF(++)* indicates application of 10-fold higher dose of NGF. *B*, Addition of anti-gp130 antibody suppressed LIF-induced expression of DINE mRNA. *C*, NGF deprivation induced DINE mRNA expression. Application of LIF together with NGF deprivation further enhanced DINE mRNA expression. All quantitative RT-PCR data were normalized by GAPDH and presented as fold increase against control. Data represent mean \pm SEM values. * $p < 0.001$ versus control by Student's *t* test. ** $p < 0.001$ versus LIF or NGF(-).

patterns reminded us that the expression profile of DINE mRNA appeared quite similar to that of galanin mRNA in injured DRGs. The double-labeling study for galanin immunostaining and DINE mRNA was therefore attempted. This double-labeling study revealed that 71% of galanin-positive neurons simultaneously expressed DINE mRNA, and 83% of DINE mRNA-expressing neurons were galanin positive in injured DRGs (Fig. 3C). Next, the embryonic expression of DINE mRNA in DRGs was examined by RT-PCR. Most prominent expression of DINE mRNA was observed at E17 (Fig. 3D).

DINE mRNA expression was induced by LIF but not other neurotrophic factors *in vitro*

Using DRG culture cells, changes of DINE mRNA expression by various neurotrophic factors, which are also increased in the axotomy model, were examined by RT-PCR. The RT-PCR study showed that neurotrophic factors, such as BDNF, GDNF, NT-3, and IGF, did not significantly change DINE mRNA expression level (Fig. 4A), whereas DINE mRNA expression was significantly increased by LIF. A similar induction was not seen by other gp130-related cytokines, such as CNTF and IL-6 (Fig. 4A). Because the culture medium contained NGF (50 ng/ml) to

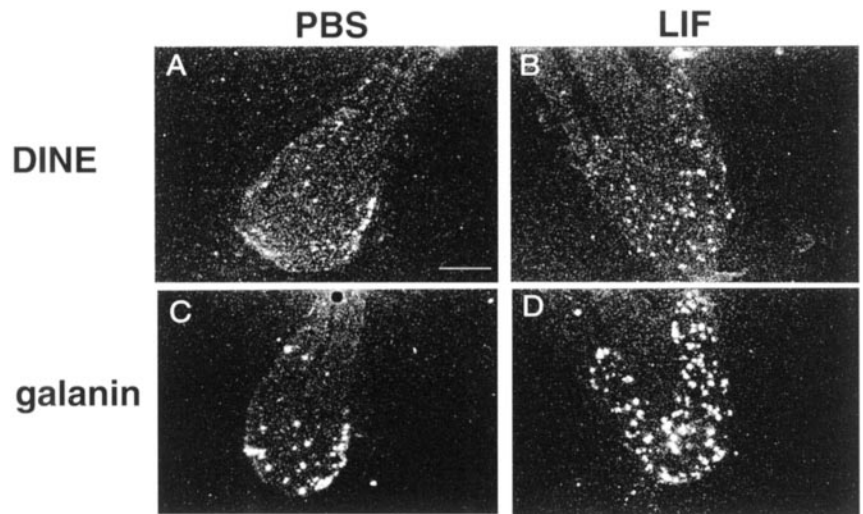


Figure 5. LIF induced both DINE and galanin mRNA expressions *in vivo*. *A* and *B* demonstrate DINE mRNA expression by *in situ* hybridization, and *C* and *D* show galanin mRNA expression. *A* and *C* were treated with PBS, whereas *B* and *D* were LIF treated. Scale bar, 500 μ m. *E*, RT-PCR confirmed increases of both DINE and galanin mRNA expressions by LIF treatment. RT-PCR data were normalized by GAPDH and presented as fold increase against control. Data represent mean \pm SEM values. * p < 0.001 versus normal by Student's *t* test.

maintain neuronal survival, a 10-fold dose NGF application NGF(++) was examined. The increased application of NGF did not affect DINE mRNA expression level (Fig. 4*A*).

Both LIF and NGF deprivation induced DINE mRNA expression *in vitro*

Because LIF was the only factor that enhanced DINE expression in above-mentioned study, a blockade of LIF receptor signaling was attempted by a use of a neutralizing antibody against the signal-transducing receptor component gp130 *in vitro*. The enhancement of DINE mRNA expression by LIF was completely blocked by the application of anti-gp130 antibody (Fig. 4*B*). To eliminate the NGF implication under the condition of LIF stimulation, we removed NGF from the culture medium. Surprisingly, this condition [NGF(-)/LIF(+)] resulted in a dramatic increase of DINE mRNA expression. Even without LIF, NGF withdrawal alone enhanced DINE mRNA expression. The application of LIF and the withdrawal of NGF showed an additive effect on DINE mRNA expression (Fig. 4*C*).

LIF and the NGF deprivation induced DINE mRNA expression in rat DRGs

As described above, the *in vitro* data strongly suggested the positive effect of LIF and the negative effect of NGF on DINE mRNA expression. We then attempted to confirm these effects in rats. Either PBS or LIF containing Spongel was placed on the epineurium of excised sciatic nerve. The control (PBS treatment) showed a slight increase of DINE mRNA expression (Fig. 5*A*). This was probably attributable to the partial injury effect by the epineurium excision. Contrary to PBS, LIF treatment demonstrated a significant increase of DINE mRNA expression. We also examined galanin mRNA expression, because galanin ex-

pression is regulated by LIF. Whereas there was a slight increase of galanin mRNA with PBS, there was a substantial increase with LIF treatment. The induction of DINE and galanin mRNAs was confirmed by RT-PCR (Fig. 5*E*). The LIF-induced expression of DINE *in vivo* was further examined by using the neutralizing antibody against gp130, which is the signaling component of LIF receptor. This neutralizing antibody is well known to inhibit gp130-mediated cytokine signaling, such as LIF-, CNTF-, and IL-6-mediated signaling. After sciatic nerve injury, either gp130 antibody or PBS was injected into the injured nerve site. When PBS treated, significant increases of DINE and galanin mRNAs were seen. Conversely, gp130 treatment dramatically suppressed expression of both mRNAs (Fig. 6*A–D*). The change by gp130 injection was confirmed by RT-PCR study as well (Fig. 6*E*).

To examine NGF deprivation effect *in vivo*, NGF neutralizing antibody was injected intraperitoneally. Both DINE and galanin mRNA expressions were barely induced by intraperitoneal injection of the control sheep IgG (Fig. 7*A,C*, respectively), although these mRNA expressions were significantly enhanced by intraperitoneal injection of neutralizing anti-NGF antibody for 2 d without nerve injury (Fig. 7*B,D*, respectively). RT-PCR study also confirmed the increases of both DINE and galanin mRNA expressions after NGF deprivation (Fig. 7*E*).

DISCUSSION

Sciatic nerve injury induced DINE mRNA expression in a group of DRG neurons

DINE is a neuron-specific metalloproteinase thought to have a neuroprotective function by promoting antioxidant machinery (Kiryu-Seo et al., 2000), although the underlying mechanism and the substrate are still unknown. Structurally, DINE resembles

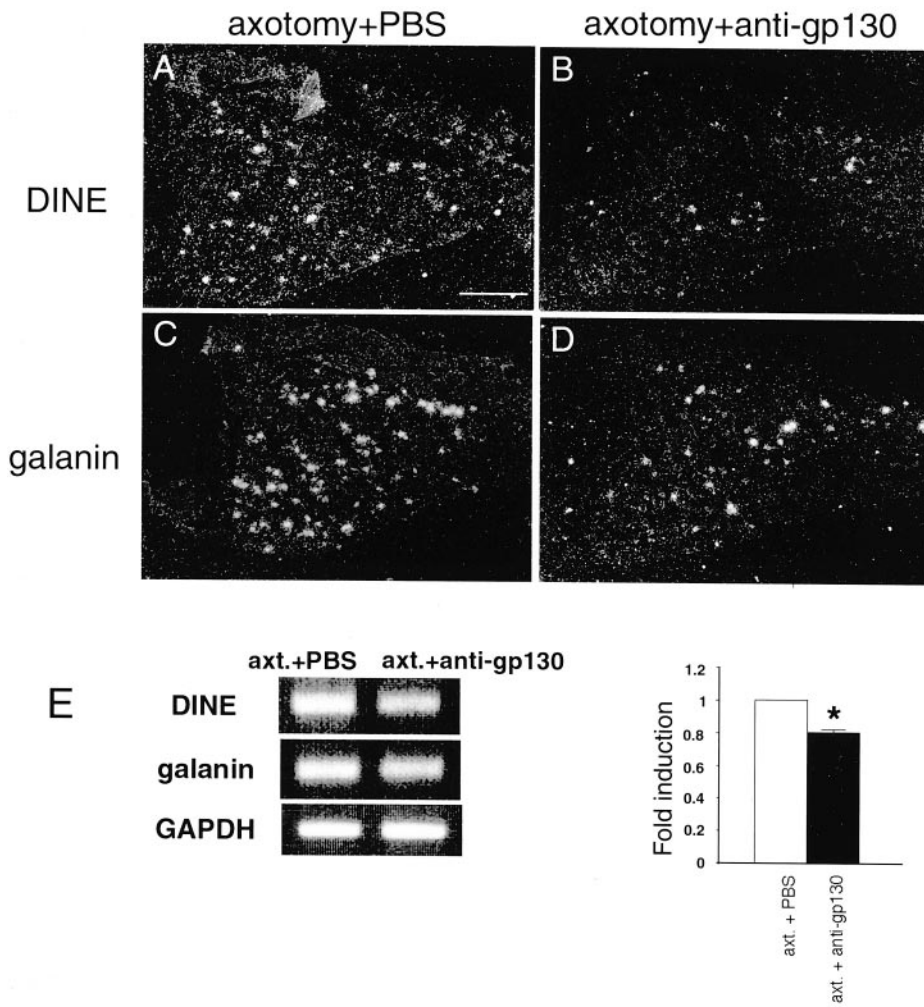


Figure 6. Intraneural injection of anti-gp130 antibody inhibited upregulations of both DINE and galanin mRNA expressions after sciatic nerve injury. *A* and *B* demonstrate DINE mRNA expression by *in situ* hybridization, and *C* and *D* show galanin mRNA expression. *A* and *C* indicate mRNA expressions after intraneural injection of PBS after nerve injury, whereas *B* and *D* demonstrate those after intraneural injection of anti-gp130 antibody after nerve injury. Scale bar, 500 μ m. *E*, RT-PCR confirmed decreases of both DINE and galanin mRNA expressions by injection of anti-gp130 antibody. RT-PCR data were also normalized by GAPDH and presented as fold increase against control. Data represent mean \pm SEM values. * $p < 0.001$ versus normal by Student's *t* test. *axt.*, Axotomy.

endothelin converting enzymes and neprilysin, which is identified as enkephalinase and currently attracting attention as a β -amyloid peptide degrading enzyme (Iwata et al., 2001). Both enzymes have an indirect neuron-rescue activity, and, in this respect, DINE is consistent with those members. Therefore, members of this family, including DINE, are suggested to have a possible protective role (Carson and Turner, 2002). In normal rat brain, DINE is enriched in hypothalamus, in which various neuropeptides are expressed, suggesting a strong relationship between DINE and neuropeptides. Expression of neuropeptides, some of which are also known to have a neuroprotective role, is dramatically regulated after nerve injury. The present study demonstrated that DINE expression was also regulated by nerve injury in a certain group of DRG cells. In non-injured DRG neurons, few DINE mRNA-expressing neurons were observed; however, after sciatic nerve injury, DINE mRNA expression was markedly induced, mainly in small DRG neurons. Small DRG neurons are identified as nociceptors and thermoceptors, and these neurons are classified mainly into two groups. One group is known as the peptide-containing NGF receptor TrkA-positive neurons (Kaplan et al., 1991), and another group is known as the nonpeptide-containing c-Ret (a GDNF receptor) and IB4-positive neurons (Molliver et al., 1997; Snider and McMahon, 1998). The double-labeling studies for DINE mRNA and TrkA or IB4 immunostaining showed that approximately one-half of DINE mRNA-positive cells were also TrkA positive, but hardly any were also

IB4 positive. Therefore, DINE-positive neurons are partly NGF dependent and not GDNF dependent. This would be consistent with the result that GDNF had no effect on the induction of DINE mRNA expression in cultured DRGs. Whereas NGF application had no effect on the induction of DINE mRNA expression, NGF deprivation significantly induced DINE mRNA expression in NGF-dependent cells. Therefore, the lack of NGF signaling from the target by axotomy, for instance, may be a trigger of DINE mRNA induction at least in part of TrkA-positive cells. However, there are DINE mRNA-positive cells that are negative to TrkA. From the aspect of growth factor receptor localization, DINE-expressing cells are not clearly characterized; nevertheless, the relatively higher localization in TrkA-positive cells and the negative dependency of NGF may suggest some functional correlation.

Nerve injury influences the expressions of neuropeptides in DRGs. For instance, expressions of calcitonin gene-related peptide (Noguchi et al., 1990) and substance P (for review, see Hökfelt et al., 1994) are downregulated after nerve injury, whereas vasoactive intestinal peptide (VIP) (Noguchi et al., 1989), galanin (Hökfelt et al., 1987), and neuropeptide Y (NPY) (Wakisaka et al., 1992) are upregulated (for review, see Hökfelt et al., 1994; Bergman et al., 1999) in DRGs. Among those conventional neuropeptides, the expression profile of DINE after nerve injury appeared relatively comparable with that of galanin and VIP. The expression of DINE mRNA was observed from 1 d

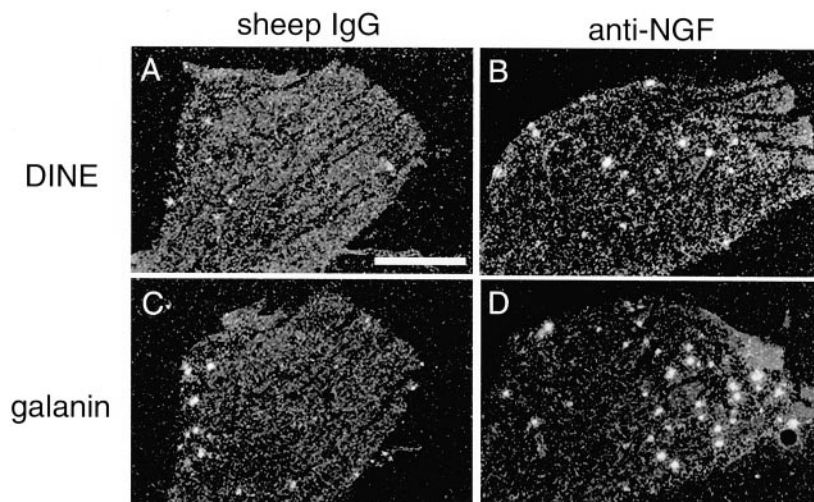
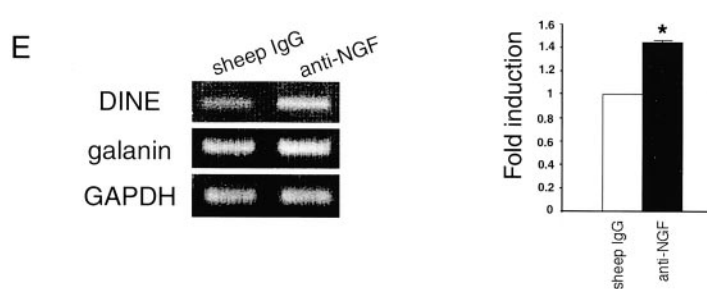


Figure 7. NGF deprivation induced both DINE and galanin mRNA expressions *in vivo*. *A* and *B* demonstrate DINE mRNA expression by *in situ* hybridization, and *C* and *D* show galanin mRNA expression. *A* and *C* demonstrate mRNA expressions after intraperitoneal injection of sheep IgG, whereas *B* and *D* show increase of mRNA expressions after injection of anti-NGF antibody. Scale bar, 500 μ m. *E*, RT-PCR confirmed increases of both DINE and galanin mRNA expressions by NGF deprivation. RT-PCR data were also normalized by GAPDH and presented as fold increase against control. Data represent mean \pm SEM values. * p < 0.001 versus normal by Student's *t* test.



after the sciatic nerve injury, reached its peak at 5 d, and maintained this expression level up to 60 d. This expression profile suggests that the expression profile of galanin is the most consistent with that of DINE. The galanin expression persisted up to 50 d after nerve injury (Brecht et al., 1997), whereas the VIP expression gradually decreased from 21 d after nerve injury (Noguchi et al., 1989). As we expected, the double-labeling study showed that DINE mRNA expression and galanin immunostaining were highly colocalized in injured DRG neurons. In addition, the expression profile of DINE mRNA during development is remarkably similar to that of galanin. The comparable expression profiles suggest that both DINE and galanin are likely to be regulated by identical factors or mechanisms after nerve injury. Although the significance of the colocalization of galanin and DINE in DRGs is unknown, both DINE and galanin have neuron rescue activity. Thus, DINE may have some association with galanin as a processing enzyme, for instance, to promote neuronal protection (Holmes et al., 2000). In addition, because galanin has a nociceptive function, DINE may also influence the nociceptive function such as allodynia (Liu et al., 2001).

LIF and NGF deprivation regulate DINE expression *in vitro* and *in vivo*

After sciatic nerve injury, a variety of neurotrophic factors and cytokines are induced and function for the survival and regeneration of injured DRG neurons. CNTF, which is normally accumulated in Schwann cells and released in response to nerve injury, could be the primary factor. The released CNTF has potent rescue activity for the injured neurons (Sendtner et al., 1992). In addition, NGF, BDNF, NT-3, GDNF, IGF-I, and LIF are synthesized and released in the distal segment of injured nerve, injured neurons, or target organs in response to nerve injury, and these factors have

potent rescue activities for injured neurons (Johnson et al., 1988; Kanje et al., 1988; Funakoshi et al., 1993; Banner and Patterson, 1994; Acheson et al., 1995; Matheson et al., 1997; Bennett et al., 1998; Groves et al., 1999; Michael et al., 1999; Zhou et al., 1999; de Pablo et al., 2000). Among these trophic factors and cytokines, which are induced after nerve injury, only LIF induced DINE mRNA expression in neonatal DRGs. The induction of DINE mRNA expression by LIF was inhibited by neutralized anti-gp130 antibody *in vitro* and *in vivo*. LIF, CNTF, and IL-6 are cytokines acting through a common signaling receptor, gp130 (Davis et al., 1993); however, both CNTF and IL-6 failed to induce DINE mRNA. These results strongly suggest that DINE expression was mediated by the interaction of LIF and LIF receptor complex. Furthermore, LIF is retrogradely transported to DRG, in particular by small-diameter nociceptive-specific neurons, but CNTF and IL-6 are not (Thompson et al., 1997). These may be reasons why DINE mRNA expression is induced by LIF but not CNTF or IL-6. However, it is likely that the cytokine dependency pattern may alter along with development and reach a peak at embryonic stage. We used neonatal DRG for *in vitro* experiment. It cannot exclude that DINE expression might have different response against the cytokines, such as CNTF and IL-6, in other case.

Interestingly, LIF is a potent factor for induction of galanin expression in cultured DRGs (Kerekes et al., 1999) and in DRGs after nerve injury (Sun and Zigmund, 1996). LIF injection into the sciatic nerve of normal animals causes increased galanin expression in DRGs, and upregulation of galanin after nerve injury is reduced after treatment with antibody against the gp130 receptor motif (Thompson et al., 1998). Collectively, LIF could be a factor, promoting both DINE and galanin expression in a group of nerve injured DRG neurons.

A previous report shows that NGF deprivation enhances galanin expression in the presence of LIF (Corness et al., 1998), and another report shows that galanin expression in cultured DRG incubated with LIF and NGF is less than that in DRG cultured with LIF alone (Ozturk and Tonge, 2001). In addition, Shadiack et al. (2001) reported that NGF antiserum induces galanin expression in intact DRG neurons. Conversely, delayed NGF infusion also reduces the number of neurons expressing VIP, cholecystokinin, NPY, and galanin after injury (Verge et al., 1995). All of these studies suggest that galanin expression is upregulated by NGF deprivation both *in vitro* and *in vivo* and that NGF suppresses galanin expression. Likewise, the present study demonstrated that DINE mRNA expression was also regulated by NGF deprivation both *in vitro* and *in vivo*. Therefore, the induction mechanism seen in galanin and DINE by NGF deprivation could be similar. In this respect, the higher colocalization of galanin and DINE in DRG neurons after axotomy could be the consequence of the similar gene expression regulation mechanism of DINE and galanin. NGF deprivation might activate or suppress some signal, but the signaling is rather hazy. In developing sympathetic neurons, both NGF deprivation and LIF treatment can activate the same downstream pathway, c-Jun N-terminal kinase (JNK) signaling, and induce apoptosis of developing neurons (Savitz and Kessler, 2000). We therefore examined whether the activation of JNK signaling [by MEKK1 (mitogen-activated protein kinase kinase kinase 1) overexpression with adenovirus] induces DINE expression in organ cultured DRGs and found that DINE mRNA expression was barely induced by JNK activation (data not shown). Consequently, NGF deprivation and JNK activation may be a distinct signal, at least for DINE expression. Although the signaling downstream of NGF deprivation should be examined further, DINE mRNA induction was regulated by at least two signals, the LIF–gp130-mediated signaling pathway and the NGF deprivation signal pathway, that were activated when the nerve was damaged.

In conclusion, DINE mRNA expression in DRGs was induced by LIF and NGF deprivation that were elicited by the nerve damage, and this expression regulation was surprisingly similar to that of galanin, leading to the higher colocalization of DINE and galanin in nerve-injured DRG neurons. Because galanin-deficient mice exhibit decreased peripheral nerve regeneration after a lesion, the large increases in galanin expression that occur in axotomized peripheral neurons have functional consequences for regeneration (Holmes et al., 2000; Zigmond, 2001). In addition, galanin has receptor subtype-specific pronociceptive and analgesic actions in the spinal cord (Liu et al., 2001). Likewise, DINE may also have potent functions for regeneration of injured sensory neurons and neuropathic pain.

REFERENCES

- Acheson A, Conover JC, Fandl JP, DeChiara TM, Russell M, Thadani A, Squinto SP, Yancopoulos GD, Lindsay RM (1995) A BDNF autocrine loop in adult sensory neurons prevents cell death. *Nature* 374:450–453.
- Banner LR, Patterson PH (1994) Major changes in the expression of the mRNAs for cholinergic differentiation factor/leukemia inhibitory factor and its receptor after injury to adult peripheral nerves and ganglia. *Proc Natl Acad Sci USA* 91:7109–7113.
- Bennett DL, Michael GJ, Ramachandran N, Munson JB, Averill S, Yan Q, McMahon SB, Priestley JV (1998) A distinct subgroup of small DRG cells express GDNF receptor components and GDNF is protective for these neurons after nerve injury. *J Neurosci* 18:3059–3072.
- Bergman E, Carlsson K, Liljeborg A, Manders E, Hökfelt T, Ulfhake B (1999) Neuropeptides, nitric oxide synthase and GAP-43 in B4-binding and RT97 immunoreactive primary sensory neurons: normal distribution pattern and changes after peripheral nerve transection and aging. *Brain Res* 832:63–83.
- Brecht S, Buschmann T, Grimm S, Zimmermann M, Herdegen T (1997) Persisting expression of galanin in axotomized mammillary and septal neurons of adult rats labeled for c-Jun and NADPH-diaphorase. *Brain Res Mol Brain Res* 48:7–16.
- Carson JA, Turner AJ (2002) β -Amyloid catabolism: roles for neprilysin (NEP) and other metalloproteinases? *J Neurochem* 81:1–8.
- Corness J, Stevens B, Fields RD, Hökfelt T (1998) NGF and LIF both regulate galanin gene expression in primary DRG cultures. *NeuroReport* 9:1533–1536.
- Davis S, Aldrich TH, Stahl N, Pan L, Taga T, Kishimoto T, Ip NY, Yancopoulos GD (1993) LIFR beta and gp130 as heterodimerizing signal transducers of the tripartite CNTF receptor. *Science* 260:1805–1808.
- de Pablo F, Banner LR, Patterson PH (2000) IGF-I expression is decreased in LIF-deficient mice after peripheral nerve injury. *NeuroReport* 11:1365–1368.
- Funakoshi H, Frisen J, Barbany G, Timmus T, Zachrisson O, Verge VM, Persson H (1993) Differential expression of mRNAs for neurotrophins and their receptors after axotomy of the sciatic nerve. *J Cell Biol* 123:455–465.
- Groves MJ, An SF, Giometto B, Scaravilli F (1999) Inhibition of sensory neuron apoptosis and prevention of loss by NT-3 administration following axotomy. *Exp Neurol* 155:284–294.
- Hökfelt T, Wiesenfeld-Hallin Z, Villar M, Melander T (1987) Increase of galanin-like immunoreactivity in rat dorsal root ganglion cells after peripheral axotomy. *Neurosci Lett* 83:217–220.
- Hökfelt T, Zhang X, Wiesenfeld-Hallin Z (1994) Messenger plasticity in primary sensory neurons following axotomy and its functional implications. *Trends Neurosci* 17:22–30.
- Holmes FE, Mahoney S, King VR, Bacon A, Kerr NC, Pachnis V, Curtis R, Priestley JV, Wynick D (2000) Targeted disruption of the galanin gene reduces the number of sensory neurons and their regenerative capacity. *Proc Natl Acad Sci USA* 97:11563–11568.
- Iwata N, Tsubuki S, Takaki Y, Shirotani K, Lu B, Gerard NP, Gerard C, Hama E, Lee HJ, Saïdo TC (2001) Metabolic regulation of brain A beta by neprilysin. *Science* 292:1550–1552.
- Johnson Jr EM, Taniuchi M, DiStefano PS (1988) Expression and possible function of nerve growth factor receptors on Schwann cells. *Trends Neurosci* 11:299–304.
- Kanje M, Skottner A, Lundborg G (1988) Effects of growth hormone treatment on the regeneration of rat sciatic nerve. *Brain Res* 475:254–258.
- Kaplan DR, Hempstead BL, Martin-Zanca D, Chao MV, Parada LF (1991) The trk proto-oncogene product: a signal transducing receptor for nerve growth factor. *Science* 252:554–558.
- Kerekes N, Landry M, Hökfelt T (1999) Leukemia inhibitory factor regulates galanin/galanin message-associated peptide expression in cultured mouse dorsal root ganglia; with a note on in situ hybridization methodology. *Neuroscience* 89:1123–1134.
- Kiryu-Seo S, Sasaki M, Yokohama H, Nakagomi S, Hirayama T, Aoki S, Wada K, Kiyama H (2000) Damage-induced neuronal endopeptidase (DINE) is a unique metalloproteinase expressed in response to neuronal damage and activates superoxide scavengers. *Proc Natl Acad Sci USA* 97:4345–4350.
- Liu HX, Brumovsky P, Schmidt R, Brown W, Payza K, Hodzic L, Pou C, Godbout C, Hökfelt T (2001) Receptor subtype-specific pronociceptive and analgesic actions of galanin in the spinal cord: selective actions via GalR1 and GalR2 receptors. *Proc Natl Acad Sci USA* 98:9960–9964.
- Matheson CR, Carnahan J, Ulrich JL, Bocangel D, Zhang TJ, Yan Q (1997) Glial cell line-derived neurotrophic factor (GDNF) is a neurotrophic factor for sensory neurons: comparison with the effects of the neurotrophins. *J Neurobiol* 32:22–32.
- Michael GJ, Averill S, Shortland PJ, Yan Q, Priestley JV (1999) Axotomy results in major changes in BDNF expression by dorsal root ganglion cells: BDNF expression in large trkB and trkC cells, in pericellular baskets, and in projections to deep dorsal horn and dorsal column nuclei. *Eur J Neurosci* 11:3539–3551.
- Molliver DC, Wright DE, Leitner ML, Parsadanian AS, Doster K, Wen D, Yan Q, Snider WD (1997) IB4-binding DRG neurons switch from NGF to GDNF dependence in early postnatal life. *Neuron* 19:849–861.
- Murphy M, Reid K, Brown MA, Bartlett PF (1993) Involvement of leukemia inhibitory factor and nerve growth factor in the development of dorsal root ganglion neurons. *Development* 117:1173–1182.
- Murphy PG, Borthwick LS, Johnston RS, Kuchel G, Richardson PM (1999a) Nature of the retrograde signal from injured nerves that induces interleukin-6 mRNA in neurons. *J Neurosci* 19:3791–3800.
- Murphy PG, Ramer MS, Borthwick L, Gaudie J, Richardson PM, Bisby MA (1999b) Endogenous interleukin-6 contributes to hypersensitivity to cutaneous stimuli and changes in neuropeptides associated with chronic nerve constriction in mice. *Eur J Neurosci* 11:2243–2253.
- Noguchi K, Senba E, Morita Y, Sato M, Tohyama M (1989) Prepro-VIP and preprotachykinin mRNAs in the rat dorsal root ganglion cells following peripheral axotomy. *Brain Res Mol Brain Res* 6:327–330.
- Noguchi K, Senba E, Morita Y, Sato M, Tohyama M (1990) Alpha-CGRP and beta-CGRP mRNAs are differentially regulated in the rat

- spinal cord and dorsal root ganglion. *Brain Res Mol Brain Res* 7:299–304.
- Ozturk G, Tonge DA (2001) Effects of leukemia inhibitory factor on galanin expression and on axonal growth in adult dorsal root ganglion neurons in vitro. *Exp Neurol* 169:376–385.
- Savitz SI, Kessler JA (2000) Leukemia inhibitory factor requires concurrent p75LNTR signaling to induce apoptosis of cultured sympathetic neurons. *J Neurosci* 20:4198–4205.
- Sendtner M, Stockli KA, Thoenen H (1992) Synthesis and localization of ciliary neurotrophic factor in the sciatic nerve of the adult rat after lesion and during regeneration. *J Cell Biol* 118:139–148.
- Shadiack AM, Sun Y, Zigmond RE (2001) Nerve growth factor antiserum induces axotomy-like changes in neuropeptide expression in intact sympathetic and sensory neurons. *J Neurosci* 21:363–371.
- Shuto T, Horie H, Hikawa N, Sango K, Tokashiki A, Murata H, Yamamoto I, Ishikawa Y (2001) IL-6 up-regulates CNTF mRNA expression and enhances neurite regeneration. *NeuroReport* 12:1081–1085.
- Snider WD, McMahon SB (1998) Tackling pain at the source: new ideas about nociceptors. *Neuron* 20:629–632.
- Sun Y, Zigmond RE (1996) Leukaemia inhibitory factor induced in the sciatic nerve after axotomy is involved in the induction of galanin in sensory neurons. *Eur J Neurosci* 8:2213–2220.
- Tandrup T, Woolf CJ, Coggeshall RE (2000) Delayed loss of small dorsal root ganglion cells after transection of the rat sciatic nerve. *J Comp Neurol* 422:172–180.
- Terenghi G (1999) Peripheral nerve regeneration and neurotrophic factors. *J Anat* 194:1–14.
- Thompson SW, Vernallis AB, Heath JK, Priestley JV (1997) Leukaemia inhibitory factor is retrogradely transported by a distinct population of adult rat sensory neurons: co-localization with trkA and other neurochemical markers. *Eur J Neurosci* 9:1244–1251.
- Thompson SW, Priestley JV, Southall A (1998) gp130 cytokines, leukemia inhibitory factor and interleukin-6, induce neuropeptide expression in intact adult rat sensory neurons in vivo: time-course, specificity and comparison with sciatic nerve axotomy. *Neuroscience* 84:1247–1255.
- Verge VM, Richardson PM, Wiesenfeld-Hallin Z, Hökfelt T (1995) Differential influence of nerve growth factor on neuropeptide expression in vivo: a novel role in peptide suppression in adult sensory neurons. *J Neurosci* 15:2081–2096.
- Wakisaka S, Kajander KC, Bennett GJ (1992) Effects of peripheral nerve injuries and tissue inflammation on the levels of neuropeptide Y-like immunoreactivity in rat primary afferent neurons. *Brain Res* 598:349–352.
- Zhou XF, Deng YS, Chie E, Xue Q, Zhong JH, McLachlan EM, Rush RA, Xian CJ (1999) Satellite-cell-derived nerve growth factor and neurotrophin-3 are involved in noradrenergic sprouting in the dorsal root ganglia following peripheral nerve injury in the rat. *Eur J Neurosci* 11:1711–1722.
- Zigmond RE (2001) Can galanin also be considered as growth-associated protein 3.2? *Trends Neurosci* 24:494–496.