variation in sex ratios of progeny, and we do not know whether temperature has a linear or a "threshold" relation to sex ratios of progeny. The high degree of variation in the sex ratios of progeny from different females may suggest polygenic sex determination, as has been hypothesized for other fishes (*13*). Although we have not proved that offspring maintain the same sexual expression until maturity, we consider sex reversals unlikely because we have never discovered any intersex individuals in culture or in the wild; cultures of *Menidia* allowed to grow well beyond the critical phase had the same sex ratio as identical cultures terminated earlier (*8*). Furthermore, sequential hermaphrodites are unknown among atherinids. Hence, in *M. menidia*, once sex is determined, it appears to be irreversible.

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6. D. P. Middaugh, *Copeia*, in press. Silversides are polygamous and spawn in large schools during daytime high tides on new and full moons and, at such times, can be easily netted. They provide no parental care after spawning.
7. These fish were raised in 60-liter aquariums and were fed *Artemia salina* nauplii to excess. In 1979 we conducted experiments involving removal of naturally spawned and reared eggs from the field just prior to hatching, but learned only that the critical period of sex determination was during the larval phase.
8. Sex cannot be determined externally. When *Menidia* had attained a minimum length of 20 mm, they were easily sexed by examination of gross morphology of gonads under a dissecting microscope and, when rarely necessary (frequency, < 1 percent), by the presence of numerous oocytes in slide preparations at higher magnification. Our criteria were nearly identical to those described for a poeciliid of similar size [F. F. Snelson, Jr., and J. D. Wetherington, *Evolution* **34**, 308 (1980)]. We validated our sexing technique by verifying that sex ratios of progeny allowed to grow to a much larger size (40 to 50 mm) were identical to the ratios in sibling sets of progeny terminated earlier (20 to 30 mm).
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4 December 1980; revised 10 March 1981

The AF64A-Treated Mouse: Possible Model for Central Cholinergic Hypofunction

Abstract. *A loss in the number of functional, sodium ion–dependent, high-affinity choline transport sites was observed in the cortex and hippocampus of mice given an intracerebroventricular injection of 65 nanomoles of AF64A (ethylcholine mustard aziridinium ion) 3 days earlier. Such an effect was not observed in the striatum. This effect of AF64A represents a long-term neurochemical deficit at cholinergic nerve terminals in some brain regions which can lead to a persistent deficiency in central cholinergic transmission. The AF64A-treated animal may thus be a model for certain psychiatric or neurological disorders that appear to involve central cholinergic hypofunction.*

We recently proposed that choline mustard analogs may be toxic to cholinergic nerve terminals and could be used in animals to model the psychobiology and pharmacology of disorders characterized by an underactivity of central cholinergic neurotransmission (*1*). We now report that intracerebroventricular injection of one of these compounds, ethylcholine mustard aziridinium ion (AF64A), into mice produces a long-lasting reduction in the number of functional, Na^+-dependent, high-affinity choline transport (HAChT) sites. The HAChT system is a rather specific marker for cholinergic nerve terminals (*2*); moreover, HAChT activity may regulate acetylcholine synthesis (*2*). Thus, our results demonstrate that AF64A causes a persistent neurochemical deficit at cholinergic nerve terminals which may lead to chronic central cholinergic hypofunction in animals.

To test whether a low dose of AF64A could affect cholinergic nerve terminals in situ, mice were lightly anesthetized with ether, and 65 nmole (25 μl) of AF64A or a vehicle solution (*3*) was unilaterally injected into the lateral ventricle with a 3/8-inch, 27-gauge Hamilton hypodermic needle attached to a 0.5-ml glass syringe (*4*). This dose of AF64A was not immediately lethal. The mice were then housed in groups for 3 days. During this period, the AF64A-injected animals developed neurological disturbances (such as hypokinesia) and various ataxic syndromes with variable frequency, and typically lost 10 to 20 percent of their original body weight. Vehicle-injected mice incurred no morbidity and gained weight at a natural rate. On the third day after treatment the mice were decapitated and their brains were dissected. Dissection was performed at room temperature to permit postmortem reversal of adaptive changes in the HAChT system (*5*). The tissue was then kept on ice until homogenization. A crude synaptosomal pellet (P_2) was pre-

Table 1. Kinetic constants of Na^+-dependent HAChT in synaptosomes from mice 3 days after AF64A or vehicle was administered into the brain, as calculated by Eadie-Scatchard analysis of transport data. Values are means ± standard errors for independent matched experiments.

Brain area	Treatment	N	V_{max}*	Percent of vehicle V_{max}	K_T† (μM)
Cortex	Vehicle	3	19.8 ± 1.9		0.39 ± 0.04
	AF64A	3	6.3 ± 0.7	32‡	0.48 ± 0.12
Hippocampus	Vehicle	3	38.8 ± 11.5		0.33 ± 0.08
	AF64A	3	20.1 ± 8.4	52‡	0.29 ± 0.05
Striatum	Vehicle	3	107.5 ± 11.8		0.46 ± 0.08
	AF64A	3	123.3 ± 25.7	114	0.56 ± 0.16

*Maximum velocity of enzyme reaction, measured as picomoles per milligram of protein per 4 minutes. †Apparent Michaelis constant for transport. ‡$P < .05$, two-tailed paired Student's t-test.

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pared from tissue pooled from three to six mice and resuspended in cold isotonic sucrose for use in HAChT measurements (*6*). The results (Fig. 1) demonstrate that AF64A noncompetitively lowered the maximal level of HAChT in the cortex and hippocampus without affecting the striatum. Table 1 summarizes a kinetic analysis of data obtained in several separate experiments, including those illustrated in Fig. 1. In mice treated with AF64A, the V_{max} of cortical HAChT decreased 68 percent and the V_{max} of hippocampal HAChT decreased 48 percent, with no change in AF64A's apparent affinity for choline. Moreover, in the striatum there were no kinetic differences in HAChT between the two treatment groups.

Our data thus demonstrate that a low dose of AF64A injected into mouse brain can cause a loss in Na^+-dependent HAChT activity in some brain regions within 3 days. Because of the 3-day interval between dosing the mice and measuring HAChT, the effect of AF64A was probably not due to carry-over of the compound from the brain tissue into the assay medium. Furthermore, by allowing an adequate postmortem delay before cooling the tissue, adaptive changes in HAChT activity were reversed to basal levels (*5*). Thus, the reduction in the V_{max} of HAChT can be ascribed to a loss in the number of functional HAChT sites in the cortex and hippocampus after AF64A injection into the brain. This could come about by an irreversible alkylation of transport sites (*7*) or as the result of a degeneration of cholinergic nerve terminals. The outcome of one or both of these interactions, if extensive enough, should be a reduction in brain acetylcholine levels (central cholinergic hypofunction). In fact, in an evaluation of neurotoxicity following peripheral administration of AF64A, we found that the compound produces a dose- and time-dependent decrease in acetylcholine levels in sublingual salivary glands and whole brains in mice (*8*). In a preliminary experiment, mice given AF64A into the brain (65 moles, intracerebroventricularly) and killed by microwave irradiation 3 days later had a 35 percent decrease in cortical acetylcholine and no change in striatal acetylcholine compared to vehicle-injected animals (*9*). Moreover, while AF64A appears to have presynaptic cholinergic neurotoxicity, postsynaptic interactions may be insignificant since AF64A does not interact with muscarinic receptors (*8*).

The differential sensitivity between the HAChT systems in striatum, cortex, and hippocampus to the presence of AF64A in the brain might be due to differences in its distribution within the tissues or to differences in the rate at which neurotoxic effects develop after the initial exposure to the compound. Alternatively, these regional brain differences might reflect an inherent biochemical heterogeneity among HAChT systems (*10*). A more detailed analysis of this last possibility may reveal important information about the neurochemical architecture of the cholinergic nerve terminal.

A neurotoxin specific for cholinergic nerve terminals would be an important tool for studying the neurobiology and pharmacology of cholinergic neurons in the central nervous system. In this context, we note that the regional decrease in brain HAChT sites brought about by AF64A resembles the pattern of presynaptic cholinergic neurochemical deterioration observed post-mortem in patients who suffered from Alzheimer-type senile dementia (*11*). Thus, animals with AF64A-induced neurotoxicity may be useful as models of certain psychiatric and neurological disorders characterized by central cholinergic hypofunction.

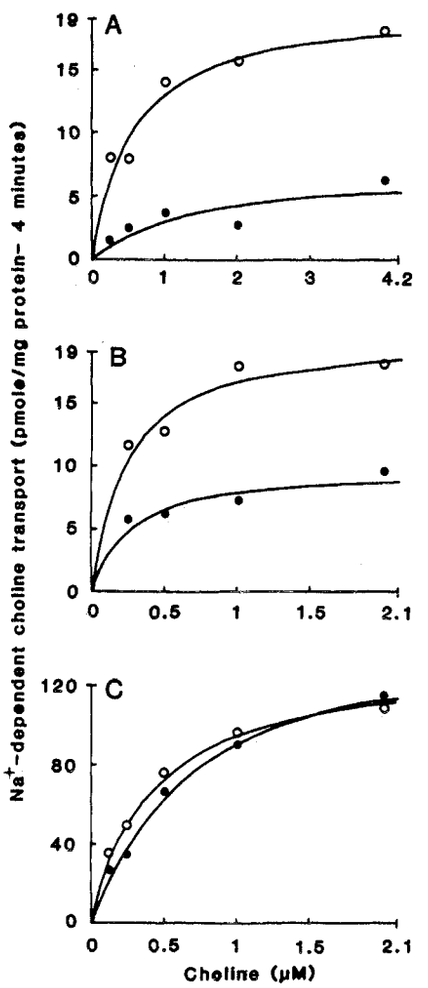

Fig. 1. Sodium ion–dependent choline transport as a function of choline concentration in incubations with synaptosomes from the cortex (A), hippocampus (B), and striatum (C) of mice 3 days after vehicle (○) or AF64A (●) was administered into the brain. The curves are the best-fit lines to a rectangular hyperbola constructed by iterative nonlinear regression analysis of the data by the PROPHET computer system (NIH, Washington, D.C.).

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12. We thank W. C. DeGroat, M. J. Zigmond, and A. G. Mallinger for their comments. Supported by PHS grants MH 26320 and MH/AG 34893. A. F. is on sabbatical leave from the Israel Institute for Biological Research, Ness-Ziona, Israel.

4 December 1980; revised 26 February 1981