which indicates that when the target cell was killed, the regenerating neuron could occasionally make an electrical synapse with its distal stump.

Since, when the target neuron is eliminated, S-interneurons regenerate to their normal region of synapse without making aberrant connections, something other than the target cell must be responsible for guiding the growing neuron and triggering it to stop growing. One likely candidate is the distal axonal stump, which generally survives during the month required for axonal regeneration, and often much longer. Regenerating neurons followed the distal stump apparently to its end and then stopped growing. The regenerating neurons may also be responding to other cues in the surrounding environment, left largely undisturbed by our surgical procedures. For example, either the ensheathing glial cell or extracellular components in the synaptic region remaining behind when the target S-cell is eliminated could be important. In the frog, when the target muscle cells are eliminated, end-plate specializations of the basal lamina provide cues to regenerating motor neurons (*3*), but neurons in the CNS of the leech do not have a basal lamina. What acts as a signpost to these growing neurons remains to be determined, but the target S-cell is not essential.

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References and Notes

1. E. Hibbard, *Exp. Neurol.* **13**, 289 (1965); H. Van der Loos, *Bull. Johns Hopkins Hosp.* **117**, 228 (1965); M. Constantine-Paton and R. R. Capranica, *J. Comp. Neurol.* **170**, 17 (1976); S. Laurberg and A. Hjorth-Simonsen, *Nature (London)* **269**, 158 (1977); P. R. Johns, M. G. Yoon, B. W. Agranoff, *ibid.* **271**, 360 (1978).
2. G. E. Schneider and S. R. Jhaveri, in *Plasticity and Recovery of Function in the Central Nervous System*, D. G. Stein, J. J. Rosen, N. Butters, Eds. (Academic Press, New York, 1974), p. 65; R. Llinas, D. E. Hillman, W. Precht, *J. Neurobiol.* **4**, 69 (1973); L. J. Misantone and D. J. Stelzner, *Exp. Neurol.* **45**, 364 (1974).
3. J. R. Sanes, L. M. Marshall, U. J. McMahan, *J. Cell Biol* **78**, 176 (1978).
4. S. Carbonetto and K. J. Muller, *Nature (London)* **267**, 450 (1977).
5. E. Frank, J. K. S. Jansen, E. Rinvik, *J. Comp. Neurol.* **159**, 1 (1975).
6. W. W. Stewart, *Cell* **14**, 741 (1978).
7. Additionally, two cells that are electrically coupled to the S-cell within each ganglion fill with Lucifer yellow and 6-carboxyfluorescein dyes. These cells are irrelevant to the present report because they do not send axons into the connective.
8. K. J. Muller and S. T. Carbonetto, *J. Comp. Neurol.* **185**, 485 (1979).
9. S. A. Scott and K. J. Muller, in preparation.
10. N. B. Gilula, O. R. Reeves, A. Steinbach, *Nature (London)* **235**, 262 (1972).
11. D. Bowling, J. Nicholls, I. Parnas, *J. Physiol. (London)* **282**, 169 (1978).
12. Crushes were made with forceps (Dumont No. 5) immediately after protease injection. Animals were allowed to recover at 16°C in leech Ringer solution (116 m*M* NaCl; 4 m*M* KCl; 1.8 m*M* $CaCl_2$; 10 m*M* tris-maleate, *p*H 7.4) for 1 day and thereafter in artificial springwater [0.5 g of solid Forty Fathoms artificial seawater (Marine Enterprises, Towson, Md.) per liter of H_2O]. Five preparations were excluded because the S-axon was not severed (three) or the protease had obviously injured other cells (two).
13. K. J. Muller, S. A. Scott, B. E. Thomas, *Carnegie Inst. Washington Yearb.* **77**, 63 (1978); I. Parnas and D. Bowling, *Nature (London)* **370**, 626 (1977); K. J. Muller and S. A. Scott, in preparation.
14. M. B. Bunge, *J. Neurocytol.* **6**, 407 (1977).
15. G. D. Bittner and D. W. Mann, *Cell Tissue Res.* **169**, 301 (1976); E. X. Albuquerque, S. S. Deshpande, L. Guth, *Exp. Neurol.* **62**, 347 (1978).
16. H. Gainer, I. Tasaki, R. J. Lasek, *J. Cell Biol.* **74**, 524 (1977).
17. We thank B. Thomas for technical assistance and J. Brown and D. Fambrough for helpful discussions. Lucifer yellow was a gift from W. Stewart. Supported in part by a National Institutes of Health fellowship to S.A.S. and NIH grant NS 15014.
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Effect of β-Endorphin on Calcium Uptake in the Brain

Abstract. *The uptake of $^{45}Ca^{2+}$ by nerve-ending fractions from brains of mice was inhibited in vitro by 10^{-9}M concentrations of β-endorphin and in mice injected intraventricularly with 7 picomoles of β-endorphin. That the effect was a specific opiate agonist response of β-endorphin was demonstrated by use of the opiate antagonist, naloxone, which reversed the action. A role for β-endorphin in the regulation of calcium flux and neurotransmitter release should be considered.*

Ever since the discovery of peptides in the brain and pituitary that have opiate-like activity [the enkephalins (*1*) and β-endorphin (*2*)], attempts have been made to determine their physiologic role by comparing their pharmacologic effects with the effects of opiate drugs, usually morphine. Of the native peptides that have been isolated, β-endorphin appears most to resemble morphine with respect to its pharmacologic profile. β-Endorphin is active as an analgesic, being 20 to 30 times more potent than morphine when injected into the lateral ventricle and three to four times more potent when administered intravenously (*3*). Furthermore, sustained infusion of β-endorphin into the periaqueductal gray region, one of the most sensitive sites in the brain to opiates, produces opiate-like dependent behavior (*4*), and cross-tolerance between β-endorphin and morphine has been demonstrated (*5*).

Perhaps one of the most important effects of opiates is their ability to inhibit neurotransmitter release; this response may be related to changes in calcium ion flux. It has been demonstrated, for example, that morphine decreases acetylcholine (*6*) and norepinephrine release (*7*). Recently, the release-inhibiting effect of morphine on acetylcholine has been reported to be antagonized by calcium (*8*). Calcium also antagonizes the analgesic action of morphine and this effect can be enhanced by manipulations that increase brain membrane permeability to calcium and can be reversed by decreasing calcium availability (*9*). There is now evidence that a single dose of opiate effects a decrease in calcium content in nerve-ending fractions of brain homogenates (synaptosomes) (*10*), and that this decrease is dependent in part on reduced calcium binding (*11*) and uptake (*12*). In contrast, the changes in calcium disposition are reversed after sustained morphine administration, and the development of tolerance and physical dependence, that is, synaptosomal calcium uptake and binding, are increased (*10–12*). In view of these considerations, we deemed it important to study the effect of β-endorphin on synaptosomal calcium uptake.

Synaptosomes were prepared from homogenates of whole brains of male CD1 mice (21 to 25 g) according to a modified method described by Cotman and Matthews (*13*). Portions (1 ml) of a synaptosomal suspension were used for the determination of $^{45}Ca^{2+}$ uptake in the presence and absence of β-endorphin in vitro and in vivo. The suspensions were allowed to stand at 30°C for 2 minutes, then we added 1 ml of $^{45}Ca^{2+}$ [specific activity 0.05 mCi/mg in a solution containing 0.1 m*M* $CaCl_2$ (final concentration)], 3 m*M* $MgCl_2$, 3 m*M* adenosine triphosphate (ATP), disodium salt, and 50 m*M* tris buffer, *p*H 7.5. At fixed intervals thereafter (0.5, 1, 2, 4, 6, and 10 minutes), the Ca^{2+} uptake was terminated by separation of the synaptosomes from the incubation medium by rapid filtration through Millipore HAW (0.45 μm pore size) and washing three times with cold "stopping" solution (100 m*M* NaCl, 3 m*M* $MgCl_2$, 0.1 m*M* $CaCl_2$, and 50 m*M* tris, *p*H 7.5). The filters with the separated synaptosomes were transferred to glass counting vials containing 10 ml of scintillation solution (*14*), and the $^{45}Ca^{2+}$ present was determined by liquid scintillation spectrometry. Other portions of the synaptosomal suspension were used for the estimation of protein and calcium content. The amount of protein from synaptosomes was determined by the method of Lowry *et al.* (*15*) with bovine

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serum albumin being used as the standard. The results are expressed as micromoles of $^{45}Ca^{2+}$ per milligram of protein. Synaptosomal Ca^{2+} content was determined directly on diluted samples by atomic absorption spectrometry (*16*). The synaptosomes in sucrose suspension were sedimented by centrifugation and the resulting pellet was resuspended by vortexing with 1.5 ml of deionized distilled water. Portions (0.5 ml) were mixed with an equal volume of 2 percent lanthanum oxide previously dissolved in 10 percent of HCl (weight to volume) and assayed for Ca^{2+}. All determinations were made in duplicate.

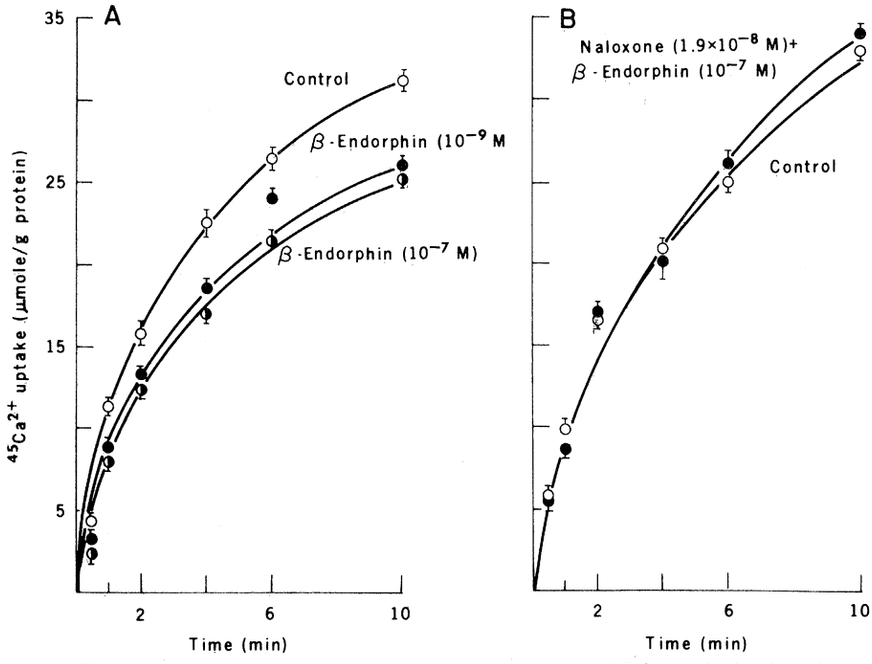

Fig. 1. The effect of β-endorphin and naloxone on synaptosomal $^{45}Ca^{2+}$ uptake in vitro. (A) β-endorphin, dissolved in sucrose buffer, was added to synaptosomes prepared from whole mouse brain homogenates 5 minutes prior to the addition of the $^{45}Ca^{2+}$ solution. (B) Naloxone was added immediately before the β-endorphin. The Ca^{2+} uptake reaction was stopped by separating the synaptosomes from the incubation medium by rapid filtration and washing with cold buffer solution.

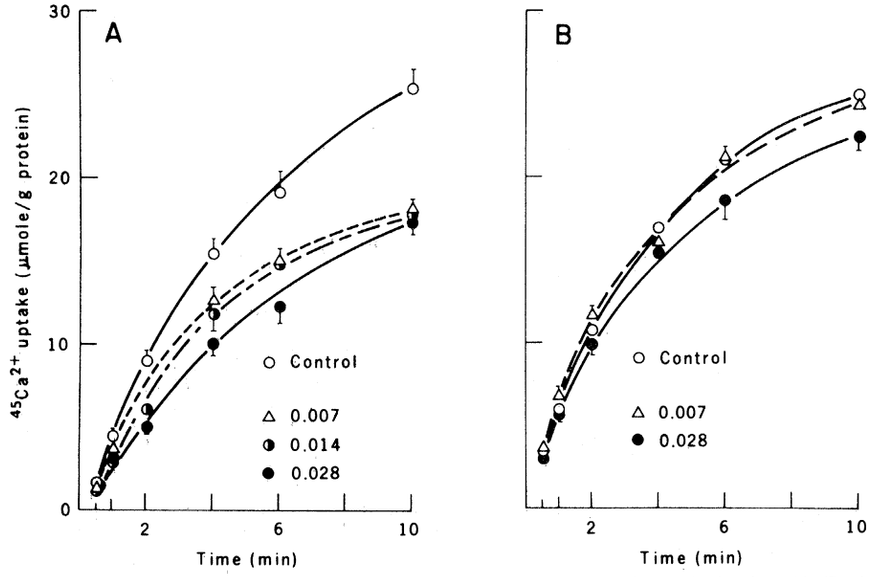

Fig. 2. The effect of β-endorphin and naloxone on synaptosomal $^{45}Ca^{2+}$ uptake in vivo. (A) Each mouse received an intracerebroventricular injection of 0.007, 0.014, or 0.028 nmole of β-endorphin (25, 50, and 100 ng, respectively) per mouse, with 9 percent NaCl being used as the vehicle. Control mice received the vehicle only. (B) Naloxone (2 mg/kg) was injected subcutaneously 10 minutes before and 20 minutes after the intraventricular injection of 0.007 or 0.028 nmole of β-endorphin. The mice were killed 30 minutes after β-endorphin administration and synaptosomes were prepared from whole-brain homogenates for determination of $^{45}Ca^{2+}$ uptake. Each point represents the mean of three determinations ± standard error.

β-Endorphin at a concentration of $10^{-9}M$ reduced significantly the $^{45}Ca^{2+}$ uptake; this concentration is 100 times lower than the concentration of morphine required to block uptake (*12*). The effect was near maximum, since increasing the concentration of β-endorphin to $10^{-7}M$ increased the inhibition of $^{45}Ca^{2+}$ by an amount that, compared to the inhibition by $10^{-9}M$ $^{45}Ca^{2+}$, was not statistically significant. The β-endorphin action on $^{45}Ca^{2+}$ synaptosomal uptake was consistently reproducible, although the absolute amount of $^{45}Ca^{2+}$ taken up by the synaptosomes from one set of experiments to another may vary as much as 30 percent. The results of a representative series of experiments are shown in Fig. 1. The effect shown in Fig. 1A was noticeable as early as 1 minute. The specificity of opiate agonist action is indicated by the fact that the inhibition of $^{45}Ca^{2+}$ uptake by β-endorphin was reversed by naloxone. When $10^{-7}M$ β-endorphin was incubated with the synaptosomes in the presence of $1.9 \times 10^{-8}M$ naloxone, the initial and maximum $^{45}Ca^{2+}$ uptake was nearly identical to that of the control group. Naloxone by itself did not affect the synaptosomal $^{45}Ca^{2+}$ uptake (data not shown).

To demonstrate the effect of β-endorphin on synaptosomal Ca^{2+} uptake in vivo, we injected unanesthetized mice intracerebrally with 0.007, 0.014, or 0.028 nmole of β-endorphin (25, 50, or 100 ng in 5 μl of 0.9 percent saline). That these doses produced significant analgesia after 10, 20, or 30 minutes was established by the tail-flick procedure. Immediately after the last interval, the mice were killed and the uptake of $^{45}Ca^{2+}$ was determined. As shown in Fig. 2A, 0.007 nmole of β-endorphin per mouse decreased the synaptosomal $^{45}Ca^{2+}$ uptake by about 30 percent and the effect was apparent within 2 minutes. Although the effect was dose-dependent, the changes in response to increasing doses of β-endorphin became less apparent when maximum inhibition was attained at 10 minutes. Administration of naloxone (2 mg/kg) subcutaneously antagonized the blockade of $^{45}Ca^{2+}$ uptake induced by the low dose of β-endorphin (0.007 nmole per mouse) and greatly reduced the response of a fourfold higher dose (see Fig. 2B). Furthermore, the inhibitory effect of β-endorphin on $^{45}Ca^{2+}$ uptake was reflected by a decrease in synaptosomal Ca^{2+} content. Both doses of β-endorphin decreased significantly the total calcium

concentrations by about 20 percent (control concentrations were 0.023 μmole per milligram of protein). The magnitude of the change observed was compatible with the decrease in $^{45}Ca^{2+}$ uptake obtained in the earlier experiments.

We then studied the effects of tolerance development on synaptosomal Ca^{2+} uptake. Male Sprague-Dawley rats (150 to 200 g) were rendered tolerant by the repeated intraventricular injection of a relatively high dose of β-endorphin. The rats, housed two to a cage, were anesthetized with ether and a stainless steel cannula was implanted in each in the fourth ventricle. Twenty-four hours later they were divided into three groups and given one of three treatments twice daily for three successive days: 5 ml of physiologic saline; 9.84 μg of morphine sulfate in 0.9 percent saline, or 9.0 μg of β-endorphin in saline. The dose of each opiate produced catalepsy that diminished with each injection and was absent after the sixth and final injection. The animals were killed 30 minutes after the last injection and synaptosomal uptake of $^{45}Ca^{2+}$ was determined.

The results indicate that the development of tolerance to β-endorphin is accompanied by enhanced synaptosomal $^{45}Ca^{2+}$ uptake. The mean uptake (± standard error) by the control groups for 10 minutes was found to be 23.9 ± 0.34 μmole per gram of protein, whereas that of the β-endorphin–treated group was 32.2 ± 0.47 μmole/g. The morphine-treated group likewise exhibited increased $^{45}Ca^{2+}$ uptake with a value of 28.9 ± 0.45 μmole per gram of protein ($P < .01$).

These data are consistent with our previous findings that morphine decreases the synaptosomal uptake of $^{45}Ca^{2+}$ prior to the development of tolerance and increases uptake when tolerance has developed (*12*). These effects are mimicked by β-endorphin and the similarities between β-endorphin and morphine suggest that β-endorphin may participate in the regulation of calcium flux and neurotransmitter release. Although the relative changes effected by β-endorphin in $^{45}Ca^{2+}$ uptake by the synaptosomes were modest, this is not surprising considering that the experiments were performed on synaptosomes derived from whole-brain homogenates. Subcellular studies indicate that long-term morphine administration increases the binding of calcium in synaptic vesicles and increases in Mg^{2+}-dependent adenosine triphosphatase activity in the same organelle (*11*, *17*). Data showing that the secretion of neurotransmitters caused by excitation is coupled with entry of Ca^{2+} (*18*) and that vesicular Mg^{2+}-dependent adenosine triphosphatase may be involved (*19*) support the concept that β-endorphin may inhibit the release of transmitters by inhibition of Ca^{2+} influx in the manner reported for morphine (*8*).

Since Ca^{2+} is necessary for the release of the enkephalins (*2*), it is reasonable to suppose that this should also hold true for the endorphins. Thus, it appears that Ca^{2+} and brain opiate peptides are interdependent and may function as part of a common system in regulating each other and the release of neurotransmitters (or neurohormones).

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References and Notes

J. Hughes, T. W. Smith, H. W. Kosterlitz, L. A. Fothergill, B. A. Morgaw, H. R. Morris, *Nature (London)* **258**, 577 (1975).
C. H. Li and D. Chung, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 1145 (19 → A. F. Bradbury, D. G. Smyth, C. R. Snell, N. J. M. Birdsall, E. C. Halme, *Nature (London)* **260**, 793 (1976).
H. H. Loh, L. F. Tseng, E. T. Wei, C. H. Li, *Proc. Natl. Acad. Sci. U.S.A.* **73**, 2895 (1976);
C. H. Li, D. Yamashiro, L. F. Tseng, H. H. Loh, *J. Med. Chem.* **20**, 325 (1977).
E. Wei and H. H. Loh, *Science* **193**, 1262 (1976).
L. F. Tseng, H. H. Loh, C. H. Li, *Biochem. Biophys. Res. Commun.* **74**, 390 (1977); J. P. Huidobro-Toro and E. L. Way, *Eur. J. Pharmacol.*, in press.
W. Schaumann, *Br. J. Pharmacol. Chemother.* **12**, 115 (1957); W. D. M. Paton, *ibid.*, p. → T. L. Yaksh and H. I. Yamamura, *Brain Res.* **83**, 520 (1975); K. Jhamandas, J. W. Phillis, C. Pinsky, *Br. J. Pharmacol.* **43**, 53 (1971).
G. Henderson, J. Hughes, H. W. Kosterlitz, *Br. J. Pharmacol.* **46**, 764 (19 → S. Arbilla and S. Langer, *Nature (London)* **271**, 559 (1978).
→ G. Sanfacon, M. Houde Depuis, R. Vanier, G. Labrecque, *J. Neurochem.* **28**, 381 (19 → K. Jhamandas, J. Sawynok, M. Sutak, *Eur. J. Pharmacol.* **49**, 309 (1978).
T. Kakunaga, H. Kaneto, K. Hano, *J. Pharmacol. Exp. Ther.* **153**, 134 (1966); R. A. Harris, H. H. Loh, E. L. Way, *ibid.* **195**, 488 (1975).
R. A. Harris, H. Yamamoto, H. H. Loh, E. L. Way, in *Opiates and Endogenous Peptides*, H. W. Kosterlitz, Ed. (Elsevier, Amsterdam, 1976), p. 361; H. L. Cardenas and D. H. Ross, *Br. J. Pharmacol.* **57**, 521 (1976).
→ R. A. Harris, H. Yamamoto, H. H. Loh, E. L. Way, *Life Sci.* **20**, 501 (1977); H. Yamamoto, R. A. Harris, H. H. Loh, E. L. Way, *J. Pharmacol. Exp. Ther.* **205**, 255 (1978).
K. V. Cerreta, F. Guerrero-Munoz, E. L. Way, *Pharmacologist*, **96**, 143 (1977); F. Guerrero-Munoz, K. V. Cerreta, E. L. Way, *ibid.*, p. 143; F. Guerrero-Munoz, K. V. Cerreta, M. L. Guerrero, E. L. Way, *J. Pharmacol. Exp. Ther.* **209**, 132 (1979).
→ C. W. Cotman and D. A. Matthews, *Biochim. Biophys. Acta* **249**, 380 (19 → J. W. Gurd, A. R. Jones, H. R. Mohler, W. J. Moore, *J. Neurochem.* **22**, 281 (1974). Synaptosomes were prepared from whole-brain homogenates (ten brains per tube diluted with ten volumes of 0.32*M* sucrose, 5 m*M* Hepes buffer, *p*H 7.5) by density gradient centrifugation on Ficoll. The synaptosomes in the crude mitochondrial fraction were purified by centrifugation at 65,000*g* for 50 minutes. The material in the region between 7.5 and 12 percent Ficoll was removed for recentrifugation at 110,000*g* for 30 minutes. The resulting pellet was separated and resuspended in 10 ml of 0.32*M* sucrose.
The scintillation fluid consisted of 2,5-diphenyloxazole (PPO; 18 g) and 1,4-bis[2-4-methyl-5-phenyloxazolyl]benzene (POPOP; 0.5 g) dissolved in ethylene glycol monomethyl ether (750 ml) and toluene (2250 ml).
O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951).
W. K. Schmidt and E. L. Way, *J. Neurochem.* **32**, 1095 (1979).
H. Yamamoto, R. A. Harris, H. H. Loh, E. L. Way, *Life Sci.* **20**, 1533 (1977).
R. P. Rubin, *Calcium and the Secretory Process* (Plenum, New York, 1974), p. 25.
A. M. Poisner and J. M. Trifaro, *Mol. Pharmacol.* **3**, 561 (1967); J. M. Trifaro and A. M. Poisner, *ibid.*, p. 572.
G. Henderson, J. Hughes, H. W. Kosterlitz, *Nature (London)*, **271**, 677 (19 → L. L. Iversen, S. D. Iversen, F. E. Bloom, T. Vargo, R. Guillemin, *Nature (London)* **271**, 679 (1978).
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Long-Term Analgesic Effects of Inescapable Shock and Learned Helplessness

Abstract. *Although exposure to inescapable shocks induced analgesia in rats, the analgesia was not manifest 24 hours later. A brief reexposure to shock, however, restored the analgesia. This reexposure to shock had an analgesic effect only if the rats had been shocked 24 hours previously. Further, long-term analgesic effects depended on the controllability of the original shocks and not on shock exposure per se. Implications of these results for learned helplessness and stress-induced analgesia are discussed.*

Whether or not an organism can control aversive events has widespread behavioral and physiological consequences. Exposure to inescapable, unavoidable shocks leads to subsequent failure to learn to escape in a different situation, decrements in shock-elicited aggression, and severe stress symptomology. None of these follow exposure to controllable (escapable) aversive events (*1*). Of the various consequences of exposure to uncontrollable aversive events, the greatest empirical and theoretical attention has been given to interference with subsequent escape learning, termed the learned helplessness effect (*1*). The mechanism or mechanisms producing it are a matter of controversy, however (*2*). Under the learned helplessness hypothesis (*1*), an organism exposed to uncon-

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