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Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion

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ABSTRACT **Estrogen is known to influence different functions in brain tissue ranging from neuronal development to plasticity and survival, but the mechanisms involved have not been defined clearly. Previous studies have shown the presence of the two estrogen receptors (ERs), ERα and ERβ, in several brain areas, but less is known about the role of estrogen in the peripheral nervous system. Here we demonstrate that dorsal root ganglion (DRG) neurons express ERα and ERβ during early postnatal development and in culture, and that the ERs localize mainly to neuronal cell nuclei. Studying the role of estrogen in DRG, we observed that low concentrations of 17β-estradiol increased survival of cultured DRG neurons deprived of nerve growth factor. 17β-Estradiol up-regulated the expression of the antiapoptotic molecule Bcl-x without affecting that of Bax, suggesting a mechanism by which the hormone counteracted neuronal death. Antiestrogens abolished the action of 17β-estradiol in the DRG neurons, which demonstrates an involvement of ERs. The results show that estrogen and ERs play an important role in the development and survival of DRG neurons.**

In the nervous system, depending on the region, about one-half of the neurons produced during fetal life are eliminated during the process of naturally occurring cell death (1). In the peripheral nervous system (PNS), target-derived neurotrophic factors, such as nerve growth factor (NGF), support the survival of specific classes of neurons in the dorsal root ganglia (DRG) (1). These neurons depend crucially on the supply of neurotrophic factors during early development, but lose this dependency at later developmental stages (2, 3). Thus, whereas embryonic and early postnatal DRG neurons require NGF, adult neurons survive even in the absence of NGF (4). The precise mechanisms by which developing DRG neurons change their requirements for neurotrophic factors are largely unknown. It has been shown that DRG neurons respond, beside NGF, to brain-derived neurotrophic factor (BDNF) (5), to glial cell line-derived neurotrophic factor (GDNF) (6), and to insulin-like growth factor I (IGF-I) (7, 8). In addition, other factors, such as neuropeptides, also influence neuronal survival and/or promote differentiation (9).

It has been proposed that cell death of neurons is regulated by the relative levels of anti- and proapoptotic proteins. In DRG the ratio between Bcl-xL (Bcl-x), promoting survival, and Bax, inducing cell death, plays a decisive role in the control of cell death (10, 11). In particular, it was shown recently that Bcl-x levels increase significantly during early postnatal development of DRG neurons, concomitantly with a decrease in Bax (12).

Estrogens represent an important class of hormones that can promote development, maturation, and function of the central nervous system (13, 14). These hormones have been shown to regulate the expression of neurotrophic factors (15), to affect the phenotype expression of neural cells (16, 17), and to control synaptic plasticity (18, 19). Recent studies indicate that estrogen administration may modulate cognitive functions such as memory and learning, showing a role for estrogen in brain functions that are not related strictly to sexual activities (20, 21). However, a detailed view of how estrogen and their cognate receptors modulate neural cells currently is unknown.

The wide distribution of estrogen receptors (ERs), including estrogen receptor α (ERα) and the recently cloned estrogen receptor β (ERβ) (22) in brain tissue supports the view for an important function of estrogen in the nervous system. The ERs are present in many brain regions already during early development (23, 24), and the expression is maintained in many cases into adulthood (25–27). In contrast to brain, little is known about ERs in the PNS. However, ERα-positive neurons recently were observed in adult rat DRG (28, 29), indicating that estrogens may play an important role in the function of DRG.

In this study, we have investigated the possible role of estrogen in developing PNS by using cultured DRG neurons prepared from postnatal day 3 (P3) rats. We demonstrate that postnatal DRG neurons express ERα and ERβ both *in vivo* and *in vitro*. 17β-Estradiol, through activation of ERs, was able to counteract cell death of DRG neurons induced by NGF deprivation. We also identified Bcl-x as a target for 17β-estradiol action in these neurons, suggesting a molecular mechanism by which estrogens promote neuronal survival.

MATERIALS AND METHODS

DRG Cultures and Survival. DRG were dissected from P3–P4 Wistar rats, and ganglia were collected in PBS containing 0.45% glucose. Trypsin (0.25%; GIBCO) was added for 30 min at 37°C, and the digestion was terminated by DMEM (GIBCO) supplemented with 10% charcoal-stripped serum (Imperial Chemical Industries). Cells were centrifuged for 3 min at 900 rpm and washed three times, and a single cell suspension was obtained by passing the tissue through a Pasteur pipette. The cells were preplated for 2 h on a 35-mm Petri dish, and nonadherent cells were seeded onto 0.1 mg/ml poly-DL-ornithine/6 μg/ml laminin-precoated (Sigma), 24-well plates at a density of 9,400 cells/cm^2. NGF (50 ng/ml) was added in addition to 0.01 mM cytosine β-D-arabino-furanoside (Sigma) to reduce the number of nonneuronal cells. After incubation overnight (37°C, 5% CO_2), fresh, serum-free medium was added, together with different concentrations of 17β-estradiol (Sigma), tamoxifen (Sigma), or ICI 182,780 (kindly provided by A. Maggi, University of Milan, Italy) as indicated in the figure legends. Three fields per well were counted in triplicate at 3–5 consecutive days by using a grid in the ocular of the microscope with stable coordinates. Every experiment was repeated at least three times. Neurons were

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Abbreviations: ER, estrogen receptor; DRG, dorsal root ganglia; NGF, nerve growth factor; PNS, peripheral nervous system; P, postnatal day.
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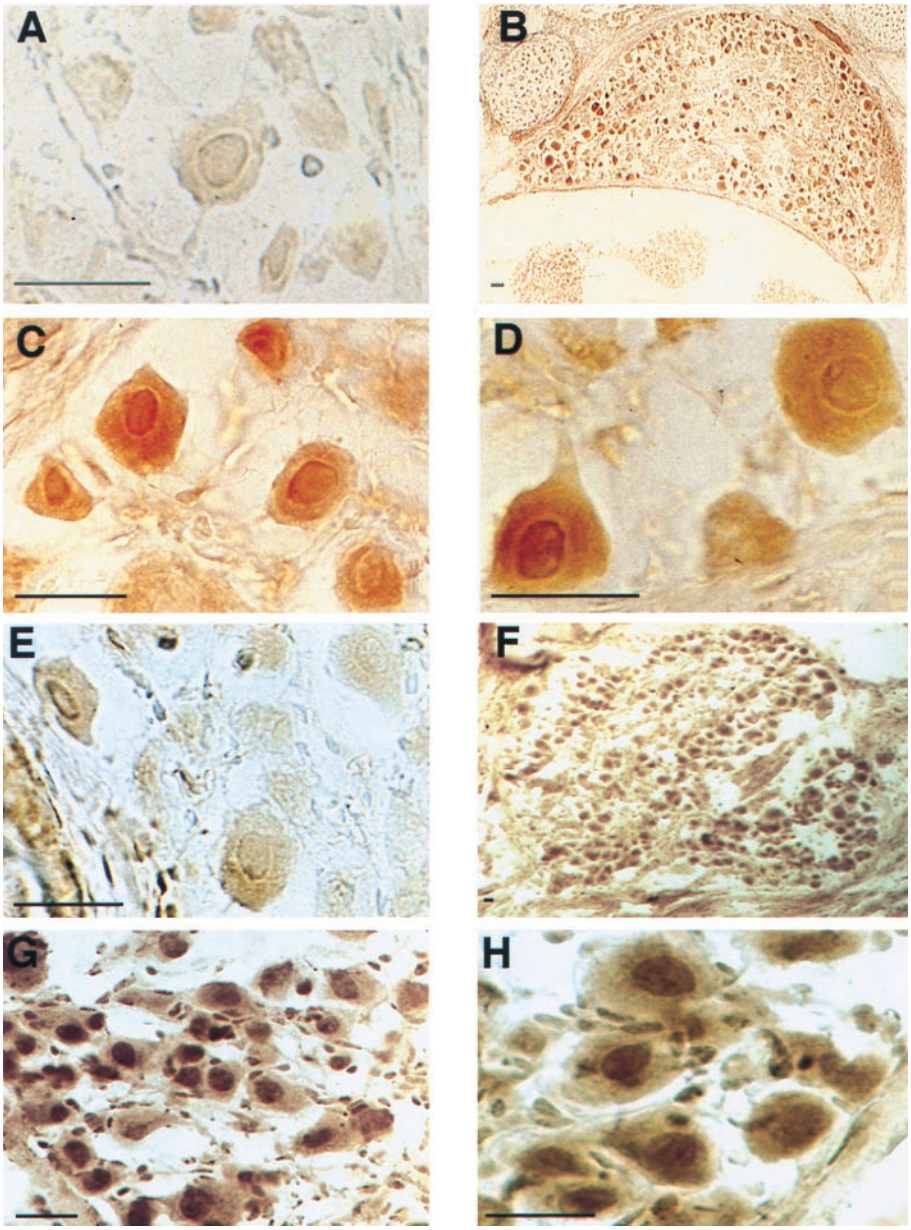

FIG. 1. ERβ and ERα are expressed in early postnatal DRG. DRG sections were prepared from P3–P4 rats and incubated with an antiserum against ERβ (*A–D*) and ERα (*E–H*) as described in *Materials and Methods*. (*A* and *E*) Control without primary antibody. (*B* and *F*) Low magnification. (*C*, *D*, *G–H*) High magnification. Nuclear staining of DRG neurons is clearly visible with some staining of the cytoplasm for ERβ. (Bar = 30 μm.)

identified by using phase-contrast optics as bright process-bearing cells. A 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed as described earlier (30). For statistical analysis, Student's *t* test was used.

Immunochemistry. To detect ERα and ERβ, a mouse mAb (7 μg/ml, clone C-542; StressGen Biotechnologies, Victoria, Canada) and an affinity-purified rabbit anti-ERβ (5 μg/ml, PA1–311; Affinity BioReagents, Neshanic Station, NJ) were used, respectively. Cells cultured for 2 days on coverslips were fixed for 15 min at 4°C by using 4% paraformaldehyde in 0.1 M phosphate buffer and blocked overnight with 5% skimmed milk in PBST (PBS/0.3% Triton X-100). Primary antibody was added for 48 h, followed by a biotinylated horse anti-mouse or a goat anti-rabbit IgG (1:250 Vector Lab) secondary antibody, which was added for 2 h at room temperature. Avidin–biotin complex (1:1,000; Vector Laboratories) was added for 1 h, and the immunoreactivity was visualized by using diaminobenzidine (Sigma) as a chromogen.

Slices, 15 μM, containing DRG were prepared from P3–P4 rats, fixed for 30 min in 4% paraformaldehyde at 4°C, washed with PBS, and incubated for 15 min with 0.3% H202 to inhibit endogenous peroxidases. The slices were blocked overnight at 4°C in PBST containing 3% rat serum, and primary antibodies were added for 3 days at 4°C. Control incubations were without primary antibodies, in addition to studies by using an excess of the corresponding peptide for ERβ.

Western Blot Analysis. Cells were plated onto six-well plates for 2 days as described above. Western blotting was performed essentially as described (31). Briefly, DRG neurons were lysed in ice-cold buffer (1% Nonidet P-40/20 mM Tris, pH 8.0/5 mM EDTA) supplemented with protease inhibitor mixture (Boehringer-Mannheim). Equal amounts of protein, determined by a protein assay (Bio-Rad), were loaded onto a 12% SDS gel, and the proteins were blotted onto poly(vinylidene difluoride)-membranes (Amersham), which were stained for equal transfer by using Ponceau staining. The membrane was

blocked for 2 h by using 5% skimmed milk in TNT buffer (10 mM Tris, pH 7.5/100 mM NaCl/0.1% Tween 20). Incubation with antibodies (Bcl-x, 1:1,000; Transduction Laboratories; Bax, 1:500, Santa Cruz Laboratories) was performed overnight at 4°C. After washing with TNT buffer, horseradish peroxidase-coupled secondary antibody (anti-rabbit, 1:1,000; Dako) was added for 2 h at room temperature. The signals were visualized by using the enhanced chemiluminescence method (Amersham). Quantification was done by using a digitized image analyses system, and values were compared with controls set to 1.

RESULTS

ERα and ERβ Are Expressed in Early Postnatal DRG and in Cultures. The expression of ERα and ERβ in postnatal, P3–P4 DRG was studied by using specific antibodies, which showed that both receptors are present in cells with the morphology of neurons (Fig. 1). The ERs were localized in nuclei, although some ERβ staining was detectable into the cytosol. Preincubation of the ERβ antibody with the corresponding peptide significantly reduced both nuclear and cytoplasmic staining.

Cultured DRG neurons also express both ERs with a clear nuclear localization (Fig. 2). ERα was detected in about 30% of the neurons, whereas about 50% were positive for ERβ. To study the functionality of ERs, the cognate ligand 17β-estradiol was added to the cultures for 10–20 min, which resulted in a down-regulation of both receptors, as was shown previously in other target cells for estrogen (32).

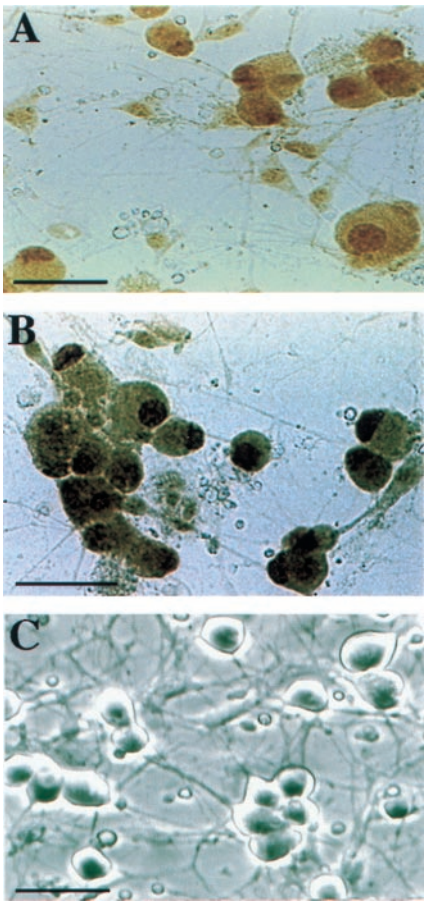

FIG. 2. DRG cultures from postnatal rats are immunoreactive for ERs. DRG neurons are positive for ERα (*A*) and ERβ (*B*). Signals are mainly in the nucleus. (*C*) Phase-contrast picture of DRG cultures. (Bar = 30 μm.)

17β-Estradiol Delays DRG Neuronal Cell Death After NGF Withdrawal. DRG neuron cultures were prepared from P3–P4 rats and treated with 1 nM 17β-estradiol to study possible survival effects of estrogen. Within 72 h after NGF withdrawal, about 75% of DRG neurons died (Fig. 3*A*). However, in the presence of 17β-estradiol, cell survival was protracted significantly although not to the same extent as with 50 ng/ml NGF (Fig. 3*A*). The effect of the hormone became apparent after 24 h, and at 48 h, 17β-estradiol treatment caused a 20–30% increase in neuron survival as compared with control (Fig. 3*A*). The maximum effect of 17β-estradiol was observed between 2 and 3 days of culture depending on the preparation, and, extending the time of study to 5 days, we noted that 17β-estradiol was still able to promote survival by about 15% (n = 3). Using the MTT test to study cell survival, that in DRG was significantly (about 25%) augmented by 17β-estradiol at day 2 (Fig. 3*B*). Studying different concentrations of 17β-estradiol, ranging from 10^{-12} to 10^{-6} M, a saturating effect, a 25% increase in survival, was observed already at 10^{-11} M.

To study the possible interplay between estrogen and NGF, DRG neurons were treated with 1 nM 17β-estradiol in the presence of different NGF concentrations. Using 1 ng/ml NGF, there was an additive effect of NGF and 17β-estradiol on cell survival (a 22% increase for 17β-estradiol vs. control, a 20% increase for NGF vs. control, and an ≈40% increase for both together vs. control; n = 3). However, this was not observed when using 10 ng/ml and 50 ng/ml NGF. To investigate whether the 17β-estradiol effect depends on NGF, we used a specific anti-NGF antibody to block NGF action (33). Although this treatment completely abolished the effect of NGF on DRG neurons, it did not interfere with that of estrogen (data not shown). This suggests that the survival-promoting effect of 17β-estradiol is not due to increased levels of NGF in the cultures.

The Effect of 17β-Estradiol on Survival Depends on ERs. To investigate the role of ERs in the 17β-estradiol-induced survival, cultured DRG neurons were incubated in the absence or presence of specific antagonists for ERs. ICI 182,780, a well characterized antiestrogen compound, completely blocked the increase in survival observed by 17β-estradiol (Fig. 4*A*). A qualitatively similar result also was obtained by using another specific ER antagonist such as tamoxifen (data not shown). Likewise, the MTT assay substantiated the findings obtained with ICI 182,780 with respect to survival (Fig. 4*B*). These results show that 17β-estradiol increases DRG neuron survival acting through one or both ER subtypes present in the DRG.

Bcl-x Is Up-Regulated by 17β-Estradiol in Postnatal DRG Cultures. To study the mechanism by which 17β-estradiol increases neuronal survival, the levels of Bcl-x and Bax in DRG were examined. In neurons treated for 48 h with 17β-estradiol, Bcl-x increased significantly (3-fold) compared with controls (Fig. 5); however, no change was observed in Bax under the same conditions (Fig. 5*B*). In contrast to 17β-estradiol, NGF treatment did not influence Bcl-x levels (Fig. 5*D*). Tamoxifen completely blocked the effect of 17β-estradiol, showing an involvement of ER. Interestingly, tamoxifen alone slightly decreased Bcl-x, which may indicate a basal activation of ERs by other factors than the ligand (34).

DISCUSSION

In the present study we demonstrate that estrogen increases survival of DRG neurons during early postnatal development and that both ERα and ERβ are expressed in the DRG. Estrogen up-regulates the expression of Bcl-x in cultured DRG, which could account for its effect on survival. The effects of estrogen on Bcl-x and neuronal survival were mediated by ER, as shown by using specific ER antagonists.

In comparison with the brain, less is known about the activities of estrogen in the PNS. There are a few reports showing the

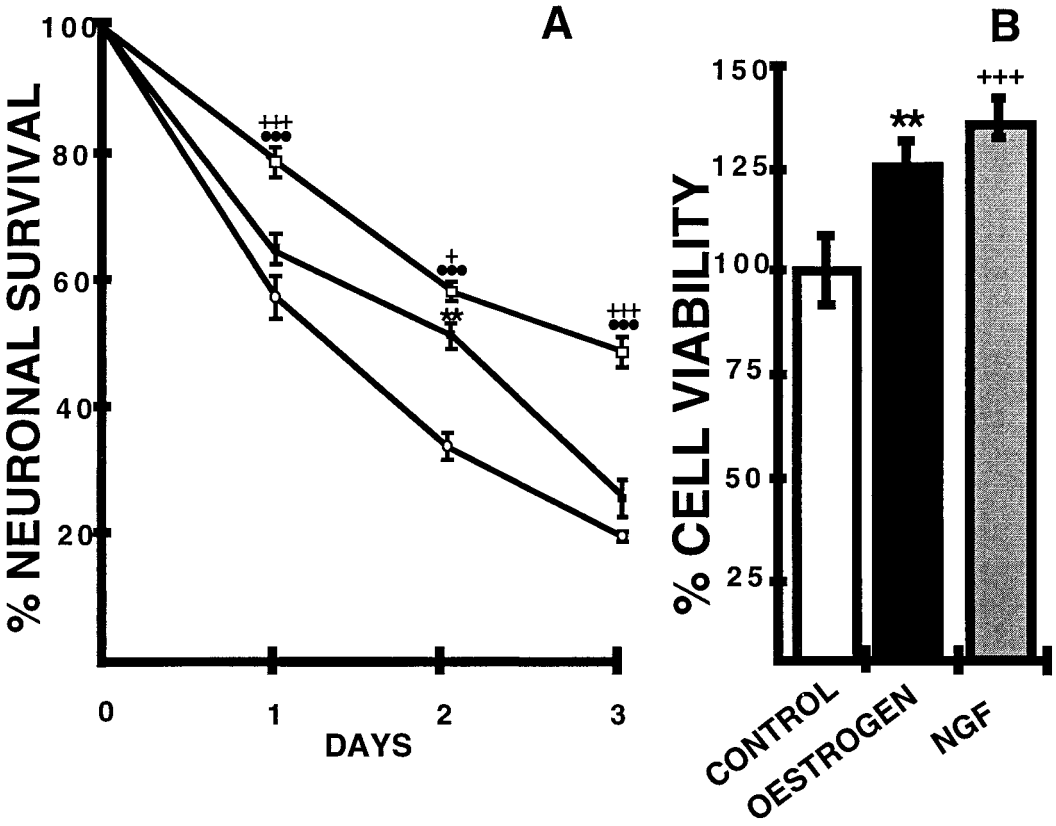

FIG. 3. 17β-Estradiol delays neuronal cell death of DRG in cultures after NGF withdrawal. (*A*) DRG cells were prepared from P4 rats and cultured with (□) or without NGF in the absence (○) or presence of 1 nM 17β-estradiol (■). Process-bearing neurons were counted each day, and the numbers were normalized with respect to day 0. The figure shows one typical experiment that was repeated three times with comparable results. Bar graph represents mean ± SEM. **, $P \leq 0.002$ for estrogen vs. control; ●●●, $P \leq 0.001$ for NGF vs. control; +++, $P \leq 0.001$ for NGF vs. estrogen; +, $P \leq 0.05$ for NGF vs. estrogen. (*B*) MTT test was done after 2 days in culture. Values represent mean ± SEM. Statistical significance is as in *A*.

presence of ERα in adult DRG (28, 29); however, no data exist on the recently cloned ERβ in the periphery. In this study, we observed the presence of both subtypes of ER in developing DRG neurons. The relative abundance of these receptors within DRG during early postnatal life suggests an important developmental function for estrogen in these neurons.

Previous studies have indicated a role for estrogen as a protective agent against various types of cell death. Clinical studies on estrogen have shown that a replacement therapy with the hormone significantly retards the manifestation of Alzheimer's disease (35, 36) and that estrogen treatment reduces the amount of β-amyloid protein (37). However,

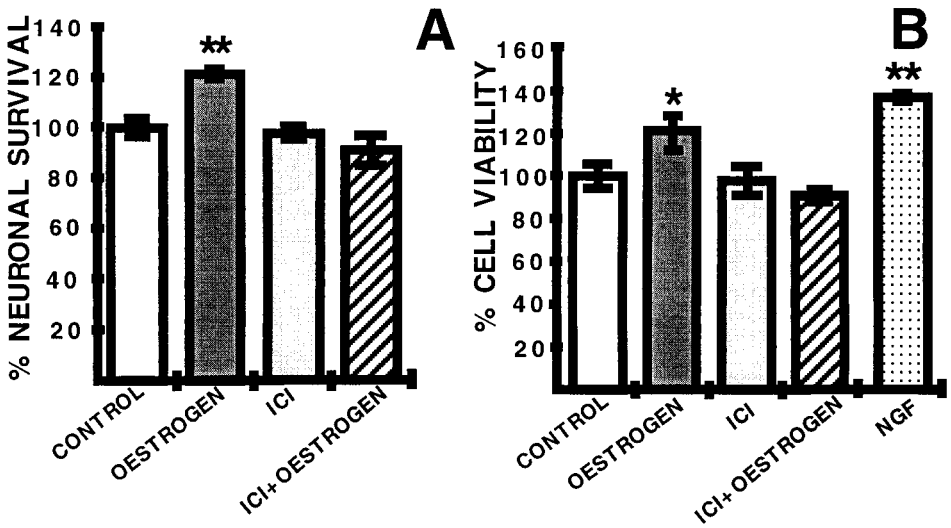

FIG. 4. 17β-Estradiol protects DRG from cell death through activation of ER. Cells were treated with 1 nM 17β-estradiol, 100 nM ICI 182,780, or 50 ng/ml NGF as indicated in the figure. DRG cell count and MTT assay were done after 3 days in culture. (*A*) Cell count. Values represent mean ± SEM. **, $P \leq 0.001$ for estrogen vs. control. (*B*) MTT assay. Values are mean ± SEM. *, $P \leq 0.005$ for estrogen vs. control; **, $P \leq 0.001$ for NGF vs. control.

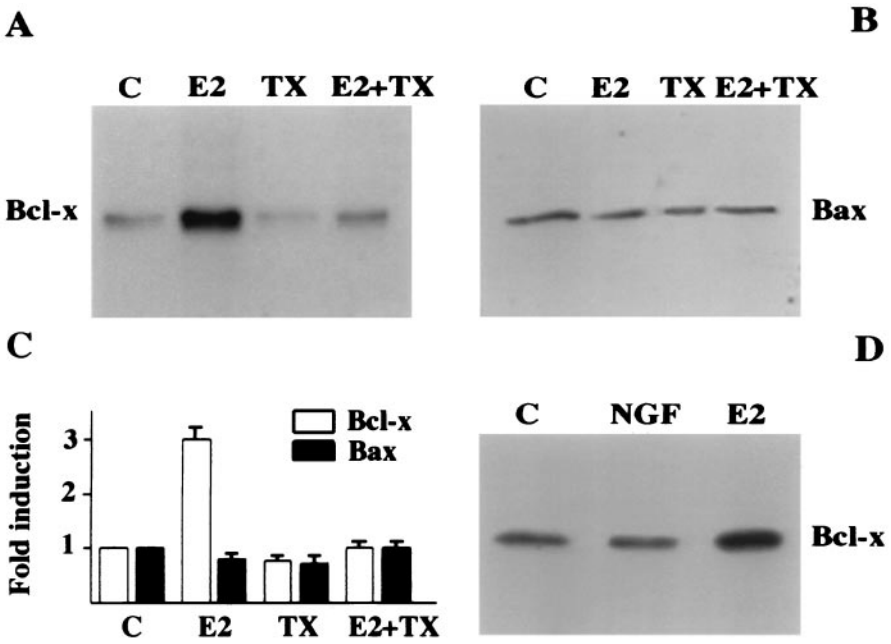

FIG. 5. 17β-Estradiol up-regulates Bcl-x without affecting Bax in postnatal rat DRG cultures. P4 DRG neurons were cultured for 2 days as indicated in the figure. Cell lysates were prepared, and an equal amount of proteins was subjected to Western blotting as described in *Materials and Methods*. (*A* and *D*) Bcl-x protein (26 kDa). (*B*) Bax protein (21 kDa). (*C*) Quantification was done by using a digitized image analyses system, and values were compared with controls set to 1 ($n = 3$). C, control; E2, 1 nM 17β-estradiol; TX, 100 nM tamoxifen; NGF, 50 ng/ml NGF.

despite the plethora of effects attributed to estrogen, the molecular mechanisms of its action often have remained controversial. In particular, in brain it is unclear whether estrogen can regulate neuronal survival through the involvement of ERs or by exerting an antioxidant or some membrane effects, which are still to be determined. In this study, we show that estrogen delays death of DRG neurons deprived of NGF. Using ICI 182,780, a specific ER antagonist, the survival effect of estrogen was found to depend on the activation of ER. DRG neurons express ERα and ERβ, both of which might be involved in the effects observed. No major abnormalities were reported in brain of mice carrying a null-mutation for ERα (38). However, the PNS has not been studied thoroughly, and the present finding on ERβ in DRG suggests that both types of ER are important in nervous tissue. Future studies will show whether the two ERs colocalize and whether they have individual roles in DRG neurons. Recently, a differential effect of ERα and ERβ was observed on the AP1 promoter in transfected cells (39).

To study the mechanism by which estrogen controls DRG neuron survival, we investigated the levels of proteins belonging to the Bcl-2 family, which are major regulators of cell death in various cell types. Bcl-x particularly is relatively highly expressed within developing DRG (12), and by using Western blots we found that Bcl-x levels in DRG were up-regulated strongly by 17β-estradiol, whereas Bax remained constant. The effect of 17β-estradiol on Bcl-x was blocked by tamoxifen, which strengthens the notion that ER activation is instrumental for the hormone to protect DRG neurons. In view of our data in DRG, it is possible that Bcl-x is a downstream target for estrogen action in other brain cells as well. It remains to be studied whether estrogen stimulates transcription of the Bcl-x gene directly or acts indirectly.

NGF is a classical neurotrophic factor for the DRG neurons and influences their survival and neuronal differentiation (1–3). The effect of 17β-estradiol was not blocked by the presence of anti-NGF antibodies, showing that 17β-estradiol does not affect the levels of NGF in these cultures. In contrast to 17β-estradiol, NGF failed to increase Bcl-x in DRG, suggesting that they act through two largely independent pathways promoting DRG neuronal survival. However, we observed an additive effect on cell survival by using 1 nM 17β-estradiol and a low concentration, 1 ng/ml, of NGF. This suggests a possibility for a crosstalk between estrogen and NGF, as shown previously, by the regulation of NGF receptor p75^{NGFR} and TrkA in adult DRG with estrogen (29).

The lack of ER-expressing neurons has made most studies on ER activity in the nervous system rather difficult. In this study, we have found that DRG neurons express considerable levels of both ERα and ERβ compared with other brain areas, making DRG a suitable system to study estrogen effects on neurons. Sensory neurons have different functions and mediate sensations such as pain and touch. In view of the abundance of ERs in DRG sensory neurons observed here, it is likely that estrogens can influence a variety of DRG functions both during development and in adult life.

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1. Oppenheim, R. W. (1991) *Annu. Rev. Neurosci.* **14,** 453–501.
2. Johnson, E. M., Jr., Gorin, P. D., Brandeis, L. D. & Person, J. (1980) *Science* **210,** 916–918.
3. Schwartz, J. P., Pearson, J. & Johnson, E. M. (1982) *Brain Res.* **244,** 378–381.
4. Johnson, E. M., Jr., Rich, K. M. & Yip, H. K. (1986) *Trends Neurosci.* **9,** 33–37.
5. Acheson, A., Conover, J. C., Fandl, J. P., DeChiara, T. M., Russel, M., Thadani, A., Squinto, S. P., Yancopoulos, G. D. & Lindsay, R. M. (1995) *Nature (London)* **374,** 450–453.
6. Molliver, D. C., Wright, D. E., Leitner, M. L., Parsadanian, A. S., Doster, K., Wen, D., Yan, Q. & Snider, W. D. (1997) *Neuron* **19,** 849–861.
7. Reinhardt, R. R., Chin, E., Zhang, B., Roth, R. A. & Bondy, C. A. (1994) *J. Neurosci.* **14,** 4674–4683.
8. Russel, J. W., Windebank, A. J., Schenone, A. & Feldman, E. L. (1998) *J. Neurobiol.* **36,** 455–467.
9. Lioudyno, M., Skoglösa, Y., Takei, N. & Lindholm, D. (1998) *J. Neurosci. Res.* **51,** 243–256.
10. Motoyama, M., Wang, F., Roth, K. A., Sawa, H., Nakayama, K., Negishi, I., Senju, S., Zhang, Q., *et al.* (1995) *Science* **267,** 1506–1510.
11. Deckwerth, T. L., Elliot, G. L., Knudson, C. M., Johnson, E. M., Jr., Snider, W. D. & Korsmeyer, S. J. (1996) *Neuron* **17,** 401–411.
12. Vogelbaum, M. A., Tong, J. X. & Rich, M. K. (1998) *J. Neurosci.* **18,** 8928–8935.
13. McEwen, B. S., Alves, S. E., Bulloch, K. & Weiland, N. G. (1997) *Neurology* **48,** S8–S15.
14. Pilgrim, C. & Hutchison, J. B. (1994) *Neuroscience* **60,** 843–855.

15. Murphy, D. D., Cole, N. B. & Segal, M. (1998) *Proc. Natl. Acad. Sci. USA* **95,** 11412–11417.
16. Agrati, P., Ma, Z. Q., Patrone, C., Picotti, G. B., Pellicciari, C., Bondiolotti, G., Bottone, M. G. & Maggi, A. (1997) *Eur. J. Neurosci.* **9,** 1008–1016.
17. Murphy, D. D., Cole, N. B., Greenberger, V. & Segal, M. (1998) *J. Neurosci.* **18,** 2550–2559.
18. Woolley, C. S., Gould, E., Frankfurt, M. & McEwen, B. S. (1990) *J. Neurosci.* **10,** 4035–4039.
19. Murphy, D. D. & Segal, M. (1996) *J. Neurosci.* **16,** 4059–4068.
20. Sherwin, B. B. (1995) *Ann. N. Y. Acad. Sci.* **743,** 213–232.
21. Packard, M. G. (1998) *Horm. Behav*. **34,** 126–139.
22. Kuiper, G. G. J. M., Enmark, E., Pelto-Huikko, M., Nilsson, S. & Gustafsson, J. J. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5925–5930.
23. Keefer, D. & Holderegger, C. (1985) *Brain Res.* **351,** 183–194.
24. Shughrue, P. J., Stumpf, W. E., MacLusky, N. J., Zielinski, J. E. & Hochberg, R. B. (1990) *Endocrinology* **126,** 1112–1124.
25. Pfaff, D. W. & Keiner, M. (1973) *J. Comp. Neurol.* **151,** 121–158.
26. Shughrue, P. J., Lane, M. V. & Merchenthaler, I. (1997) *J. Comp. Neurol.* **388,** 507–525.
27. Li, X., Schwartz, E. & Rissman, E. F. (1997) *Neuroendocrinology* **66,** 63–67.
28. Yang, Y., Ozawa, H., Lu, H., Yuri, K., Hayashi, S., Nihonyanagi, K. & Kawata M. (1998) *Brain Res.* **791**, 35–42.
29. Sohrabji, F., Miranda, R. C. & Toran-Allerand, C. D. (1994) *J. Neurosci.* **14**, 459–471.
30. Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Thoenen, H. & Lindholm, D. (1995) *J. Neurochem.* **65,** 2241–2259.
31. Korhonen, L., Hamnér, S., Olsson, P.-A. & Lindholm, D. (1997) *Eur. J. Neurosci*. **9,** 2489–2496.
32. Santagati, S., Gianazza, E., Agrati, P., Vegeto, E., Patrone, C., Pollio, G. & Maggi, A. (1997) *Mol. Endocrinol.* **11,** 938–949.
33. Lindholm, D., Hartikka, J., da Penha Berzaghi, M., Castrén, E., Tzimagiorgis, G., Hughes, R. A. & Thoenen, H. (1994) *Eur. J. Neurosci.* **6,** 244–252.
34. Patrone, C., Gianazza, E., Santagati, S., Agrati, P. & Maggi, A. (1998) *Mol. Endocrinol.* **12,** 835–841.
35. Tang, M. X., Jacobs, D., Stern Y, Marder, K., Schofleld, P., Gurland, B., Andrews, H. & Mayeux, R. (1996) *Lancet* **348,** 429–432.
36. Henderson, V. W., Paganini-Hill, A., Emanuel, C. K., Dunn, M. E. & Buckwalter, J. G. (1994) *Arch. Neurol.* **51,** 896–900.
37. Xu, H., Gouras, G. K., Greenfield, J. P., Vincent, B., Naslund, J., Mazzarelli, L., Fried, G., Jovanovic, J. N., Seeger, M., Relkin, N. R., *et al.* (1998) *Nat. Med.* **4,** 447–451.
38. Shughrue, P. J., Lubhahn, D. B., Negro-Vilar, A., Korach, K. S. & Merchenthaler, I. (1997) *Proc. Natl. Acad. Sci. USA* **94,** 11008–11012.
39. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J.-J., Kushner, P. & Scanlan, T. S. (1997) *Science* **277,** 1508–1510.