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10. We have not yet ascertained whether other fragments of β-LPH, or the entire β-LPH itself would be active in our in vivo bioassay. However, L. H. Lazarus, N. Ling, R. Guillemin [*Proc. Natl. Acad. Sci. U.S.A.* **73**, 2156 (1976)] and B. M. Cox, A. Goldstein, C. H. Li (*ibid.*, p. 1821), and L. Graf, A. Z. Ronai, S. Bajusz, G. Csez, J. Szekely [*FEBS Lett.* **64**, 181 (1976)] have reported a lack of "opioid" activity of β-LPH in their in vitro bioassays.
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Regulation of Acetylcholine Synthesis: Does Cytoplasmic Acetylcholine Control High Affinity Choline Uptake?

Abstract. *When brain synaptosomes are obtained from animals that have been injected intravenously with [$^2H_4$]choline 1 minute before being killed, their high affinity [^3H]choline uptake is correlated inversely with their acetylcholine content and directly with the rate at which they synthesize [$^2H_4$]acetylcholine. The control of such choline uptake by the cytoplasmic acetycholine concentration is proposed as a mechanism regulating acetylcholine synthesis in cholinergic nerve terminals.*

The relatively small changes in acetylcholine (ACh) concentrations that are caused by alterations in neuronal impulse flow (*1*, *1a*) suggest that a regulatory feedback system controls the rate of ACh synthesis in response to changing demands. The nature of this regulatory mechanism has been the subject of much investigation and speculation. It has recently been proposed that precursor availability may regulate ACh synthesis, since the increase in plasma and brain choline concentrations following oral or parenteral administration of choline is associated with an increase in brain ACh concentration (*2*). Feedback mechanisms that have been suggested include inhibition of choline acetyltransferase (E.C. 2.3.1.6) by ACh (*3*); maintenance by choline acetyltransferase of a mass action relationship between choline, acetyl coenzyme A (acetyl CoA), ACh and coenzyme A (CoA) (*4*); and, most recently, regulation of the high affinity choline uptake system (*5*, *6*) which appears to be coupled to ACh synthesis (*7*) and release (*1a*, *8*). On the basis of the properties of synaptosomal fractions isolated from animals subjected to various treatments before being killed, it was suggested (*5*, *6*) that the high affinity choline transport system is regulated by neuronal impulse flow.

One of the simpler mechanisms by which some of the properties of synaptosomes in vitro could be dependent upon their immediate antemortem history would be through the content of choline and ACh of the synaptosomes at the time they were prepared by homogenation of brain tissue. We present evidence that the choline and ACh content of synaptosomes reflect cholinergic events in the brain at the time the synaptosomes were prepared. We have confirmed that the high affinity choline uptake by synaptosomes is influenced in a predictable manner by the way in which the animal is treated before it is killed; we show that this uptake is consistently related to the synaptosomal ACh content and synthesis rate. We propose that the factor controlling the high affinity uptake of [^3H]choline by synaptosomes in these experiments may be their content of ACh.

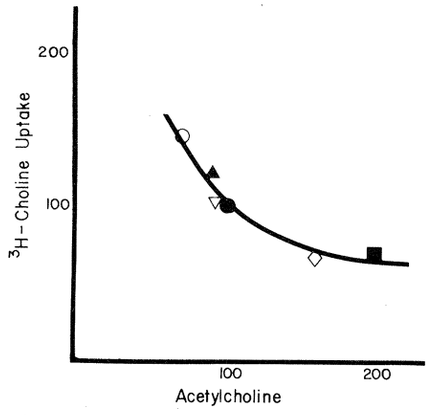

Fig. 1. Acetylcholine concentration and [^3H]choline uptake by synaptosomes prepared from animals subjected to various treatments before being killed. Experimental details were as indicated in Tables 1 and 2. The rate of the high affinity uptake of 0.54 μ*M* [^3H]choline was measured as described by Atweh *et al.* (*6*) and is presented as a percentage of the rate of uptake by synaptosomes prepared from saline-treated mice. The concentration of ACh in the synaptosomes is presented as a percentage of the concentration in synaptosomes prepared from saline-treated mice. Symbols: • saline; ○ atropine; ▲, pentylenetetrazole; ▽, electroshock; ◇, pentobarbital; and ■, oxotremorine, at the doses given in Table 2. There is a highly significant correlation (r = .970; P = .0014) between [^3H]choline uptake and the reciprocal of the ACh concentration.

Synaptosomes prepared from mouse brain at various time intervals after the animals are killed show changes in their content of choline and ACh (Table 1) which parallel those observed previously in whole brain (*9*). A progressive increase in choline content and a decrease in ACh content are observed in both systems. When [$^2H_4$]choline is injected intravenously 1 minute before the animals are killed, the [$^2H_4$]ACh formed in brain provides an estimate of relative turnover rate of ACh (*10*, *10a*). The mole fractions of [$^2H_4$]choline and [$^2H_4$]ACh in synaptosomes parallel the corresponding values in whole brain (Table 1, columns 5 and 9).

To examine the effect that synaptosomal choline and ACh content may have on high affinity [^3H]choline uptake in vitro, mice were treated with drugs known to influence cholinergic activity and with electroshock. Synaptosomes were prepared from the whole brains and were used to measure: (i) the rate of [^3H]choline uptake under conditions favoring high affinity uptake, (ii) total choline and ACh concentrations in the freshly isolated synaptosomes, and (iii) mole fractions of [$^2H_4$]choline and [$^2H_4$]ACh in synaptosomes prepared from animals into which [$^2H_4$]choline was injected intravenously 1 minute before they were killed. The relationships between these variables were consistent, regardless of the measures used to perturb the normal state before death (Table 2).

High affinity choline uptake (Fig. 1) was reduced in synaptosomes prepared from animals treated with pentobarbital or oxotremorine, as reported earlier (*6*). The synaptosomal acetylcholine content was in both cases significantly increased, like that in whole brain (*11*). In contrast, prior treatment of the animals with atropine increased the high affinity choline uptake by synaptosomes; such synaptosomes contained lower concentrations of ACh, again in agreement with reported effects in whole brain (*11*). Treatment with pentylenetetrazole or electroshock produced similar but smaller changes in both the high affinity uptake of choline and the concentration of ACh.

The mole fraction of [$^2H_4$]ACh in the synaptosomes was inversely related to the total ACh content ($r = -.940$; $P = .01$). A similar inverse relationship between turnover and concentration has been reported previously in whole brain, although the perfused superior cervical ganglia and isolated phrenic nerve diaphragm preparation show little or no variation in ACh content under stimulus-induced increases in turnover rate (*8*). These experiments suggest that changes in brain ACh turnover rate induced experimentally in vivo result in reciprocal changes in ACh levels, that these in turn modulate the high affinity choline uptake system, and that all three of these effects can be observed in synaptosomes prepared after the experimental manipulation. Inhibition of high affinity choline uptake by externally applied ACh has been reported (*12*), and Whittaker (*13*) has reported that high affinity choline uptake by squid optic lobe synaptosomes was reduced after they were incubated with acetylcholine (0 to 10 m*M*). The present experiments suggest that naturally occurring levels of cytoplasmic ACh may control high affinity choline uptake in synaptosomes from mouse brain.

It is possible that an effect of intrasynaptosomal ACh concentration on high affinity choline uptake could be mediated through a corresponding change in the concentration of choline, particularly if the relative concentrations of choline and ACh in the cytoplasm were determined by mass action (*4*). In this case choline concentration within the endings

Table 1. Acetylcholine and choline concentrations in whole mouse brain (nanomoles per gram wet weight) and in synaptosomal fraction (nanomoles per milligram of protein $\times 10^2$) at various time intervals after death. Mice (25 to 35 g) were decapitated 1 minute after intravenous injection of [$^2H_4$]choline (20 μmole/kg; 5 ml/kg). The brains were excised and incubated in a moist, 37°C chamber for the indicated intervals and were then either: (i) homogenized in 85 percent acetone and 15 percent 1*N* formic acid, or (ii) homogenized in 0.32*M* sucrose for the preparation of synaptosomes by the method of Atweh *et al.* (*6*). Concentrations of acetylcholine and choline were determined by combined gas chromatography and mass spectrometry as described previously (*16*). Since 1 g of brain contains approximately 0.1 g of protein, figures for the whole brain and the synaptosomal fraction are directly comparable. Each value represents the mean plus or minus standard error of four determinations.

Time interval (seconds)	Acetylcholine				Choline			
	[$^2H_0$]-ACh	[$^2H_4$]-ACh	Total ACh	Mole fraction [$^2H_4$]ACh	[$^2H_0$]-choline	[$^2H_4$]-choline	Total choline	Mole fraction [$^2H_4$]choline
				Whole brain				
0	17.06 ± 1.03	0.49 ± .01	17.55 ± 1.04	0.028 ± .001	51.64 ± 1.51	2.22 ± .01	53.86 ± 1.50	0.041 ± .001
2	13.18 ± .37	0.31 ± .02	13.36 ± .40	0.020 ± .001	105.3 ± 8.2	2.22 ± .18	106.9 ± 8.1	0.020 ± .001
10	9.81 ± .42	0.21 ± .01	10.02 ± .42	0.021 ± .002	227.7 ± 25.0	2.54 ± .96	230.2 ± 25.5	0.007 ± .001
60	3.60 ± .49	0.046 ± .017	3.64 ± .50	0.010 ± .003	987 ±171	2.69 ± .58	990 ±171	0.003 ± .001
				Synaptosomal fraction				
0	13.84 ± .54	0.74 ± .12	14.58 ± .58	0.039 ± .013	72.1 ± 4.8	1.08 ± .16	73.2 ± 4.3	0.017 ± .005
2	14.65 ± 1.43	0.22 ± .067	14.86 ± 1.37	0.015 ± .005	113.3 ± 1.6	2.50 ±1.06	116.8 ± 0.8	0.022 ± .009
10	11.39 ± .36	0.062 ± .025	11.45 ± .32	0.006 ± .003	237.0 ± 16.7	1.55 ± .29	238.5 ± 17.0	0.006 ± .001
60	3.92 ± .30	0.067 ± .034	3.94 ± .33	0.016 ± .009	414.1 ± 19.9	1.65 ± .36	415.7 ± 19.7	0.004 ± .001

Table 2. Acetylcholine and choline in synaptosomal preparations from mouse brain. Drugs were administered intraperitoneally in saline at the indicated times before the animals were killed. One minute before the animals were killed [$^2H_4$]choline (20 μmole/kg) was injected intravenously, and the whole brain synaptosomes were prepared as indicated in Table 1. For the electroshock experiments the [$^2H_4$]choline was administered 55 seconds prior to applying a current of 50 ma r.m.s. for 35 msec between the orbits. At 60 seconds the mice were decapitated. After resuspending the P_2 pellet and removing a portion for [$^2H_4$]choline uptake determination, the remaining suspension was centrifuged at 17,000*g* to yield a washed synaptosomal pellet (P_2') and supernatant. The ACh and choline values were determined from these two fractions by combined gas chromatography and mass spectrometry (*12*). All levels are expressed as nanomoles per milligram of protein. No ACh was found in the supernatant fractions. Each value represents the mean plus or minus standard error. The number in parentheses indicates the number of animals per group. ND, not determined.

Treatment	Time (minutes)	Synaptosome fraction (P_2')				Supernatant	
		Total ACh	Mole fraction [$^2H_4$]ACh	Total choline	Mole fraction [$^2H_4$]choline	Total choline	Mole fraction [$^2H_4$]choline
Saline	30	0.146 ± .006 (30)	0.0393 ± .0132	0.708 ± .043	0.0171 ± .0049	1.057 ± .125	0.0189 ± .0032
Pentobarbital (262 μmole/kg)	30	0.230 ± .021* (10)	0.0147 ± .0044*	0.516 ± .044	0.0084 ± .0010	0.904 ± .087	0.0077 ± .0008
Oxotremorine (5.9 μmole/kg)	30	0.288 ± .020* (9)	0.0104 ± .0014*	0.811 ± .064	0.0136 ± .0005	0.959 ± .123	0.0139 ± .0021
Atropine (28.8 μmole/kg)	20	0.102 ± .005* (8)	0.0598 ± .0053*	0.534 ± .056	0.0136 ± .0011	0.813 ± .097	0.0209 ± .0100
Pentylenetetrazole (543 μmole/kg)	5	0.132 ± .010 (9)	ND	0.607 ± .107	ND	0.894 ± .087	ND
Electroshock		0.133 ± .018 (4)	0.0356 ± .0039	0.570 ± .065	0.0113 ± .0006	1.428 ± .134	0.0070 ± .002

*$P < .001$ (Student's *t*-test).

would fall when (unbound) ACh was depleted; this, in turn, would favor passive carrier-mediated transport of choline into the ending under the influence of an electrochemical gradient (*14*) even if the extracellular concentration of choline were considerably less than the cytoplasmic concentration. An apparent change in high affinity choline uptake under the conditions used could also occur as a result of drug-induced changes in the isotopic dilution of labeled choline by endogenous choline, although Simon and Kuhar (*5*) have stated that endogenous choline levels in the incubation medium "were extremely low and no difference was found between synaptosomal fractions from pentobarbital treated animals and those from control animals." For these reasons, choline concentrations in both the synaptosomal pellet and the incubation medium were measured and are reported in Table 2. The synaptosomal choline concentration and mole fraction of $[^2H_4]$choline were not significantly changed by any of the treatments. It may nevertheless be fallacious to infer from these results that the choline concentration within cholinergic nerve endings does not change under these experimental conditions. While it seems reasonable to suppose that most of the ACh is contained in cholinergic synaptosomes and therefore reflects cholinergic function, choline is a universal constituent of all cells and there is evidence that concentrations of endogenous and isotopic choline in whole brain (or parts of it) may not be representative of those in cholinergic nerve endings (*15*). The constant synaptosomal choline concentrations which we report do not therefore preclude the occurrence of adaptive changes in choline concentration in cholinergic endings in response to changes in ACh turnover rate and concentration.

The concentration of endogenous choline found in the supernatant was less than 0.04 μM, which is unlikely to influence these experimental results significantly; however, this would be sufficient to obfuscate the interpretation of experiments in which lower concentrations of $[^3H]$choline are used and endogenous choline concentration in the supernatant is not measured.

In summary, we have shown that changes in high affinity choline uptake by synaptosomes which appear to result from experimental manipulation of cholinergic activity before the animal is killed are associated also with changes in synaptosomal ACh content and turnover. The simplest mechanism for modulation of choline uptake by antemortem neuronal activity appears to be an effect of synaptosomal ACh concentration on high affinity choline uptake. Whether this is a direct effect on ACh or is due to a secondary change in synaptosomal choline levels remains to be established.

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14. M. P. Blaustein and J. M. Goldring [*J. Physiol. (London)* **247**, 589 (1975)] have adduced evidence that pinched-off presynaptic nerve terminals suspended in a medium containing 5 mM K^+ have membrane potentials (55 to 60 mv) which would sustain an equilibrium concentration ratio of choline of ~ 1 : 10 favoring the cytoplasm. The $[K^+]$ in the present experiments is 4.75 mM.
15. For example, NH_4^+ appears to reduce substantially the amount of $[^2H]$choline entering the brain following an intravenous injection without altering the rate of $[^2H]$ACh synthesis [S. H. Butcher, L. L. Butcher, A. K. Cho, *Life Sci.* **18**, 733 (1976)], suggesting an effect of NH_4^+ on the nonspecific component of choline uptake which is not associated with ACh synthesis. Others have reported that cholinergic denervation of the hippocampus results in little or no reduction in the entry of $[^3H]$choline, despite other evidence of degeneration of cholinergic terminals [V. H. Sethy, R. H. Roth, M. J. Kuhar, M. H. VanWoert, *Neuropharmacology* **12**, 819 (1973); Atweh and Kuhar (*10a*)]. There is also evidence that choline is compartmentalized in synaptosomal preparations [P. G. Guyenet, P. LeFresne, J. C. Beaujovan, J. Glowinski, in *Cholinergic Mechanisms*, P. G. Waser, Ed. (Raven, New York, 1975), p. 137].
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Tail-Pinch Stimulation: Sufficient Motivation for Learning

Abstract. *A paper clip applied to the tails of rats induced gnawing and eating, which decreased in latency and increased in duration with experience. With sustained pressure to the tail, rats learned a new habit in order to gain access to wood chips on which to gnaw. That these are also properties of behavior elicited by electrical brain stimulation suggests that both manipulations may act through the same mechanism. These results support the hypothesis that a nonspecific arousing stimulus can be a sufficient condition for establishing learned habits.*

Mild pressure to the tails of rats elicits eating, gnawing, biting, and licking (*1*), which resemble behaviors elicited by electrical stimulation of the hypothalamus (*2*). The particular behavior elicited by tail-pinch depends on the particular goal object available (*3*); this is also true of electrically induced behavior (*4*). Further similarities between behavior elicited by tail-pinch and that elicited by hypothalamic stimulation can be discovered by examining the learning that contributes to the plasticity of the behavior.

Electrically induced behavior appears gradually and requires some experience with the stimulation before a reliable response can be elicited (*5*); the strength of this response increases with additional experience with the stimulation (*5*). In addition, when the hypothalamus is stimulated, an animal will learn a new approach response that will allow it to perform the appropriate consummatory behavior (*6*). In the presence of electrical brain stimulation, animals will learn to find a variety of appropriate objects with which to engage in consummatory behavior. Cats learned a Y-maze in order to attack a rat during hypothalamic stimulation (*7*). Satiated rats receiving hypothalamic stimulation learned the new