amount of plastic substrate that can be exploited for such learning purposes. According to this hypothesis the plastic substrate for vocal learning is renewed once yearly, a growing, then shedding of synapses, much the way trees grow leaves in the spring and shed them in the fall.

The shrinkage of brain nuclei in adulthood, resulting from a loss of dendritic processes, may be likened to a rejuvenating process that reduces the size of a network to an earlier developmental age. Of course, such a process can be labeled "rejuvenation" only if it is followed by a new wave of dendritic proliferation and synapse formation. If rejuvenation of brain circuitry ever becomes possible in humans, being able to induce a retraction of neurites may be found to be the indispensable first step, to be followed by their regrowth. We may now have an animal model for this kind of phenomenon.

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5. All work was done with males from the Rockefeller University Field Research Center closebred strain of Belgian Waserschlager canaries. These birds were kept indoors; light:dark schedules followed the photoperiod of central New York State.
6. The purpose of breeding these birds was to ensure that they would undergo the hormonal changes normally associated with reproductive maturity and the shift from spring to summer and fall condition.
7. Birds were killed by ether overdose.
8. Blood samples obtained from the right auricle were allowed to clot and retract overnight at 2°C. Serum was then separated by centrifugation and stored at −40°C until analysis. Plasma androgen (testosterone + probably dihydrotestosterone) levels were measured by radioimmunoassay (RIA) [V. L. Gay and J. T. Kerlan, *Arch. Androl.* **1**, 239 (1978); I. Lieberburg, L. C. Krey, B. S. McEwen, *Brain Res.* **178**, 207 (1979)].
9. Birds were perfused through the left ventricle with 0.9 percent saline followed by 10 percent Formalin in 0.9 percent saline. After perfusion, the testes were removed and placed in 10 percent Formalin in 0.9 percent saline. The head was placed in a stereotaxic head-holder, and the brain was blocked rostrally in the transverse plane with a knife so as to reproduce the plane of section of our canary atlas [T. C. Stokes, C. M. Leonard, F. Nottebohm, *J. Comp. Neurol.* **156**, 337 (1974)]. The brain was then removed from the skull and stored in 10 percent Formalin in 0.9 percent saline for 1 to 2 weeks, then transferred to a 30 percent sucrose in 10 percent Formalin solution for 2 to 4 days. After 24 hours in sucrose solution the brain sinks to the bottom of the jar, and it was then that brains were weighed. For weighing, brains were removed from the sucrose solution and gently placed on tissue paper to absorb excess wetness, then weighed in the same solution, including the rostral section that had been blocked off. After sucrose, the brains were placed in the gelatin-albumen embedding medium, where they remained for 2 to 4 weeks. Embedding and sectioning proceeded as described in the canary atlas mentioned above. Frozen sections were cut with a repeat interval of 50, 50, and 25 μm. One of the 50-μm series was collected sequentially into 50 percent ethyl alcohol, mounted on chrome-alum (chromium potassium sulfate) coated slides and stained with cresyl violet, a Nissl substance stain for cell bodies.
10. Volume of brain structures was reconstructed through the use of a microprojector and polar planimeter (*11,12*).
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13. F. Nottebohm, T. M. Stokes, C. M. Leonard, *J. Comp. Neurol.* **165**, 457 (1976). The volume of each right and left nucleus HVc and RA was reconstructed separately.
14. In the pigeon, Rt is part of the tectofugal visual pathway [H. J. Karten and A. M. Revzin, *Brain Res.* **2**, 368 (1966); A. M. Revzin and H. J. Karten, *ibid.* **3**, 264 (1966-1967)], and SpM receives input from the telencephalon and projects to the cerebellum [H. J. Karten and T. E. Finger, *ibid.* **102**, 335 (1976)]. In canaries and zebra finches, part of the telencephalic input to SpM may come from RA or from part of the archistriatum close to RA [F. Nottebohm, D. B. Kelley, J. A. Paton, unpublished observations; M. E. Gurney, thesis, California Institute of Technology (1980)]; if this projection comes in fact from RA, it is a small one. Nucleus SpM has not yet been studied in songbirds to ascertain its role, if any, in song control. For purposes of the present study only the volumes of the left Rt and left SpM were reconstructed. From earlier observations (*11,12*), we know that the right and left volumes of these nuclei are symmetrical. When normalizing the volume of HVc and RA, each bird's total (left + right) HVc and RA volume was divided by twice the volume of its left Rt.
15. To reconstruct the caudal forebrain volume at the level of HVc, we measured the summed area of three telencephalic sections. For each bird, one of these sections was the one that showed the largest cross section through HVc; the other two sections were taken, respectively, 200 μm more rostral and 200 μm more caudal. For each bird the right and left reconstructed volumes were added, obtaining a unitary value for caudal forebrain volume.
16. For each bird, the testicular weights used for obtaining means and standard deviations were gotten by adding the weights of the right and left testis: spring, 253.2 ± 41.9 mg; fall, 1.8 ± 0.9 mg. There was a parallel difference in serum androgen levels, as measured by RIA (*8*). The spring and fall androgen levels were, respectively, 1.65 ± 1.24 ng/ml and 0.13 ± 0.22 ng/ml [$t(19) = 4.21$, $P < .05$].
17. For right-left comparisons [Wilcoxon matched-pairs signed-ranks test, spring RA and HVc, $T(9) \geq 16$, $P > .05$; fall RA and HVc, $T(12) \geq 20$, $P > .05$].
18. Significance of seasonal differences for all brain measures listed in Table 1 was tested with two-tailed *t*-tests; significance was rejected at $P > .05$.
19. Table 1 shows that Rt may show seasonal changes in size. Thus, use of Rt volume to normalize each bird's HVc and RA values may be an overly stringent way of looking at the magnitude of seasonal changes in HVc and RA; it is reassuring that marked seasonal differences persist under those circumstances.
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23. Song "learning" and song "forgetting" are used here to refer not so much to the acquisition and loss of an auditory memory, but rather to the conversion of that memory into a motor program, with the consequent matching of an auditory model. The learning of such a motor program seems to require brain space (*12*).
24. Male zebra finches learn their single song during the first 3 months after hatching [K. Immelmann, in *Bird Vocalizations*, R. A. Hinde, Ed. (Cambridge Univ. Press, London, 1969), p. 61]. No new songs are learned in adulthood. In adult male zebra finches, the volume of HVc and RA shows no specific volume changes even many months after castration [A. P. Arnold, *Brain Res.* **185**, 441 (1980)]. Since the amount of singing in male zebra finches is testosterone-dependent [E. Pröve, *J. Ornithol.* **115**, 338 (1974); *Z. Tierpsychol.* **48**, 47 (1978); A. P. Arnold, *J. Exp. Zool.* **191**, 309 (1975)], we may infer that in this species a drop in testosterone levels and a reduction in pathway use do not, by themselves, lead to gross changes in the volume of HVc and RA. The physiology of a spring to fall seasonal change probably involves more than a change in levels of gonadal hormones, so that a better test of the prediction offered is still required.
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27. I thank L. Crane for helping with the logistics of these experiments and for removing the brains. S. Kasparian did the RIA measurements of plasma androgen levels, with the help of C. Harding and L. C. Krey, in laboratory space made available by B. McEwen. T. J. DeVoogd, J. A. Paton, and M. E. Nottebohm offered helpful editorial comments. Supported by PHS grant 5R01 MH 18343 and by Rockefeller Foundation grant RF 70095 for research in reproductive biology.

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Site-Specific, Sustained Release of Drugs to the Brain

Abstract. *A dihydropyridine–pyridinium salt type of redox system is used in a general and flexible method for site-specific or sustained delivery (or both) of drugs to the brain. A biologically active compound linked to a lipoidal dihydropyridine carrier easily penetrates the blood-brain barrier. Oxidation of the carrier part in vivo to the ionic pyridinium salt prevents its elimination from the brain, while elimination from the general circulation is accelerated. Subsequent cleavage of the quaternary carrier–drug species results in sustained delivery of the drug in the brain and facile elimination of the carrier part.*

The delivery of drugs to the brain is often seriously limited by transport and metabolism factors and, more specifically, by the functional barrier of the endothelial brain capillary wall called the blood-brain barrier (*1*). Site-specific delivery and sustained delivery of drugs to the brain are even more difficult, and no useful simple or general methods to achieve them are known. We now report a general method, useful for site-specific and controlled delivery of various drugs, which is achieved by affecting the bidirectional movement of the drugs in and out of the brain with a dihydropyridine ⇌ pyridinium salt redox system.

The dihydropyridine ⇌ pyridinium salt type of redox delivery system was first successfully used for delivery to the brain of *N*-methylpyridinium-2-carbal-

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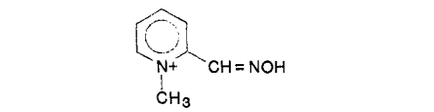
doxime chloride (2-PAM) (**1**) (*2*, *3*)—the drug of choice for the treatment of organophosphate poisoning—as its 5,6-dihydropyridine derivative (Pro-2-PAM) (**2**), which exists as a stable imminium salt (**3**). The lipoidal **2** (pK_a = 6.32) easily penetrates the blood-brain barrier where it gets oxidized to the active **1**.



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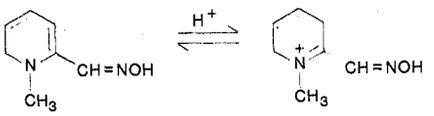


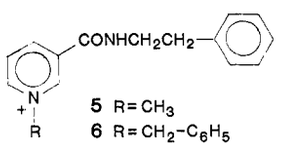
2 **3**

A dramatic increase in the brain delivery of **1** by the use of **3** was thus achieved, resulting in a reactivation of phosphorylated brain acetylcholinesterase in vivo (*4*). Subsequent brain influx-efflux studies with **1** and **2** in vivo (*5*) have indicated that the barrier properties of the blood-brain barrier, at least for such small quaternary salts as **1**, are not the same on the two sides of the barrier. Although **1** does not penetrate the brain at all, it is excreted rapidly after its fast formation in the brain (half-life approximately 1 minute for oxidation of **2** to **1** in vivo). It was suggested (*5*), however, that since large quaternary salts formed in the brain will not be easily excreted, a drug delivery system based on dihydropyridine ⇌ pyridinium salt carrier system should function as a specific and sustained release method, as indicated in scheme 1.

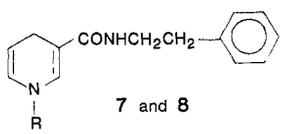
According to this scheme, a drug [D] is coupled to a quaternary carrier $[QC]^+$ and the obtained $[D\text{-}QC]^+$ is reduced chemically to the lipoidal dihydro form [D-DHC]. After administration of this compound in vivo, it is quickly distributed throughout the body, including the brain. The dihydro form [D-DHC] is then oxidized (rate constant, k_1) (by the NAD ⇌ NADH system) to the original $[D\text{-}QC]^+$ (ideally inactive) quaternary salt, which because of its ionic, hydrophilic character should be eliminated rapidly from the body, while the blood-brain barrier should prevent its elimination from the brain ($k_3 >> k_2$; $k_3 >> k_7$). Enzymatic cleavage of the $[D\text{-}QC]^+$ that is locked in the brain will result in a sustained delivery of the drug species [D], followed by its normal elimination (k_5), metabolism. A properly selected carrier $[QC]^+$ will also be eliminated rapidly from the brain ($k_6 >> k_2$). Because of the facile elimination of $[D\text{-}QC]^+$ from the general circulation, only small amounts of drug are released in the body ($k_3 >> k_4$); [D] will be released primarily in the brain ($k_4 > k_2$). The overall result ideally will be a brain-specific, sustained release of the desired drug.

Table 1. Rates of oxidative conversion in biological fluids (at 37°C) of 1-methyl-3-(*N*-β-phenethyl)carbamoyl-1,4-dihydropyridine (**7**) and 1-benzyl-3-(*N*-β-phenethyl)carbamoyl-1,4-dihydropyridine (**8**) to the corresponding quaternary pyridinium salts **5** and **6**. Undiluted heparinized human whole blood, 20 percent fresh human plasma, and 2 percent rat brain and liver homogenates were used. The conversions of **7** to **5** and **8** to **6** were followed by changes in their characteristic ultraviolet spectra (maximum wavelength, 350 nm for **7** and **8**; 262 for **5** and **6**), against appropriate reference samples. Symbols: *N*, number of rats; *k*, rate constant; *r*, correlation coefficient; $t_{1/2}$, half-life.

Medium	Process (*N*-substituent)							
	7 → **5** (methyl)				**8** → **6** (benzyl)			
	N	*k* (10^{-4} sec^{-1})	*r*	$t_{1/2}$ (minutes)	*N*	*k* (10^{-4} sec^{-1})	*r*	$t_{1/2}$ (minutes)
Human plasma	13	1.8	.998	64.2	12	0.74	.999	156
Whole blood	5	8.4	.952	13.7	5	4.7	.974	24.4
Brain homogenate	8	4.1	.996	28.2	13	2.1	.999	55
Liver homogenate	7	8.0	.999	14.4	5	7.5	.998	15.3



5 R = CH_3
6 R = CH_2-C_6H_5

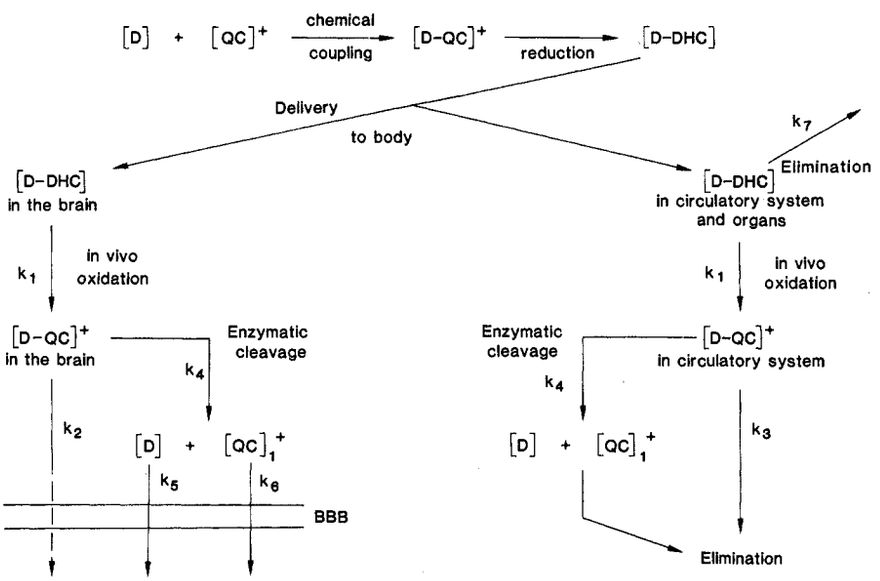


7 and **8**

Phenylethylamine (**4**) was chosen as a model for a drug [D], and it was coupled with nicotinic acid. Quaternization to the salts $[D\text{-}QC]^+$ (**5** and **6**) was followed by reduction by sodium dithionite (*6*) to the corresponding derivatives [D-DHC] (**7** and **8**).

Studies in biological fluids in vitro indicated facile oxidative conversion of the dihydro forms **7** and **8** to the quaternary derivatives **5** and **6** (Table 1).

Because of its relative ease of oxidation, the *N*-methyl derivative **5** was selected for studies in vivo. The dihydro derivative **7** was administered intravenously to rats. The rats were killed at various times after administration of **7**, and the brain and blood were analyzed for the quaternary compound **5**. The results (Fig. 1) strongly support the concept shown in scheme 1. Thus, the quaternary salt **5** disappears quickly from the blood, but the concentration of **5** increases steadily in the brain, reaching a maximum at about 80 minutes after administration. The analysis of the next,



Scheme 1; *BBB*, blood-brain barrier.

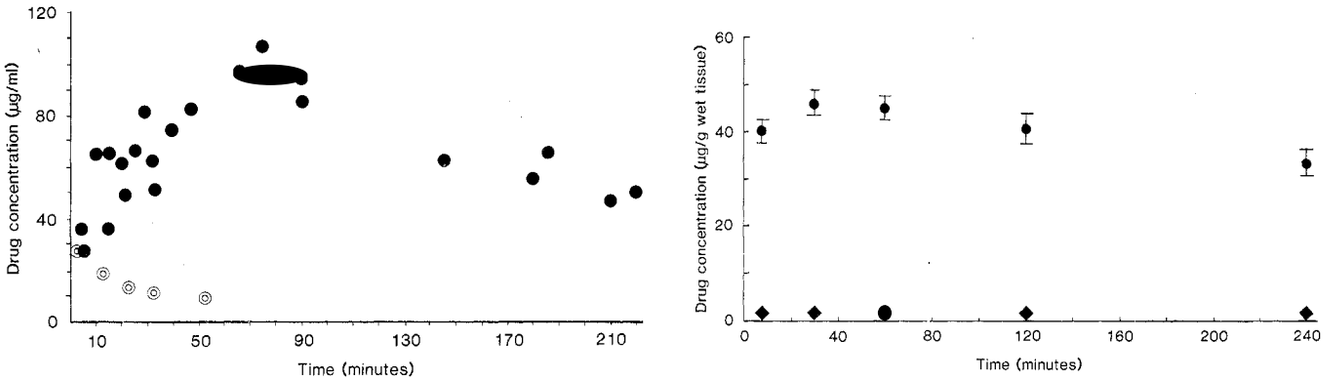
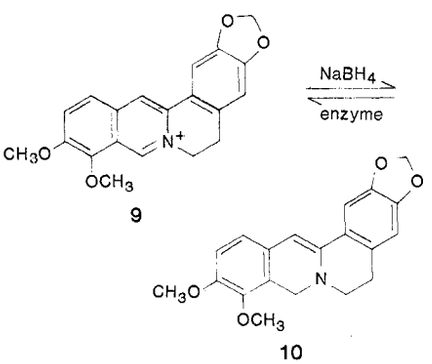


Fig. 1 (left). The concentrations of 1-methyl-3-(*N*-β-phenethyl)carbamoylpyridinium salt (**5**) in the brain (●) and in the blood (○) of rats after administration of the 1-methyl-3-(*N*-β-phenethyl)carbamoyl-1,4-dihydropyridine (**7**). A group of rats (average weight, 350 g) was injected through the jugular vein with a freshly prepared solution (0.5 g/ml in dimethyl sulfoxide) at a dose of 125 mg/kg. After the appropriate time period, 1 ml of blood was withdrawn from the heart, and the animal was perfused with 20 ml of saline. The animal was decapitated; the brain was removed, weighed, and homogenized in 2 ml of water; and 8 ml of acetonitrile was added to the homogenate. After centrifugation, the amount of the quaternary salt (**5**) was determined by high-performance liquid chromatography. The mobile phase was acetonitrile in phosphate buffer (*p*H 6.2) at a ratio of 3:2, with a flow rate of 1 ml/min. A reverse-phase Bondapak C_{18} column and an ultraviolet detector at 254 nm were used. The amounts given were calculated from the recovery standard curve obtained by introducing known amounts of **5** in brain homogenates. The blood samples were diluted with 3 ml of normal saline followed by 16 ml of acetonitrile and then were centrifuged and analyzed as above. Fig. 2 (right). The concentration in rat brain of berberine (**9**) when administered as berberine (◆) or as dihydroberberine (●). The method described in the legend of Fig. 1 was used. Compound **10** was administered as the hydrochloride salt at 55 mg/kg. Analysis was performed with high-performance liquid chromatography (mobile phase, acetonitrile in phosphate buffer at a ratio of 1:1; flow rate of 2 ml/min; retention time for **9**, 4 minutes, and for **10**, 9 minutes; Bondapak C_{18} reverse-phase column).

descending portion indicates a half-life of 2.15 hours for the disappearance of **5**. In a separate experiment with a homogenate of freshly perfused rat brain, the rate of cleavage of the quaternary salt **5** to the drug model **4** was determined. The obtained half-life of about 3 hours indicates that the descending portion of the curve corresponds mainly to the sustained delivery process of the drug species. Thus all criteria set forth in scheme 1 were met: one single intravenous injection of a drug coupled to a dihydropyridine carrier system resulted in accumulation of the corresponding drug–quaternary carrier species in the brain. This was followed by a sustained release of the drug in the brain, and possibly only in the brain, because at the same time the drug–quaternary carrier system was being rapidly eliminated from the blood. In addition, the carrier species formed after the release of the drug, trigonelline, is a nontoxic natural product. Thus the delivery system did not present any additional toxicity problem. When the quaternary derivative **5** was administered at an equivalent dose level in dimethyl sulfoxide, none could be detected in the brain of rats.

Various types of drugs could possibly be delivered to the central nervous system in a controlled, sustained manner by use of the above or analogous redox carrier systems. Current studies include the use of *N*-methylnicotinic acid (trigonelline) esters and amides (as above), and their pyridine ring–substituted derivatives, for delivery of amino- or hydroxyl-containing drugs—including small peptides—to the brain.

A different use of our redox delivery system starts with a large, biologically active quaternary salt. The first compound studied was berberine (**9**), which has rather high activity in vitro against



several types of cancer, including Ehrlich and lymphoma ascites (*7, 8*), but very low activity in vivo (*9*). This difference may be due to the inability of the ionic berberine to penetrate lipoidal membranes. To determine whether such an ionic species could be delivered to the brain and accumulate there, we prepared the corresponding dihydroberberine (**10**) using sodium borohydride [dihydroberberine was previously isolated from the disproportionation of the 8-hydroxyberberine derivative (*10*)]. The dihydro form **10** was transformed to the stable hydrochloride form which was then used for studies in vitro and in vivo. In human plasma, **10** is oxidized back to **9** at 37°C, with a half-life of 25.1 minutes; the half-life in rat brain homogenate was 15.2 minutes.

As in the system **5** ⇌ **7**, berberine (**9**) itself does not penetrate the brain of rats at all, but very large amounts of berberine were found in rat brain after intravenous administration of the dihydroberberine (**10**) (Fig. 2). The high brain levels are maintained for a long time, an indication of site-specific delivery to the brain of large quaternary salts that would be expected to be eliminated rapidly from the general circulation.

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