defective REV vectors have titers reported to be a hundred times that of ME111 (*29*), injection of embryos with replication-defective REV vector could provide a very efficient means of producing germline mosaic chickens. Defective retroviral vectors derived from other viruses such as avian leukosis virus might also be effective (*30*). The progeny of such mosaics could carry proviral insertions at many different sites within the genome.

The embryos used for these studies contained at least 10^4 cells, some of which formed the epiblast at the outer surface of the blastoderm (*31*). Primordial germ cells reside in this layer of cells, but are indistinguishable from somatic stem cells before migrating to the germinal crescent (*19–22*). Our experiments show that injection of the nonreplicating REV vector ME111 beneath the unincubated chicken embryo blastoderm resulted in infection of precursors to both blood and semen. Analysis of blood DNA from progeny of G_0 birds with vector-positive semen confirmed germline transfer of vector sequences. Since replicating helper virus was not detected in the G_0 mosaics used for breeding, nor in their G_1 progeny, the vector sequences present in these animals resulted from infection immediately following injection of the G_0 embryos. This approach provides a way to study cell lineage relations during differentiation (*32, 33*) and vector-mediated gene expression. Chicken embryo epiblast cells have been cultured in vitro (*34*), and generation of chicken chimeras has been accomplished by injecting stem cells into the blastocoel of recipient embryos (*35*). The susceptibility of germline stem cells to REV infection in vivo suggests that a similar approach might be used on stem cells cultured in vitro, paralleling work with murine stem cell lines used to generate transgenic mice (*15*).

Our results identify the unincubated chicken embryo as a source of germline stem cells susceptible to infection by REV vectors and demonstrate the first use of replication-defective REV vectors to transfer heritable, nonviral, genetic information into the chicken germline. The ease and efficiency of this procedure provide both researchers and commercial breeders with a practical method for genetic manipulation of the chicken.

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24. Before injection, freshly laid eggs were held horizontally with respect to their long axis for 5 hours or more at 18° to 20°C so that the blastoderm would lie just beneath the topmost area of the shell. Egg shells were wiped with 70% ethanol before and after a 5- to 8-mm hole was made in the shell with a Dremel (Model 280-5) moto-tool fitted with an aluminum oxide grinding stone (Dremel #924). The shell membrane was removed with a scalpel just before injection. Injection was performed with a Narishige micromanipulator and a Drummond 100-μl digital microdispenser fitted with a glass needle of 50- to 60-μm outer diameter. Needles were pulled using a Kopf Model 720 vertical pipette puller. Ten microliters of an overnight harvest of cell culture media containing ME111 (~10^4 TKTU/ml) were injected beneath the surface of the exposed blastoderm. Injection was monitored using a Wild M5A dissecting microscope. Eggs were resealed with a patch of shell membrane placed over the hole and allowed to dry. The patch was then covered with Devcon Duco cement. Eggs were placed at 37.8°C in a Natureform incubator and allowed to hatch.
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36. We thank H. Temin for providing the C3 helper cells, M. Emerman for advice on the ME111 vector, N. Davidson for continuing interest in this work, A. Berk for critical reading of the manuscript, and J. Bennett and J. Heuston for manuscript preparation.

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Selective Loss of Hippocampal Granule Cells in the Mature Rat Brain after Adrenalectomy

ROBERT S. SLOVITER,* GUY VALIQUETTE, GARY M. ABRAMS, EDWARD C. RONK, ANNE L. SOLLAS, LINDA A. PAUL, SIMON NEUBORT

Adrenalectomy of adult male rats resulted in a nearly complete loss of hippocampal granule cells 3 to 4 months after surgery. Nissl and immunocytochemical staining of hippocampal neurons revealed that the granule cell loss was selective; there was no apparent loss of hippocampal pyramidal cells or of γ-amino butyric acid (GABA)–, somatostatin-, neuropeptide Y–, calcium binding protein–, or parvalbumin-containing hippocampal interneurons. The hippocampal CA1 pyramidal cells of adrenalectomized animals exhibited normal electrophysiological responses to afferent stimulation, whereas responses evoked in the dentate gyrus were severely attenuated. Corticosterone replacement prevented both the adrenalectomy-induced granule cell loss and the attenuated physiological response. Thus, the adrenal glands play a role in maintaining the structural integrity of the normal adult brain.

ALTHOUGH HORMONES INFLUENCE the survival of neurons in the developing brain (*1*), none have been shown to be necessary for maintenance of the structural integrity of the mature brain. The hippocampus, a brain region involved in learning, memory, and a number of neurological disease states (*2, 3*), is a target of adrenal steroids (*4*) and is thought to play a role in the endocrine functions of the adrenal-hypothalamic-pituitary axis (*5*). Adrenal hormones may exacerbate neurotoxic insults to the hippocampus (*6*), and adrenalectomy can protect hippocampal pyramidal cells

R. S. Sloviter, Neurology Research Center, Helen Hayes Hospital, New York State Department of Health, West Haverstraw, NY 10993, and Departments of Pharmacology and Neurology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.
G. Valiquette and G. M. Abrams, Neurology Research Center, Helen Hayes Hospital, West Haverstraw, NY 10993, and Department of Neurology, Columbia University, New York, NY 10032.
E. C. Ronk, A. L. Sollas, L. A. Paul, S. Neubort, Neurology Research Center, Helen Hayes Hospital, West Haverstraw, NY 10993.

*To whom correspondence should be addressed.

from age-related loss (7). In an initial study on the effect of adrenal hormones on hippocampal structure and function, we discovered unexpectedly that adrenalectomy of adult rats results in a selective loss of hippocampal granule cells.

Adult male Long-Evans rats were adrenalectomized (ADX) (n = 52) and given free access to food and a 0.9% NaCl solution. To prevent death that occurs in many animals during the 3-week period after adrenalectomy, corticosterone was added to the drinking solution of all animals during this period (*8*). As controls we used naïve and sham-operated, age-matched animals. The efficacy of surgery was evaluated by the measurement of serum corticosterone concentrations by radioimmunoassay (*9*). Randomly chosen subsets of sham-operated and ADX animals remained on corticosterone (20 μg per milliliter of drinking solution) for the duration of the experiment to determine the effect of corticosterone replacement. At various intervals after adrenalectomy, brain tissue from experimental and control animals was evaluated with Nissl and immunocytochemical stains (*10, 11*).

Nissl staining revealed a selective, bilateral loss of hippocampal granule cells in ADX animals, while immediately adjacent pyramidal cells appeared normal (Fig. 1). This granule cell loss was nearly complete throughout the septal and middle thirds of the hippocampus 3 to 4 months after adrenalectomy and was always greater in these areas than in the temporal third. In the example in Fig. 1B, the area of the dentate gyrus (molecular layer, granule cell layer, and hilus) was approximately 30% of that in control animals. At earlier survival periods, cell loss was variable and incomplete. In addition, granule cell loss appeared first in the inner (dorsal) blade of granule cells and always at the most lateral end of the blade. Hippocampal structure appeared normal in ADX animals given corticosterone (Fig. 1A) (n = 14), sham-operated controls (n = 8), and naïve animals (n = 8). Brain structure outside the hippocampus appeared normal in ADX animals although the possibility of less obvious cell loss in nonlaminar brain regions or at longer survival times cannot be excluded on the basis of this initial qualitative analysis.

We then used immunocytochemistry to visualize specific hippocampal cell populations (*11, 12*). Calcium binding protein (CaBP) is present normally in hippocampal granule cells, CA1 pyramidal cells, and a variety of interneurons (Fig. 2A) (*12*). After adrenalectomy, CaBP-like immunoreactivity (LI) was undetectable in the granule cell region throughout the rostral two-thirds of the hippocampus (Fig. 2B). However, the CaBP immunoreactive CA1 pyramidal cells and interneurons of all hippocampal regions appeared similarly stained in ADX and control animals. Hippocampal GABA-, somatostatin-, neuropeptide Y–, and parvalbumin-immunoreactive interneurons were present in their normal locations (*11*), despite the near total loss of the dentate granule cells (Fig. 2, C through F). The axosomatic plexus of inhibitory axon terminals formed by the GABA-immunoreactive neurons still surrounded surviving granule cells or presumed hilar mossy cells (Fig. 2D), and the somatostatin-immunoreactive plexus, normally present in the outer dentate molecular layer, was evident despite the loss of granule cells and shrinkage of the molecular layer (Fig. 2F). Thus, the effect of adrenalectomy on hippocampal granule cells is selective.

Electrophysiological experiments were performed on ADX and control animals to identify the physiological changes that accompany the loss of granule cells and to determine if the pyramidal cells, which appeared normal, responded normally to afferent stimulation. We used evoked potentials in response to perforant path stimulation as an index of hippocampal functional activity (*13*). ADX rats, ADX and corticosterone-treated rats, sham-operated control rats, and normal rats were anesthetized with ether and given the anesthetic urethane (1.25 g/kg, intraperitoneally). Animals were placed in a stereotactic device with a stimulating electrode in the perforant path and a glass recording electrode filled with 4*M* NaCl in the cortex above the hippocampus (*13*). Potentials evoked by perforant path stimulation were recorded at different depths within the hippocampus to sample the activity evoked from different hippocampal cell populations.

Compared to corticosterone-treated ADX rats, sham-operated rats, and normal controls, ADX rats (n = 5) exhibited abnormal responses (Fig. 3). Whereas normal potentials were evoked from CA1 pyramidal cells, granule cell responses to the same afferent stimuli were absent despite verification that the recording electrode tip was in the area dentata (Fig. 1B, asterisk). Corticosterone-treated ADX animals and sham-operated rats, all of which exhibited normal hippocampal morphology, appeared normal physiologically (n = 5).

Not all rats that underwent adrenalecto-

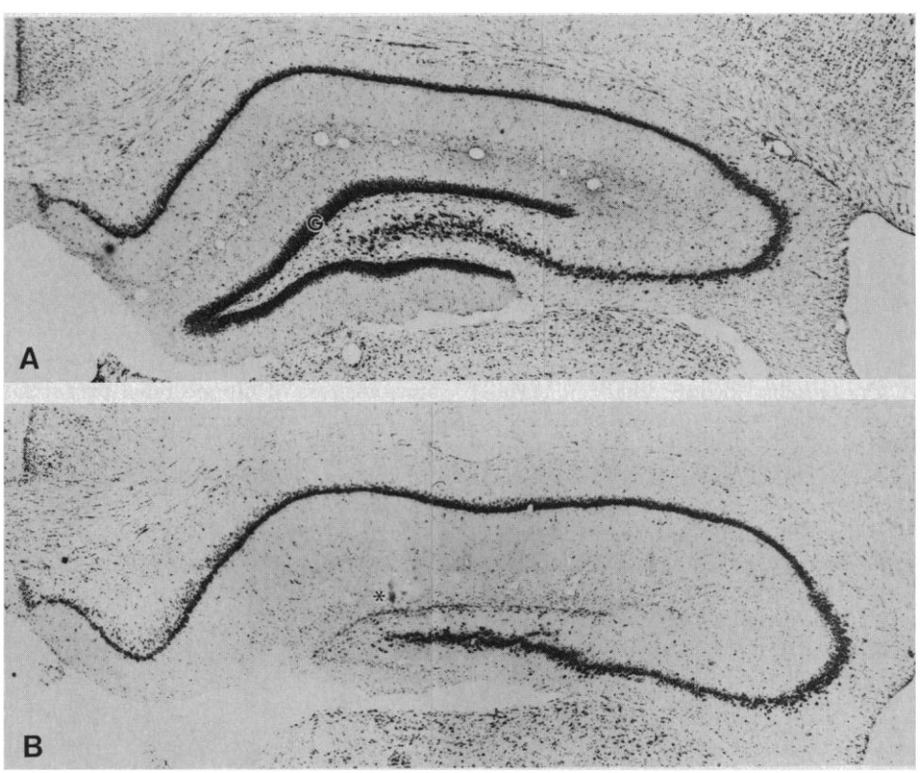

Fig. 1. Nissl-stained hippocampus from (A) ADX, corticosterone-treated and (B) ADX rats. (**A**) Normal morphology in an ADX animal (ADX-6) given corticosterone for 4 months after adrenalectomy. The granule cell layer (G) contains the principal cells of the dentate gyrus. (**B**) ADX rat ADX-14, 4 months after adrenalectomy. Note the selective loss of the granule cells and survival of other hippocampal neurons. The area of the dentate gyrus (molecular layer, granule cell layer, and hilus) in (B) is approximately 30% of that in (A). The asterisk in (B) marks the recording electrode track showing the location at which the potentials in Fig. 3D were recorded. Fixation was as in (*10*). Magnification, ×94.

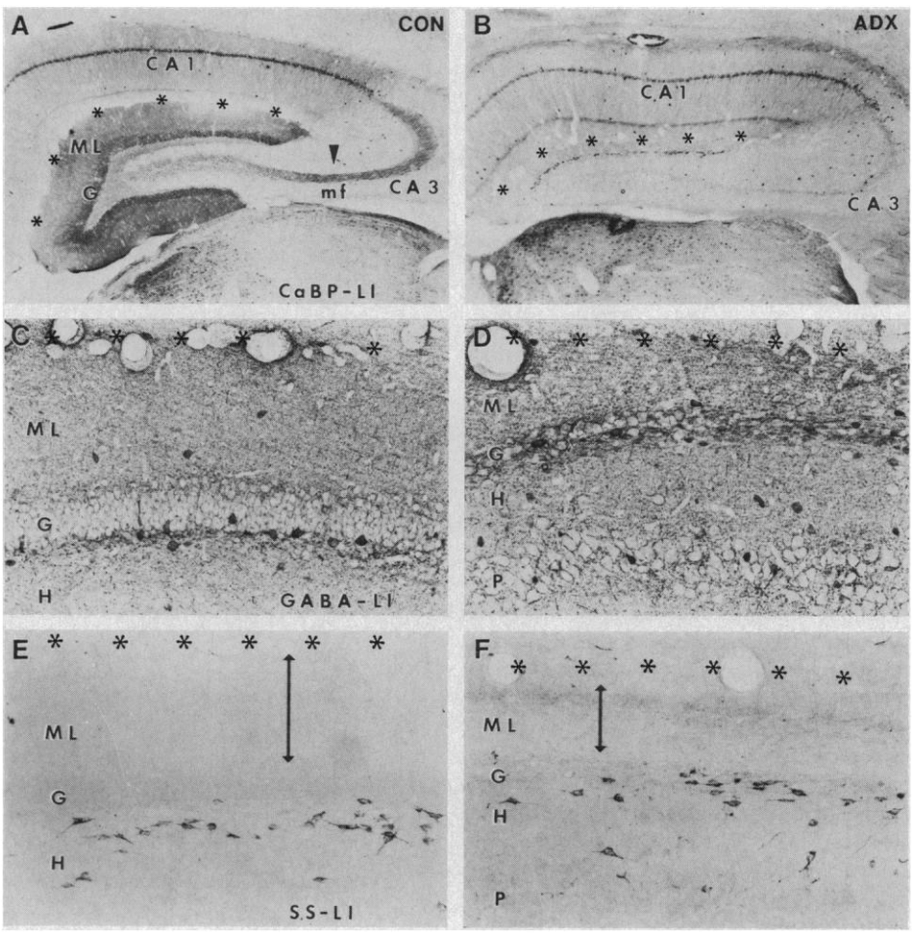

Fig. 2. CaBP-, GABA-, and somatostatin (SS)-like immunoreactivity (LI) in corticosterone-treated and -untreated ADX rats. (**A**) CaBP-LI in corticosterone-treated, ADX animal ADX-6 (Fig. 1A). Note the darkly stained granule somata (G) and dendrites in the molecular layer (ML), their axon collaterals beneath the granule cell layer, and their stained axon bundle, the mossy fibers (mf), which innervate area CA3. Also note the stained CA1 pyramidal cells and the location of the hippocampal fissure (asterisk), which is the border between the area dentata and hippocampal area CA1. The pattern of staining in corticosterone-treated, ADX rats was indistinguishable from normal animals processed simultaneously. (**B**) ADX animal ADX-14 (Fig. 1B). Note the loss of dentate and mossy fiber staining, and shrinkage of the molecular layer, which extends the distance from the hippocampal fissure (asterisk) to the few remaining stained granule cells or interneurons. The difference in the darkness of staining of area CA1 in (A) and (B) is neither consistent nor meaningful. (**C**) Normal pattern of GABA-LI in area dentata in corticosterone-treated animal ADX-6 [which includes the molecular layer, granule cell layer, and hilus (H)]. Note unstained granule cells surrounded by GABA-immunoreactive fibers and darkly stained GABA-immunoreactive neurons in all layers. (**D**) Rat ADX-14. Note loss of granule cells and shrinkage of molecular layer, but survival of GABA-immunoreactive neurons and fibers. P, CA3 pyramidal cell layer. (**E**) SS-immunoreactive dentate neurons in corticosterone-replaced rat ADX-6. (**F**) SS-LI in ADX rat ADX-14. Note survival of hilar SS-immunoreactive cells despite loss of adjacent granule cells. Similar results were obtained with antisera raised against parvalbumin and neuropeptide Y (not shown). Arrows denote the width of the molecular layer. Magnifications, ×72 in (A) and (B); ×359 in (C) through (F).

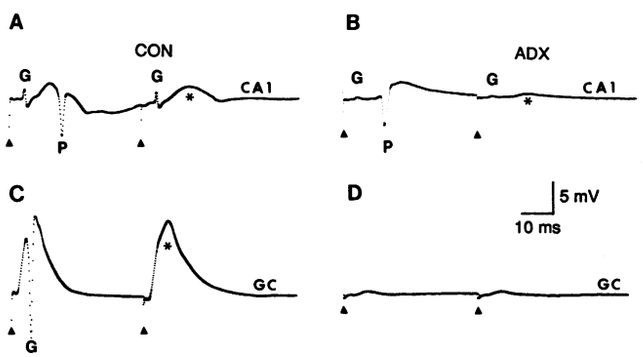

Fig. 3. Hippocampal responses to twin pulse perforant path stimulation in normal and ADX rats. (**A**) Control (CON) evoked potentials recorded in the CA1 pyramidal cell layer. Perforant path stimulation at 8 Hz evokes granule cell population spikes (G) recorded in CA1 as positive spikes. The CA1 pyramidal cell population spike (P) follows the granule cell spike. The response to twin pulses reflects the state of inhibition (*13*). Note that the pyramidal cell spike evoked by the first stimulus (arrowheads denote the stimulus artifacts) prevents the pyramidal cells from discharging in response to an identical second stimulus (the asterisk denotes the absent spike). This illustrates the presence of inhibition in the normal animal. (**B**) The response recorded in area dentata of ADX rat ADX-14 to identical stimuli as used in (A). (**C**) Normal (CON) granule cell response recorded in the granule cell layer to twin pulse perforant path stimulation at 2 Hz. (**D**) The evoked potential recorded in the area dentata of rat ADX-14. Same stimulus parameters as in (C). The location of the electrode tip in what remained of the granule cell layer was verified histologically (Fig. 1B, asterisk). Positivity up.

my exhibited hippocampal damage (18 of 52 rats exhibited no apparent cell loss). Investigation of the source of this variability revealed that, in many cases, adrenalectomy may have been incomplete despite undetectable serum corticosterone concentrations. Two factors, well known in clinical adrenal insufficiency, were important in this regard: body weight and serum sodium and potassium concentrations. One subgroup of ADX animals gained weight more slowly than control animals and exhibited lowered serum sodium and elevated potassium (*14*). These animals showed granule cell loss. The other subgroup exhibited normal weight gain, normal sodium and potassium levels, and no granule cell loss (*14*). Because human adrenocortical insufficiency causes weight loss, sodium wasting, and potassium retention (*15*), it seems likely that these latter animals were not completely adrenalectomized. The possibility that rats with biologically significant adrenal function can still exhibit undetectable serum corticosterone was supported by the results of corticosterone measurements in ADX animals given the hormone in their drinking solution.

Rats that had been ADX were given free access to 0.9% NaCl-containing corticosterone to determine if hormone treatment would prevent the hippocampal cell loss. Although these animals drank the solution, gained weight normally, and exhibited normal serum sodium and potassium concentrations (*16*) and no hippocampal damage, they still had undetectable serum corticosterone. To test the possibility that corticosterone was undetectable because of rapid metabolism or rapid distribution into hormone-deficient tissues, we removed the drinking solution during the night and made it available in the morning. Blood samples were taken 1 to 2 hours later and were found to contain measurable serum corticosterone concentrations (*16*). Thus, we tentatively concluded that the absence of hippocampal

damage in some ADX animals was due to incomplete adrenalectomy or the presence of ectopic adrenal cells or other tissues that produce compounds with adrenal hormone activity.

Our finding that adrenalectomy selectively destroys granule cells is particularly unexpected because these cells are the hippocampal neurons most resistant to ischemic insult, hypoxia, or seizure activity (*3, 13*). The mechanism by which adrenalectomy causes the selective loss of hippocampal granule cells is unknown. The simplest explanation is that the granule cells need adrenal hormone receptor stimulation to remain viable. The possibility that granule cells die when their mineralocorticoid (Type I) or glucocorticoid (Type II) receptors (*4*) are not occupied by endogenous ligand is provocative. These cells may require a protein or other factor that is regulated by adrenal hormone receptor activation. Conversely, granule cell loss could be due to a process secondary to adrenalectomy, and the prevention of this loss by corticosterone may not be the result of a direct action of corticosterone within the brain.

Progress toward understanding the role of the hippocampus in normal learning and memory and in pathological conditions has been hampered by the difficulty in producing selective hippocampal lesions. Adrenalectomy may be a useful method to remove hippocampal granule cells selectively and then determine how learning, memory, and other functions are altered.

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8. Naive, ADX, and sham-operated male Long-Evans rats, weighing 150 to 174 g, were purchased from Harlan Sprague-Dawley. Animals were treated in accordance with the guidelines of the New York State Department of Health and the National Institutes of Health. In preliminary experiments, many ADX animals did not survive the 3-week period after surgery. Therefore, in this study all animals were given corticosterone (Sigma) (20 μg/ml) in the drinking solution (0.9% NaCl) starting within 1 week after surgery. After 1 week on hormone, the corticosterone concentration was halved and, after another week, halved again. After 3 weeks, corticosterone was removed from the drinking solution, and all animals survived.
9. Immediately before perfusion-fixation, 4 ml of blood was removed from the femoral vein under urethane anesthesia (1.25 g/kg, intraperitoneally). This sampling time was chosen to avoid the possibility that the loss of blood might cause ischemia that could contribute to the hippocampal damage. Corticosterone was measured in serum with a radioimmunoassay kit purchased from Radioimmunoassay Systems Laboratory, Carson, CA. All ADX animals used in this study exhibited undetectable corticosterone concentrations (less than 25 ng of serum per milliliter; $n = 52$). By comparison, sham-operated controls exhibited corticosterone concentrations within the normal stressed range (sham-operated: 313 ± 85 ng/ml, range: 205 to 410, $n = 4$; normal: 350 ± 50 ng/ml, range: 270 to 410, $n = 7$; means ± SD).
10. Rats were perfused through the heart with ice-cold 2% paraformaldehyde in 0.1*M* sodium acetate buffer, *p*H 6.5, for 3 min (no saline wash) followed by 30 min with ice-cold 2% paraformaldehyde and 0.1% glutaraldehyde in 0.1*M* sodium borate buffer, *p*H 8.5. After storage of the intact rat overnight at 4°C, the brain was removed and cut on a Vibratome. Sections 20-μm thick were mounted onto glass slides, dried, dehydrated, rehydrated, and stained with 0.75% cresyl violet. Alternate 50-μm sections of each brain were stained immunocytochemically (*11*).
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14. ADX rats that exhibited granule cell loss (ADX-loss) weighed less at the end of the experiment than undamaged ADX rats (ADX–no loss); ADX-loss: 409 ± 15 g, $n = 10$; ADX–no loss: 496 ± 15 g, $n = 10$; mean ± SEM, $P < 0.005$. Serum sodium and potassium concentrations were as follows. Sodium: ADX-loss, 136 ± 2 mmol/liter, $n = 6$; ADX–no loss: 145 ± 2 mmol/liter, $n = 3$; sham-operated: 145 ± 1 mmol/liter, $n = 4$; normal: 144 ± 1 mmol/liter, $n = 4$; $P < 0.001$, two-tailed t test; mean ± SD. Potassium: ADX-loss: 7.7 ± 0.5 mmol/liter, $n = 6$; ADX–no loss: 5.5 ± 0.7 mmol/liter, $n = 3$; sham-operated: 5.0 ± 0.4 mmol/liter, $n = 4$; normal: 5.0 ± 0.2 mmol/liter, $n = 4$; $P < 0.001$). Sodium and potassium were measured with a Technicon RA-1000 system.
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16. Corticosterone-treated ADX animals exhibited normal sodium and potassium concentrations (sodium, 143 ± 3 mmol/liter; potassium, 5.6 ± 0.6 mmol/liter, $n = 4$). One to 2 hours after drinking, detectable serum corticosterone was present (53 ± 21 ng/ml, $n = 6$).

17. We thank D. W. Dempster, J. H. Goodman, R. Lindsay, S. C. Rubin, R. K. S. Wong, and E. A. Zimmerman for useful discussions and criticism of the manuscript and M. Mukhtar of the Helen Hayes Hospital Clinical Laboratory for measuring sodium and potassium concentrations. Antisera raised in rabbits against CaBP or parvalbumin were generously provided by J. W. Pike and K. G. Baimbridge, respectively. A. J. Malcolm of the MRC Regulatory Peptide Group, Vancouver, Canada, provided monoclonal somatostatin antibody. Supported by an equipment gift from the Helen Hayes Hospital Citizen's Advisory Council and by NIH grant NS18201.

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Evidence That the Leucine Zipper Is a Coiled Coil

ERIN K. O'SHEA, RHEBA RUTKOWSKI, PETER S. KIM

Recently, a hypothetical structure called a leucine zipper was proposed that defines a new class of DNA binding proteins. The common feature of these proteins is a region spanning approximately 30 amino acids that contains a periodic repeat of leucines every seven residues. A peptide corresponding to the leucine zipper region of the yeast transcriptional activator GCN4 was synthesized and characterized. This peptide associates in the micromolar concentration range to form a very stable dimer of α helices with a parallel orientation. Although some features of the leucine zipper model are supported by our experimental data, the peptide has the characteristics of a coiled coil.

THE ESSENTIAL FEATURES OF THE proposed leucine zipper (*1*) are that the region containing the leucine repeat has a helical structure, that the leucines align along one face of the helix, and that interdigitation of the leucine side chains facilitates dimerization. The leucine repeat is found in several biologically interesting proteins (*1*) including two transcriptional regulators (yeast GCN4 and mammalian C/EBP) and three nuclear transforming proteins (Jun, Fos, and Myc). These DNA binding proteins do not contain recognizable helix-turn-helix (*2*) or zinc-finger (*3*) motifs. A 30-residue region immediately adjacent to the leucine repeat is required for specific DNA binding in both GCN4 (*4*) and C/EBP (*5*), suggesting that the proposed leucine zipper may have a fundamental role in arranging the DNA binding surface of these proteins (*1*).

We tested the leucine zipper model and began structural studies of these proteins by focusing on the leucine repeat itself. GCN4,

Whitehead Institute for Biomedical Research, Nine Cambridge Center, Cambridge, MA 02142, and Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139.