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Effects of Handling and Etherization on Incorporation of [^{3}H] Lysine into Protein of Mouse Brain and Liver[1]

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ENTINGH, D. J. AND T. DAMSTRA. *Effects of handling and etherization on incorporation of [^{3}H] lysine into protein of mouse brain and liver.* PHARMAC. BIOCHEM. BEHAV. 5(2) 111–116, 1976. – A standardized handling experience of 20 sec duration elevated the amount of radioactive lysine incorporated into brain total proteins during a 10 min labeling period begun either 10 min or 30 min after the handling. Etherization for 20 sec produced similar metabolic changes. Incorporation of [^{3}H] lysine into liver proteins was minimally affected by handling, while slightly altered by etherization. The metabolic changes detected in the brain after handling did not appear to be side-effects of changes in blood-borne radioactivity. The results indicate that laboratory stresses often thought to be of minor importance can have large effects on ordinary assays of protein metabolism in brain.

Stimulation Protein Handling Etherization Brain Liver Lysine Radioactivity

APPROXIMATIONS of rates of protein synthesis have come to play increasingly important roles in demonstrations that various chemical agents and experimental manipulations have effects upon the biology of central nervous system neurons. One such approximation, the rate of incorporation of radioactive amino acids into brain proteins, has been reported to be altered by hormones [19,21], spreading depression [3], electrical stimulation [11], sensory stimulation [2,18], and behavioral training paradigms [10,18].

Since so many different treatments have been reported to alter the incorporation of amino acids into brain proteins, it is reasonable to hypothesize that at least some portion of all of these changes may be induced by a common or nonspecific mechanism. Indeed, from behavioral studies [2, 5, 6, 18], it is clear that many forms of environmental stimulation trigger brief episodes of altered protein or amino acid metabolism in brain.

Thus it is important to determine what minimal experimental manipulations might trigger such effects. We report here the effects of two ordinary laboratory procedures, handling and etherization, upon the rate of incorporation of [^{3}H] lysine into brain proteins in mice.

METHOD

Animals

Male C57Bl/6J mice (Jackson Laboratories), 7 to 8 wk old were individually housed for 3 days before use. The temperature of the animal room was 24 ± 1°C, and food and water were always available.

Behavioral Treatments

All experiments were conducted between 9 a.m. and 12 a.m. to minimize diurnal variation in plasma corticosterone [1].

Each Nonhandled mouse was brought, in its home cage, into the biochemistry laboratory 10 min before it was injected with [^{3}H] lysine. After the injection it was immediately returned to its home cage and killed 5, 10, or 20 min later.

Each Handled mouse was brought into the biochemistry laboratory, lifted from its cage by the tail, and immediately placed in a 700 ml beaker. The floor of the beaker was covered to a depth of 1 cm by absorbent cotton, overlayed by 1 mm-mesh wire gauze. A plastic lid was placed on the

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beaker while the mouse was in it. After it had been in the beaker for 20 sec, the mouse was slid gently back into its home cage. Either 10 or 30 min later the mouse was injected with [^{3}H] lysine, returned immediately to its home cage, and killed 5, 10, or 20 min later.

Etherized mice were treated identically to Handled mice, except that the atmosphere of the beaker in which they were placed was saturated with ethyl ether at room temperature.

Injections

Each mouse was lifted from its cage by its tail, immobilized in one hand, injected subcutaneously with 30 μCi of L [4,5-^{3}H] lysine monohydrochloride (1 μCi/μl, 5.5 Ci/mmole, Amersham/Searle), and placed in its home cage.

Biochemical Methods

Rate of incorporation of lysine. The method of Rees *et al.* [18] was used for brain and liver in all experiments. Each mouse was decapitated by guillotine. The brain, minus olfactory bulbs, was quickly removed, rinsed in ice-cold saline and homogenized in 5 ml of ice-cold 0.05 *M* sodium borate. A piece of the right lobe of the liver (100 to 200 mg wet weight) was removed from each mouse, weighed, rinsed in ice-cold saline, and homogenized in 5 ml of the same homogenizing medium.

For estimates of Total radioactivity (less tritiated water) 0.2 ml of homogenate was pipetted into a scintillation vial and dried. For estimates of Protein radioactivity (TCA-insoluble fraction) 0.2 ml of homogenate was delivered to a 2.3 cm filter paper disc. The discs were dried and washed successively with blank discs in 5% trichloroacetic acid (TCA), 3 times; ether: 95% ethanol (1:1) at 40°C; and twice in ether. The discs were dried and placed in scintillation vials.

One ml of Soluene-100 was added to each vial. Vials were heated at 50°C for 8 hr, allowed to stand at room temperature for at least 24 hr, and 10 ml of scintillation fluid was added to each vial. Counts/min were converted to disintegrations/min (DPM) using external standardization. The median value of 3 replicates was taken as the DPM for each sample after appropriate blank DPM had been subtracted.

To correct for variability in the amount of radioactive precursor available for incorporation into protein, incorporation values were expressed as the average (over time) percentage of total radioactivity incorporated into the TCA-insoluble fraction per minute:

$$\text{Incorporation} = (\text{DPM}_{\text{protein}} \times 100)/(\text{DPM}_{\text{total}} \times \text{min}).$$

In one experiment the amount of protein in brain homogenates was determined by the method of Lowry *et al.* [15].

Correction for radioactivity from blood entrapped in brain samples. In the last of the 3 experiments described here (time-course experiment), a subtractive correction was made for the presence in the brain homogenates of radioactive blood trapped in the brain capillaries. The volume of blood trapped in decapitated brains was measured [7] and found to be 0.017 ± 0.003 μl blood/mg wet brain in 5 nonhandled mice and 0.015 ± 0.004 in 4 handled mice. The contamination was taken to be 0.016 in further calculations for both groups.

Blood was taken from the severed neck of each mouse in the time-course experiment, and samples of 5 μl were spotted on paper filter discs. Insoluble and total DPM in these samples were measured by the method described above for brain and liver samples. Corrected DPM in brain tissue were expressed as:

$$\text{DPM}_{\text{corrected}}/\text{mg brain} = \text{DPM}_{\text{homogenate}}/\text{mg brain} - 0.016 \times \text{DPM}/\mu\text{l blood}.$$

Separate corrections were made for the insoluble and soluble (total − insoluble) blood contamination, since the rate of incorporation of [^{3}H] lysine was much lower in blood than in brain.

No attempt was made to make similar corrections for blood-contamination of liver samples.

Statistics

The statistical significance of differences between group means was assessed by two-tailed *t* tests, corrected for unequal sample variances.

RESULTS

In the first experiment, effects of handling and etherization upon the rate of incorporation (RI) of lysine into brain and liver proteins were examined with a delay of either 10 or 30 min between the manipulation of the animal and the injection of [^{3}H] lysine (Table 1). The incorporation time was 10 min. Both etherization and handling significantly elevated the RI in brain, at both 10 and 30 min delay. Etherization had a somewhat greater effect on RI in brain than did handling, at both delays. For both etherization and handling, the elevation of brain RI was greater at the shorter delay between manipulation and injection.

The total radioactivity in brain was significantly depressed by handling at 30 min delay, but not altered by the other 3 manipulations.

For liver, RI was elevated slightly by etherization, 10 min delay, but not significantly changed by the other 3 manipulations. In every case, changes in RI for liver were smaller than those for brain. Total DPM/mg liver are not shown in Table 1 because liver samples were not weighed in this experiment.

The second experiment served as a replication of the effects of handling on RI of brain and liver; with 10 min handling-injection delay and 10 min incorporation time. We were primarily interested in eliminating the possibility that all of the changes depicted in Table 1 for RI in brain could have been due to erroneous measures of the RI in nonhandled mice. Liver samples were weighed here.

The results of the second experiment (Table 2) substantiated those of the first in all respects. Nine months had elapsed, and different workers performed the handling and biochemical processing in the 2 experiments. A slight increase in Total DPM in brain was seen in both experiments. RI was elevated by 22% in the second experiment compared to 27% in the first.

In the liver, handling induced a 10% increase in total DPM (borderline significance) and a significant elevation of 8% in incorporation rate.

The third experiment examined the influence of handling on the time-course of labeling in blood, liver, and brain. The purpose was to determine (1) if the uptake of [^{3}H] lysine into these tissues was well-behaved across time, (2)

TABLE 1

EFFECTS OF HANDLING OR ETHER ON INCORPORATION OF [^{3}H] LYSINE INTO PROTEIN (TCA-INSOLUBLE FRACTION) DURING 10-MIN INCORPORATION PERIOD. MEAN ± SEM; 5 MICE PER GROUP

Treatment	Delay between treatment and injection, min	Total DPM mg brain	Brain % Total incorporated per min	% Difference in rate of incorporation	Liver % Total incorporated per min	% Difference in rate of incorporation
Nonhandled	—	434 ± 18	0.94 ± 0.02	[0]	3.06 ± 0.12	[0]
Handled	10	443 ± 48	1.19 ± 0.07	+27*	3.21 ± 0.09	−5
Handled	30	378* ± 19	1.12 ± 0.07	+19*	2.82 ± 0.12	−8
Ether	10	426 ± 37	1.33 ± 0.08	+41†	3.51 ± 0.14	+15*
Ether	30	408 ± 34	1.20 ± 0.06	+28†	3.07 ± 0.16	0

Significance of difference from nonhandled group: *$p<0.05$; †$p<0.01$.

TABLE 2

EFFECTS OF HANDLING ON [^{3}H] LYSINE DURING 10-MIN INCORPORATION PERIOD. HANDLED MICE WERE INJECTED 10 MIN AFTER BEING HANDLED. MEAN ± SEM; 8 MICE PER GROUP

	Nonhandled	Handled	% Difference
A. Brain (DPM/mg wet tissue)			
Total DPM	445 ± 6	459 ± 12	−3
Insoluble DPM	41 ± 1.1	53 ± 2.6	+28§
Incorporation*	0.96 ± 0.02	1.17 ± 0.03	+22§
B. Brain (DPM/mg protein)			
Insoluble DPM	135 ± 4	169 ± 8	+25‡
C. Liver (DPM/mg wet tissue)			
Total DPM	615 ± 18	694 ± 17	+10
Insoluble DPM	204 ± 12	243 ± 9	+19†
Incorporation*	3.32 ± 0.09	3.57 ± 0.07	−8†

*Incorporation = % of Total DPM incorporated into protein (insoluble) per min.
†$p<0.05$; ‡$p<0.01$; §$p<0.001$.

whether the effects on RI in the brain also occurred in the blood, and (3) the degree to which the RI changes observed in brain might be modulated by the radioactivity in the blood trapped in capillaries of the brain at decapitation. The delay between handling and injection was again set at 10 min. Handled and nonhandled mice were killed at 5, 10, or 20 min after injection.

The time course of labeling in the three tissues, shown in Figure 1, conformed to our general expectations. Radioactivity in blood appears to have peaked prior to 5 min, but these data would also be consistent with a peak between 5 and 10 min. At 5 min, the concentration of radioactive lysine is highest in blood, and lowest in brain. Handling elevated total DPM in blood ($p<0.05$ at all time points) and in liver (N.S. at all times), but depressed total DPM in brain ($p<0.05$ only at 20 min).

Handling had no effect on the rate of incorporation of [^{3}H] lysine into proteins of either blood or liver, except for a small significant depression of RI in blood at 20 min. Thus it seems unlikely that blood-borne changes can directly account for the increased RI seen in the brains of handled mice ($p<0.001$ at all time points).

The correction for radioactivity in blood trapped in brain amounted to 4% or less of the insoluble DPM in brain at all time points. The blood contribution to soluble DPM in brain was about 13% at 5 min, 7% at 10 min, and 4% at 20 min. Thus, while insoluble DPM in trapped blood had essentially no effect on handling-induced changes in brain-insoluble-DPM at 5 and 10 min, the presence of trapped soluble DPM from blood tended to slightly lower the apparent magnitude of changes in RI in brain in the first two experiments.

Therefore, under the present circumstances, the radioactivity contributed to the brain homogenates by trapped blood can be said to have only minor effects on the phenomenon of major interest, and thus produce no

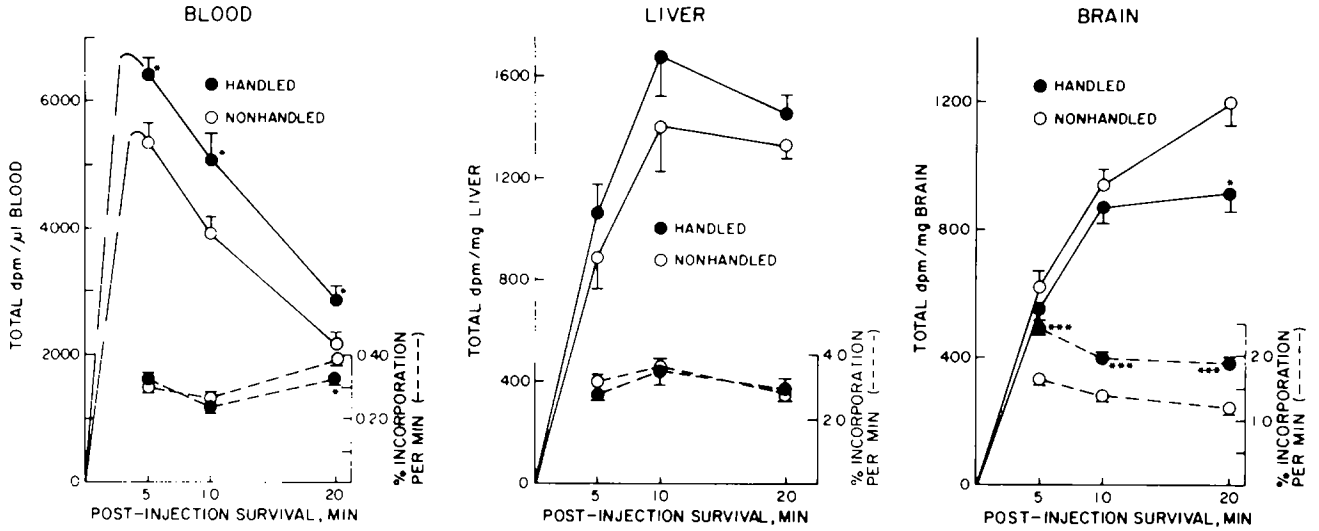

FIG. 1. Time course of effect of handling on incorporation of [^{3}H] lysine into blood, liver, and brain. Brain data have been corrected for radioactivity due to entrapped blood. Handled mice were injected 10 min after being handled. Mean ± S.E.M.; 5 mice per group.

difficulties of interpretation here.

The time-course data provided an opportunity to calculate the rate of incorporation of [^{3}H] lysine into protein using a variety of methods. The data from brain were examined by 3 methods:

Method 1 (Endpoint):

For a given time of sacrifice, t_i,

$$I(t_i) = \text{Insoluble}\ (t_i)/\text{Total}\ (t_i) \times t_i.$$

This was identical to the method used to generate the results shown in Tables 1 and 2, and Fig. 1.

Method 2 (Integrated Pool):

$$I(t_i) = \text{Insoluble}\ (t_i)/\int_0^{t_i} \text{Soluble dt}.$$

This method takes into account the time course of labeling of the soluble pool in brain, to find the average rate of incorporation of soluble [^{3}H] lysine between the time of injection and a particular time of sacrifice.

Method 3 (Interval, Integrated Pool):

$$I(t_i) = (\text{Insoluble}\ (t_i) - \text{Insoluble}\ (T_{i-1}))/\int_{t_{i-1}}^{t_i} \text{Soluble dt}.$$

This method finds the average rate of incorporation between 2 intervals of sacrifice, e.g., between 5 and 10 min. During the first interval, 0 to 5 min, the result is identical to that from Method 2.

Note that under Method 1, Incorporation is calculated for individual animals, and then averaged, while for Methods 2 and 3, Incorporation is calculated from the average values of soluble DPM and insoluble DPM for groups of animals. Thus the latter 2 methods do not lend themselves to accurate estimation of standard errors of measurement.

Results of the 3 modes of calculation are given in Table 3. All 3 methods indicate that rate of labeling is highest during the earlier portions of the 20 min labeling period; this trend is clearest under Method 3. This pattern of more rapid incorporation early in the pulse has been described before [13] and is usually attributed to the labeling of proteins with halflives on the orders of 5 to 10 min.

All methods indicate that handling elevates incorporation throughout the labeling period by 40 to 50 per cent. The progressive decline of this difference over time (Method 3) suggests either that the longer-lived proteins are relatively less effected, or that the physiological change affects most proteins, but diminishes with time.

None of the measures studied were correlated significantly with time of day.

DISCUSSION

The results show that brief handling or etherization of mice elevates the proportion of [^{3}H] lysine accumulated in total brain proteins.

(1) Is this a change in rate of protein synthesis? This question can not be answered unequivocally from these data. The approximation used here, rate of incorporation of [^{3}H] lysine into brain protein, will be elevated by increased protein synthesis, but also would be increased by a depressed rate of protein catabolism or by a reduction of the size of the free lysine pool at the sites of protein synthesis. The problem of empirically undetectable fluctuations in precursor specific activity is shared by all tracer studies of *in vivo* protein metabolism [5,6].

The metabolic disturbance, whatever its nature, has several interesting characteristics. Its magnitude is fairly large, an increase of 31% averaged across all mice studied with 10-min pulses, started 10 min after the experience, and 41% after etherization. Several characteristics suggest changes in protein synthesis or degradation rates occurred. Total radioactivity found in brain tended to be unchanged shortly after handling (Table 1, 2), although a real depression in total radioactivity might have occurred later (Table 1, "Handling-30 min"; Fig. 1, "Brain"). The time-course data (Fig. 1) indicate there were no major temporal irregularities in total radioactivity in any of the tissues between injection and 10 min. Moreover, the calculation of proportions of precursor radioactivity incorporated into brain protein over time (Table 3) are similar to

TABLE 3

INCORPORATION OF [^{3}H] LYSINE INTO BRAIN PROTEINS; COMPARISONS OF THREE METHODS FOR CALCULATING INCORPORATION FROM DATA SHOWN IN FIG. 1. METHODS ARE DESCRIBED IN TEXT

Time (Min)	Incorporation x 100 Nonhandled	Handled	% Difference H/NH
Method 1 (End Point):			
5	1.66 ± 0.06	2.48 ± 0.04	+50
10	1.40 ± 0.06	1.98 ± 0.09	+41
20	1.21 ± 0.04	1.82 ± 0.09	+51
Method 2 (Integrated Pool):			
5	3.60	5.67	+58
10	2.72	4.10	+51
20	2.14	3.11	+45
Method 3 (Interval, Integrated Pool):			
5	3.60	5.67	+58
10	2.36	3.46	+46
20	1.81	2.47	−36

those reported by other authors [13] in suggesting that a substantial portion of the labeled protein has a half-life on the order of only a few minutes. It is clear the pattern of metabolism of the injected [^{3}H] lysine has been altered, but we emphasize that simple biochemical interpretations of these data should be avoided.

(2) Are these effects specific to brain tissue? Both etherization and handling induced moderately large changes in incorporation rate in brain. In liver, etherization induced a smaller change in liver at the 10 min etherization-injection delay, but no change at the 30 min delay (Table 1). Liver changes induced by handling were small or absent (Tables 1,2, Fig. 3). Handling had no effect on incorporation of lysine into blood proteins (Fig. 1). As explained in RESULTS, blood trapped in brain tissue in the earlier experiments tended to reduce the apparent size of the "brain effect" of handling. On balance, the data strongly suggest a biochemical change in the vicinity of brain tissue. However, these data do not distinguish between neurons, glia, or the blood-brain interface as primary reactive loci.

(3) What is the physiological trigger? These experiments were not designed to answer this question directly. Taking hints from an earlier study in our laboratory [18] where more intense sensory stimulation (e.g., 20 brief footshocks over 15 min) elevated incorporation of [^{3}H] lysine in proteins of most brain regions by about 15% and the liver by 50 to 60%, it seems most likely that the present changes in incorporation are rapid responses to some circulatory hormones. Adrenal corticosteroids seem not to be responsible for the sequelae of the more intense stimulation [17]. ACTH (adrenocorticotrophic hormone) and MSH (melanocyte-stimulating hormone) are likely candidates for further study, since both hormones exhibit anabolic effects on cerebral proteins [19], have been reported to influence behavior at very low doses [4] and are elevated markedly in plasma by footshock and to a lesser degree by "psychogenic stress" [14,20]. Brief etherization is a potent stimulant of plasma ACTH and MSH levels [8,12], and may affect the pituitary by mechanisms different from those triggered by physical trauma [8].

If a circulatory hormone is involved, it is clear from comparing these data with those of Rees *et al.* [18] that the brain reacts more strongly to mild stimulation (handling) while liver reacts more intensely to stronger stimulation (footshock, loud sounds).

(4) What is the function of such changes? Why the metabolism of brain amino acids or proteins should react to stress is not clear. The functional roles in the biological economy of brain cells or tissue are simply not understood for changes such as these.

Pevzner [16] has suggested that the rate of cerebral RNA synthesis is an inverted-U function of the amount of sensory input. Previous results suggest that the present changes may represent the early, moderate-intensity, end of a similar function for brain protein synthesis, with stronger and more repetitious stimulation [18] inducing smaller increases in synthesis, and intense exercise [22] producing decreases in synthesis.

The behavioral or psychological importance of this reactivity is also uncertain. While it is clear from the above discussion that we find it heuristically useful to consider these events as "reactions to stress," we are unable to suggest useful direct measures of the intensity (as viewed by the mice) of the stressors studied. We believe that the standardized handling treatment employed here is a relatively weak stressor compared to many of the treatments routinely applied to laboratory rodents. It consisted of one slow and brief lift by the tail, followed by gentle sliding from the holding beaker. The modal reaction of the mice to the tail-lift was a spinal flexion that directed the head and forepaws toward the tail, with some flailing of all limbs.

Nonetheless, for future hypotheses and experiments it is important to note the following. (A) Handling can be an aversive stimulus to mice. (B) Handling is also a somewhat novel stimulus to these mice, since it is likely that they had not been handled by humans more than 10 times previously (mainly at weekly intervals to change their bedding). Stimulus novelty may have potent information-storage sequelae in mammals [5]. Certain effects of sensory stimulation upon brain and liver protein synthesis [18] and adrenal cortical function [9] habituate to repeated stimulation; others do not. (C) The effect of handling was detectable against a baseline of any similar effects that might have been induced by the more vigorous and stressful

(all mice struggled, most squealed) stimulation associated with injections of [^{3}H] lysine. The differential effect might be due to an interaction between the two manipulations.

(5) Implications for previous and future studies. The methods used here were highly similar to those used in many investigations of influences of hormones, drugs, sensory input, and behavioral training upon cerebral protein metabolism [5,6]. Without citing a variety of specific instances, it is clear that the changes seen after the present simple laboratory manipulations are of the same order of magnitude as those reported for more complicated treatments.

If the present findings are found to generalize across mouse strains, species, sex, age, etc., then great care will have to be taken in equating the amount of handling or etherization applied to various experimental groups. Of greater practical and theoretical importance is the very real possibility that various influences upon cerebral amino acid and protein metabolism combine in nonlinear fashion. Thus, stimulation as trivial as handling may push metabolic rates or measures to a ceiling that obscures or reverses otherwise positive influences of additional physiological input. "Trivial" here is used only to imply that experimental designs usually require some handling, and if ceiling effects occur, a simple equation of handling between groups may lead to instances of false negative results.

Extreme speculation suggests that effects similar to those seen here may have introduced a particular artifact into fundamental studies of basal rates of cerebral protein synthesis. It is a common finding in such studies that the average rate of incorporation of amino acids into brain total proteins tends to decrease as the length of the isotope pulse increases, particularly over the range of 5 to 60 min. This has usually been interpreted as evidence for a substantial fraction of brain proteins having half-lives on the order of tens of minutes. Given the present results, it seems possible that elevated incorporation early in a pulse might reflect stress-induced physiological departures from baseline rates of synthesis.

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