Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 12308–12312, December 1994
Neurobiology

Key role for pregnenolone in combination therapy that promotes recovery after spinal cord injury

(attenuation/dehydroepiandrosterone/bacterial lipopolysaccharide/indomethacin/axonal regeneration)

LLOYD GUTH*†, ZIYIN ZHANG*, AND EUGENE ROBERTS‡

*Department of Biology, The College of William and Mary, Williamsburg, VA 23187; and ‡Department of Neurobiochemistry, Beckman Research Institute of the City of Hope, Duarte, CA 91010

Contributed by Eugene Roberts, August 8, 1994

ABSTRACT **Controlled compressive injury to rat spinal cord was chosen to test therapies that might attenuate the progression of tissue destruction and locomotor deficits that characteristically occur after spinal injury. A highly significant reduction of damage was achieved by immediate postinjury treatment with a combination of the following: an antiinflammatory substance, indomethacin; a stimulator of cytokine secretion, bacterial lipopolysaccharide; and the parent steroid, from which all other steroids arise, pregnenolone. This treatment reduced histopathological changes, spared tissue from secondary injury, and increased restoration of motor function. Remarkably, 11 of 16 of the animals treated with the above combination were able to stand and walk at 21 days after injury, 4 of them almost normally. The results were far superior to those obtained in controls or in animals to which the substances were given separately or in combinations of two. This approach may prove to be applicable to nervous system injury, in general, and to injury in other tissues.**

Development of a pharmacological therapy to attenuate consequences of traumatic spinal cord injuries has proven to be difficult. Such injuries trigger a characteristic self-perpetuating progression of degenerative processes that eventually result in tissue destruction and often in permanent paraplegia or quadriplegia (1–5). Any single therapy is not likely to be effective in attenuating these autodestructive processes because so many factors are involved (6–8). The key to attenuation of damage and eventual successful regeneration of injured spinal cord may lie in optimization of relations among affected neurons and surrounding nonneural cells by (*i*) stimulating release of growth-promoting cytokines and lymphokines by nonneural cells while suppressing the effects of cytotoxic ones, (*ii*) maintaining sufficient activity in injured neurons to achieve adequate genomic transcription for production of neuron-produced trophic substances, and (*iii*) facilitating coordinative processes that enable neural, metabolic, and immune systems, separately and together, to cycle freely through their operational modes to achieve structural and functional reconstitution.

Combined treatment of rats with spinal cord injury with an antiinflammatory substance, indomethacin (IM), and a stimulator of cytokine secretion, bacterial lipopolysaccharide (LPS), reduced the amount of cavitation and increased cellular in-growth into the lesion to a greater extent than did treatment with either substance alone, but restoration of motor function was marginal (1). In the present study, we examined the effects of two steroids that have pleiotropic effects in neural and nonneural tissues, dehydroepiandrosterone sulfate (DHEAS) (9–11) and pregnenolone (PREG) (12–15), with a view to achieving further enhancement of functional recovery after injury.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Test Animals and Surgical Procedures. Sprague–Dawley female rats (170–200 g) were anesthetized with 4% (wt/vol) chloral hydrate (400 mg/kg, intraperitoneally), and the dorsal skin was shaved and scrubbed with benzalkonium chloride (0.13% aqueous). Laminectomy was performed aseptically at T8, leaving the dura intact. A no. 5 Dumont jewelers forceps was introduced between dura and bone, and the spinal cord was compressed for 2 sec. Pellets were implanted subcutaneously into a pocket prepared by lateral extension of the rostral margin of the incision or into a second incision made just lateral to the rostral margin of the first one when two pelleted substances were used. Urine was expressed twice daily by gentle manual compression of the abdomen for 7–10 days until bladder control was restored.

Substances Tested. Pellets (Innovative Research of America, 43606) containing 50 mg of PREG, 50 mg of DHEAS, or 25 mg of sodium acetylsalicylic acid (AS) gave continual release of drug for 21 days (i.e., 2.38 mg of PREG or DHEAS or 1.19 mg of AS per day). Rats receiving LPS were given daily intraperitoneal injections of 0.2 mg of LPS from *Salmonella enteritidis* (Sigma) in 0.1 ml of sterile pyrogen-free water. Those receiving IM were given daily intraperitoneal injections of 0.2 mg of IM sodium trihydrate (Indocin).

Histological Procedures. On the 21st experimental postoperative day, the rats were anesthetized and euthanized by intracardial perfusion with 15 ml of saline followed by 250 ml of Bouin's fixative. The vertebral column was removed and the spinal cord was dissected from the vertebral column, dehydrated, and embedded in Paraplast (Fisher). Serial longitudinal sections were cut at 8 μm in either the coronal (experiment 1) or the sagittal (experiment 2) plane. Every fifth slide was stained with hematoxylin/eosin (H&E) for examination of cellular detail and for image analysis. Adjacent slides were impregnated with Protargol for visualization of nerve fibers.

Image analysis was performed using the BioQuant system (BQ Meg IV, Biometrics) as described (1). Only those sections containing a portion of central canal in proximity to the lesion were studied to ensure that observations were made at the same level in all samples. For quantitative estimation of sparing of ventrally located white matter (axons), sections were cut longitudinally in the sagittal plane (experiment 2). The five sections chosen from each spinal cord included the one nearest the central canal (i.e., the midsagittal section) and sections at 90 μm and 180 μm, respectively, on either side of it. The sum of the widths of undamaged ventrally located white matter from the five

Abbreviations: LPS, lipopolysaccharide; IM, indomethacin; PREG, pregnenolone; PREG-S, pregnenolone sulfate; DHEAS, dehydroepiandrosterone sulfate; AS, acetylsalicylic acid; H&E, hematoxylin/eosin.

†To whom reprint requests should be addressed.

Table 1. Motor function and histological evaluation of spinal cord damage in longitudinal sections cut in the coronal plane after standardized crush at T8

Group	Treatment	No. of rats		Tarlov scale rating, 10 days		Tarlov scale rating, 21 days		Area of lesion, mm^2		(Area of cavity/area of lesion) × 100	
		Total	Survivors	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	Control	6	6	2.00	0.29	2.25	0.36	1.62	0.11	57.7	5.0
2	IM	8	6	2.33	0.38	2.58	0.33	1.61	0.14	64.0	4.9
3	LPS	5	4	3.12	0.52	3.50	0.61	1.64	0.08	38.2†	4.8
4	DHEAS	6	6	2.00	0.18	2.92	0.24	1.68	0.10	55.4	3.0
5	PREG	6	6	2.17	0.40	3.25	0.36	1.84	0.13	54.6	4.7
6	LPS/IM	8	4	2.50	0.35	3.20	0.30	1.63	0.14	29.8*	5.2
7	DHEAS/LPS/IM	8	6	2.75	0.28	3.29	0.39	1.12†	0.12	28.3*	3.4
8	PREG/LPS/IM	7	6	4.08*	0.36	4.50*	0.34	1.15‡	0.08	25.6*	2.4

Groups 1, 2, 3, and 6 were implanted with 21-day release placebo pellets; groups 4 and 7 were implanted with the same size pellets containing 50 mg of DHEAS; and groups 5 and 8 were implanted with pellets containing 50 mg of PREG. Data for the number of rats were recorded only for animals surviving the entire experimental period of 21 days. *P* values are for comparison with controls. *, $P < 0.01$; †, $P < 0.05$; ‡, $P < 0.001$.

sections measured at the epicenter of the lesions was designated as "spared tissue."

Functional Assessment. Walking abilities of operated rats were evaluated daily in double-blind fashion independently by two investigators (L.G. and Z.Z.) without knowledge of treatment group or of previous ratings. The rating system was a modification of the Tarlov scale (16): 0, complete hindlimb paralysis; 1, barely perceptible hindlimb movements; 2, clearcut hindlimb movements not resulting in weight bearing or locomotion; 3, hindlimbs used to crawl but not to support weight of body; 4, hindlimbs used to support weight of body for standing and walking, but with clearcut disability; 5, posture and walking ability nearly normal.

Statistical Analysis. Assessment of locomotor function according to the above scale was analyzed statistically using analysis of variance (ANOVA) with multiple comparisons made by the Student–Newman–Keuls procedure. The scale of 1–5 consists of an ordered metric level of measurement where the relative ordering of intercategory distances is known, even though their absolute magnitude cannot be measured. Such data can be treated as though it had been measured at equal intervals and the analysis of variance is an appropriate and sensitive method of statistical analysis (17). Nonparametric ANOVA (Dunn's procedure) was performed in those instances when the parametric procedure was inappropriate (e.g., when the Bartlett test revealed statistically significant differences in variance between treatment groups).

RESULTS

Combined treatment with PREG/LPS/IM led to statistically significant improvement in motor function in comparison with controls; the mean daily Tarlov score for the group was higher than those of all the other groups for every day between postoperative days 7 and 21 (Table 1). It was especially noteworthy that four of six animals in the PREG/LPS/IM group walked almost normally at day 21, whereas none of the animals in the other treatment groups achieved this degree of functional restitution. There was a trend toward improved function with other treatments, but the differences did not attain statistical significance.

Lesion sizes were closely similar and statistically indistinguishable in the DHEAS/LPS/IM and PREG/LPS/IM groups and were significantly reduced in comparison with other treatment groups. Cavitation within the lesions was less than in the controls both in these two groups and in the groups receiving LPS alone or LPS/IM. Cavitation was not significantly reduced in groups receiving only IM, DHEAS, or PREG, suggesting that LPS was required for reducing cavitation.

A larger-scale experiment (Table 2) then was performed in four groups of rats: control, PREG/LPS, PREG/LPS/IM, and PREG/LPS/AS. In the latter experimental group, AS replaced IM as an antiinflammatory agent. In each of the three experimental groups, there was significantly greater improvement in motor function than in the controls at days 10 and 21. Six of 10 animals of the PREG/LPS/IM group were able to stand and walk well at the end of the experiment, whereas only 1 of the 10 rats in the PREG/LPS/AS group and none in the PREG/LPS or control groups achieved this degree of locomotor recovery. The locomotor function of the PREG/LPS/IM group was significantly better than that of the others ($P < 0.01$, Fisher's exact test).

Area of lesion and extent of cavitation of the lesion (Table 2) were significantly less than that in the controls only in the groups receiving PREG/LPS/IM. This latter treatment also spared from injury a large amount of tissue in the ventral white matter of the spinal cord. The somewhat lesser attenuative effects of PREG/LPS/AS may be attributable to the

Table 2. Motor function and histological evaluation of spinal cord damage in longitudinal sections cut in the sagittal plane after standardized crush at T8

Group	Treatment	No. of rats		Tarlov scale rating, 10 days		Tarlov scale rating, 21 days		Area of lesion, mm^2		(Area of cavity/area of lesion) × 100		Spared tissue, μm	
		Total	Survivors	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
1	Control	11	9	1.67	0.09	2.39	0.10	1.89	0.12	52.6	3.8	53	15.4
2	PREG/LPS	11	10	2.22*	0.12	3.15*	0.20	1.77	0.14	51.7	4.6	131	33.1
3	PREG/LPS/AS	10	10	2.80‡	0.19	3.50‡	0.16	1.63	0.19	44.5	3.8	214†	71.9
4	PREG/LPS/IM	10	10	3.12‡	0.27	4.12‡	0.26	1.33†	0.14	35.7†	4.0	724‡	209

Variances were not homogeneous. Therefore, ANOVA was performed after logarithmic transformation of data. Data for number of rats are recorded only for animals surviving the experimental period of 21 days. *P* values are for comparison with controls. *, $P < 0.01$; †, $P < 0.05$; ‡, $P < 0.001$.

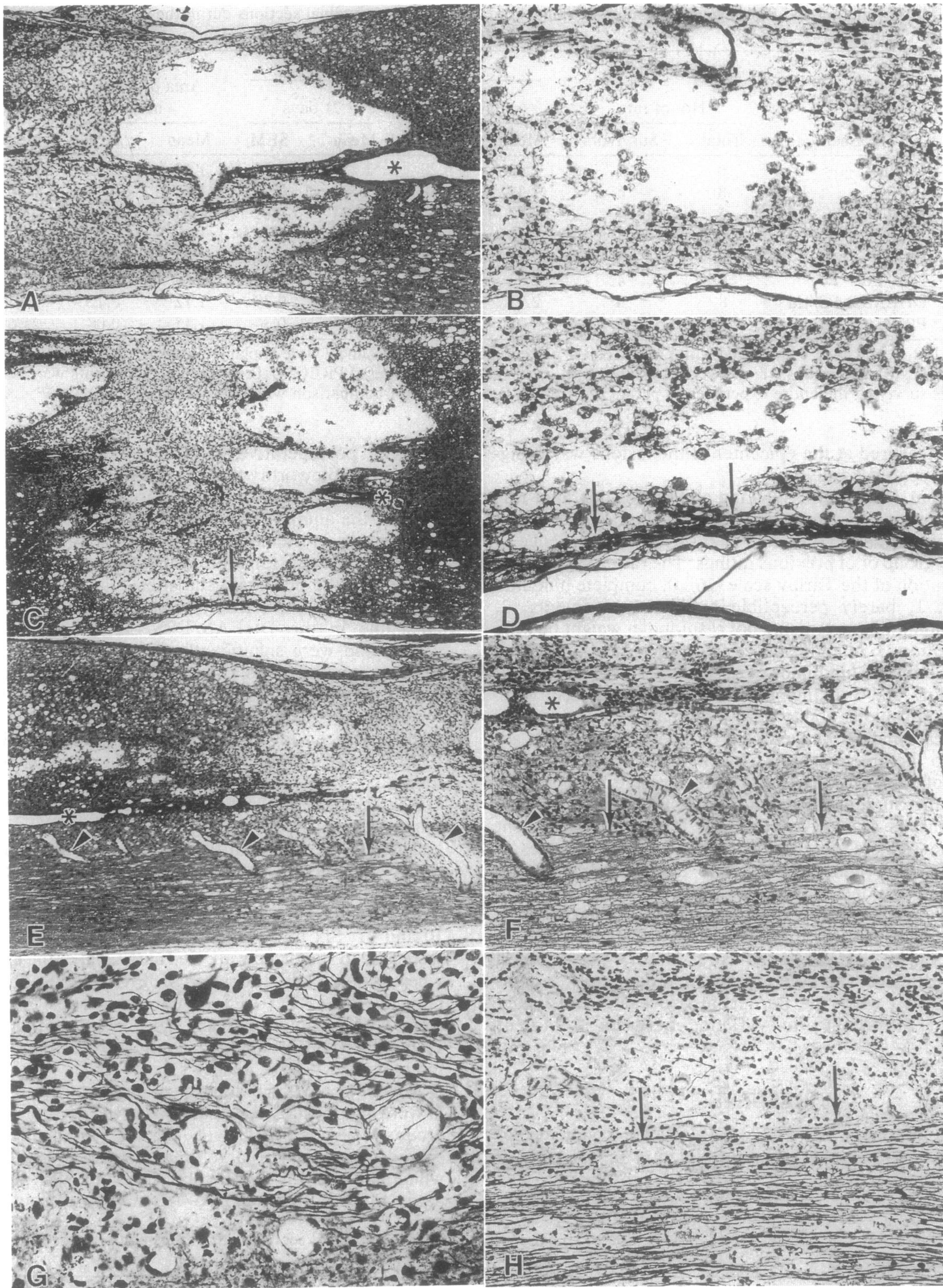

FIG. 1. Remarkable injury-attenuating effects of PREG/LPS/IM shown in midsagittal sections of spinal cord through site of lesion at 21 days postoperative. (*A*) Control. (H&E; ×40.) The central canal, seen in the undamaged tissue at the right, shows a characteristic dilatation (*) near the lesion. (*B*) Control. (H&E; ×100.) (*C*) PREG/LPS. (H&E; ×40.) The largest cavities are dorsal to the level of the central canal, indicated by an asterisk (*). There is a thin rim of residual spinal tissue at the ventral margin of the lesion (arrow). (*D*) PREG/LPS. (H&E; ×100.) (*E*) PREG/LPS/IM. (H&E; ×36.) The tissue ventral to the central canal (*) is characterized by sparing of white matter (arrow) and in-growth of blood vessels (arrowheads). (*F*) PREG/LPS/IM. (H&E; ×90.) This higher magnification of a portion of *E* shows ependymal cells migrating into the lesion from the central canal (*). Staining properties of the spared ventral white matter (arrows) are typical of myelinated nerve fibers. Blood vessels entering the ventral gray matter include large capillaries (left arrowhead) and arterioles (right arrowhead). (*G*) PREG/LPS/IM. (Protargol; ×180.) A modest number of nerve fibers with characteristics of regenerating axons course within the lesion. (*H*) PREG/LPS/IM. (Protargol; ×90.) This section, which is serial to that shown in *F*, shows the spared tissue in the ventral white matter (arrows) to consist of normal-appearing longitudinally oriented nonvaricose nerve fibers.

relatively low dose of AS used. Thus, the results indicate that the antiinflammatory agents probably are necessary for maximal effect.

The degree of protection against tissue damage obtained with PREG/LPS/IM is illustrated in Fig. 1. Closely similar results to those obtained with the latter were seen with DHEAS/LPS/IM (data not shown). There were remarkable histological differences between the controls (Fig. 1 *A* and *B*) and the PREG/LPS/IM group (Fig. 1 *E* and *F*). The extent of cavitation seen in the controls (Fig. 1*A*) was somewhat reduced by treatment with PREG/LPS (Fig. 1*C*) and was greatly reduced by treatment with PREG/LPS/IM (Fig. 1*E*). The cells of the control and PREG/LPS groups consisted primarily of rather loosely arranged macrophages (Fig. 1 *B* and *D*), while the matrix of the PREG/LPS/IM group (Fig. 1*F*) was more densely cellular and populated by proliferated glial cells in addition to macrophages. The most distinctive histological feature in rats of the PREG/LPS/IM group was the extensive amount of spared tissue seen ventral to the central canal (Fig. 1*F*), which was considerably greater than in the control (Fig. 1*B*) and PREG/LPS-treated (Fig. 1*D*) rats. This spared tissue was well-vascularized by large sinusoidal capillaries that derived from the ventrum and coursed dorsally toward the central canal and contained regularly arranged normally appearing axons (Fig. 1*H*); while the lesion immediately dorsal to this tissue contained a modest number of regenerating axons (Fig. 1*G*) that grew in relation to the ependymal cells, astrocytes, Schwann cells, and blood vessels that had invaded the lesion.

Combining the results in Tables 1 and 2, 27 of 29 rats (93%) receiving no treatment or DHEAS or PREG alone survived for 21 experimental days, as did 36 of 38 of those receiving PREG/LPS or when PREG/LPS was given with IM or AS (94.7%). However, only 14 of 21 (66.7%) survived in the groups receiving LPS or IM alone or in combination. The latter two groups in aggregate had significantly fewer numbers of survivors ($P < 0.03$, Fisher's exact test). Thus, there are toxic effects of LPS and IM individually that may be additive when these substances are given together and against which PREG and DHEAS offered protection. DHEAS has been shown to protect mice from toxicity of high doses of LPS (18).

DISCUSSION

Upon injury, numerous signals emanate from cells that flock to the injured terrain (neutrophils, macrophages, and lymphocytes) and from resident nonneuronal cells (ependymal cells, microglia, and astrocytes) that have undergone reactive changes as a result of injury. These changes, collectively called the "inflammatory response," are potentially self-limiting and set the stage for recovery by removing obstructive debris, by loosening extracellular matrix so that cells and substances can move freely, and by facilitating defenses against destructive microorganisms. But components of the inflammatory response also are destructive in injured spinal cord and lead to a progressive tissue destruction that often results in permanent structural and functional damage (1–6). For example, there is activation in tissues of membrane-located hydrolyzing enzymes that liberate free fatty acids from phospholipids (5), among which is the polyunsaturated fatty acid arachidonic acid, from which in turn are formed and released inflammatory metabolites such as eicosanoids and thromboxane A_2. The latter process can be interrupted pharmacologically by inhibition of cyclooxygenase activity with agents such as IM and AS (see refs. 6 and 19). PREG, itself, has antiinflammatory effects not attributable to formation of cortisone (15), which might be relevant to its synergistic action with LPS/IM in attenuating damage.

Although growth-regulatory substances are liberated from macrophages and other cells at sites of spinal cord injury, this endogenous secretion apparently is inadequate to fully promote wound healing. From among a number of possibilities, we chose LPS to enhance secretion of cytokines (see ref. 1). Immediate postinjury treatment with LPS/IM in the present and previous experiments achieved modest attenuation of histopathological damage, but there was little recovery of motor function. Addition of PREG to the above two substances in the present study gave truly remarkable enhancement of attenuation of histopathological damage and improvement in motor function (see above).

PREG, synthesized from cholesterol and serving as parent substance for the formation in tissues of a panoply of steroids and steroid derivatives including all of the known steroid hormones (20, 21), was used in this study. We posit that administration of this naturally occurring substance might best enable an injured system to self-organize into effective recovery by giving rise to the greatest number of other steroids (20, 21), substances that are known to facilitate coordinative processes within and among neural, metabolic, and immune systems (e.g., refs. 13–15 and 22–25). Pregnenolone sulfate (PREG-S), formed from PREG, may help maintain neuronal activity in the postinjury state. For adequate recovery to take place, there must be sufficient activity in affected neurons to achieve adequate genomic transcription for production of neuron-specific substances required for repair of the whole injured system (26–30). PREG-S is both a negative modulator of the γ-aminobutyric acid inhibitory receptor complex and a positive modulator of the *N*-methyl-D-aspartate excitatory receptor complex (31–33). By affecting both neurotransmitter systems in the above manner, PREG-S could exert remarkable synergistic amplification of excitatory transmission. Additionally, since PREG-S greatly enhances Ca^{2+} entry during stimulation of *N*-methyl-D-aspartate receptors, there would be increased release of consequent Ca^{2+}-triggered cascades of reactions to achieve the plastic changes required for organized recovery to take place (34). The greater efficacy of PREG-S than DHEAS in the latter regard may be one of the reasons that PREG restored motor function and DHEAS did not in the present study.

Levels of PREG many times higher than in plasma ordinarily are found in nervous tissue (35), where PREG is synthesized from cholesterol in oligodendrocytes and Schwann cells independently of the peripheral endocrine system (36). We presume that PREG, itself, and the steroids formed from it play key coordinative roles in many aspects of function in normal and injured spinal cord and posit that injury compromises both the formation and the required cellular distribution of these substances. The results of immediate postinjury administration of PREG, in combination with other substances such as discussed above, when optimized with respect to dose and type of agent, may supply a key to development of successful therapies for attenuating the damage that occurs after spinal cord injury in humans. If this proves correct, our findings may well be applicable to other types of neural damage (e.g., stroke) and possibly to injuries of nonneural tissues, as well.

We gratefully acknowledge the technical assistance of Katherine A. Barton of The College of William and Mary and the critical review by Dr. Oswald Steward of the University of Virginia. The work of L.G. and Z.Z. was supported by a grant (NS21460) from the National Institute of Neurological Disorders and Stroke. The work of E.R. was supported by institutional funds.

1. Guth, L., Zhang, Z., DiProspero, N. A., Joubin, K. & Fitch, M. T. (1994) *Exp. Neurol.* **126**, 76–87.
2. Balentine, J. D. (1978) *Lab. Invest.* **39**, 236–253.

3. De La Torre, J. C. (1981) *Spine (Philadelphia)* **6**, 315–335.
4. Guth, L., Barrett, C. P., Donati, E. J., Deshpande, S. S. & Albuquerque, E. X. (1981) *J. Comp. Neurol.* **203**, 297–308.
5. Anderson, D. K., Demediuk, P., Saunders, R. D., Dugan, L. L., Means, E. D. & Horrocks, L. A. (1985) *Ann. Emerg. Med.* **14**, 816–821.
6. Benveniste, E. N. (1992) *Am. J. Physiol.* **263**, C1–C16.
7. Roberts, E. (1987) *Prog. Brain Res.* **71**, 209–227.
8. Guth, L., Barrett, C. P., Donati, E. J., Smith, M. V., Lifson, M. & Roberts, E. (1985) *Exp. Neurol.* **88**, 44–55.
9. Parker, L. N. (1989) *Adrenal Androgens in Clinical Medicine* (Academic, New York).
10. Regelson, W., Loria, R. & Kalimi, M. (1988) *Ann. N.Y. Acad. Sci.* **521**, 260–273.
11. Roberts, E. (1990) *Prog. Brain Res.* **86**, 339–355.
12. Flood, J. F., Morley, J. E. & Roberts, E. (1992) *Proc. Natl. Acad. Sci. USA* **89**, 1567–1571.
13. Roberts, E. (1995) *Biochem. Pharmacol.* **49**, in press.
14. Selye, H. (1942) *Rev. Can. Biol.* **1**, 573–632.
15. Henderson, E., Weinberg, M. & Wright, W. A. (1950) *J. Clin. Endocrinol.* **10**, 455–474.
16. Wrathall, J. R., Pettegrew, R. K. & Harvey, F. (1985) *Exp. Neurol.* **88**, 108–122.
17. Abelson, R. P. & Tukey, J. W. (1970) in *The Quantitative Analysis of Social Problems*, ed. Tufte, E. R. (Addison-Wesley, Reading, MA), pp. 407–417.
18. Danenberg, H. D., Alpert, G., Lustig, S. & Ben-Nathan, D. (1992) *Antimicrob. Agents Chemother.* **36**, 2275–2279.
19. Mitchell, J. A., Akarasereenont, P., Thiemermann, C., Flower, R. J. & Vane, J. R. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 11693–11697.
20. Prasad, V. V. K. & Lieberman, S. (1990) *Ann. N.Y. Acad. Sci.* **595**, 1–16.
21. Lieberman, S., Greenfield, N. J. & Wolfson, A. (1984) *Endocr. Rev.* **5**, 128–148.
22. Weksler, M. E. (1993) *Eur. J. Clin. Pharmacol.* **45**, S21–S23.
23. Araneo, B. A., Woods, M. L., II, & Daynes, R. A. (1993) *J. Infect. Dis.* **167**, 830–840.
24. Araneo, B. A., Shelby, J., Li, G.-Z., Ku, W. & Daynes, R. A. (1993) *Arch. Surg.* **128**, 318–325.
25. Wisniewski, T. L., Hilton, C. W., Morse, E. V. & Svec, F. (1993) *Am. J. Med. Sci.* **305**, 79–83.
26. Ghosh, A., Carnahan, J. & Greenberg, M. E. (1994) *Science* **263**, 1618–1623.
27. Swaab, D. F. (1991) *Neurobiol. Aging* **12**, 317–324.
28. Will, B. E., Rosenzweig, M. R., Bennett, E. L., Hebert, M. & Morimoto, H. (1977) *J. Comp. Physiol. Psychol.* **91**, 33–50.
29. Metsis, M., Timmusk, T., Arenas, E. & Persson, H. (1993) *Proc. Natl. Acad. Sci. USA* **90**, 8802–8806.
30. Barres, B. A. & Raff, M. C. (1993) *Nature (London)* **361**, 258–260.
31. Majewska, M. D. (1992) *Prog. Neurobiol.* **38**, 379–395.
32. Wu, F.-S., Gibbs, T. T. & Farb, D. H. (1991) *Mol. Pharmacol.* **40**, 333–336.
33. Bowlby, M. R. (1993) *Mol. Pharmacol.* **43**, 813–819.
34. Irwin, R. P., Maragakis, N. J., Rogawski, M. A., Purdy, R. H., Farb, D. H. & Paul, S. M. (1992) *Neurosci. Lett.* **141**, 30–34.
35. Lanthier, A. & Patwardhan, V. V. (1986) *J. Steroid Biochem.* **25**, 445–449.
36. Akwa, Y., Young, J., Kabbadj, K., Sancho, M. J., Zucman, D., Vourc'h, C., Jung-Testas, I., Hu, Z. Y., LeGoascogne, C., Jo, D. H., Corpechot, C., Simon, P., Baulieu, E. E. & Robel, P. (1991) *J. Steroid Biochem. Mol. Biol.* **40**, 71–81.