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Effect of L-Fucose on Brain Protein Metabolism and Retention of a Learned Behavior in Rats[1]

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WETZEL, W., N. POPOV, B. LÖSSNER, S. SCHULZECK, R. HONZA AND H. MATTHIES. *Effect of L-fucose on brain protein metabolism and retention of a learned behavior in rats.* PHARMAC. BIOCHEM. BEHAV. **13**(6) 765–771, 1980.—The intraperitoneal or intraventricular application of L-fucose (100 mg/kg or 75 μg, respectively) prior to training in shuttle box avoidance as well as in shock-motivated brightness discrimination in rats significantly improved the retention of learned behavior 24 hr later. The application of D-fucose was without influence on retention. In naive animals, intraventricularly applied L-fucose (75–200 μg) caused an increase in the rate of protein synthesis in the hippocampus, resulting in a significant increase in total proteins of this brain structure, mainly attributed to the Tris-insoluble protein fractions. The results are discussed in terms of an activation of glycoprotein formation by increasing supply with L-fucose.

L-Fucose Shuttle-box Y-maze Memory retention Hippocampal proteins Rats

RECENTLY, there has been increasing evidence of the particular role of glycoproteins in adaptive functions of neuronal membrane components determining the efficiency of interneuronal connections [1, 6, 18] and the resulting information processing and storage [3, 4, 19, 28].

Radioactive L-fucose was proven to be a suitable precursor for the investigation of glycoprotein biosynthesis, since it is not converted to other sugars [2, 9, 38] and does not seem to be a significant constituent of glycolipids and mucopolysaccharides [12, 16, 27, 40]. Thus, substantial changes in L-fucose incorporation into brain tissue after different learning experiments and environmental exposures were considered as a reflection of altered glycoprotein synthesis [7, 10, 11, 32, 34, 36, 37].

The mechanisms determining the formation of individual glycoprotein molecules as well as the significance of the terminal position of fucose at the glycan chains anchored to the corresponding polypeptides to form glycoproteins are poorly understood (for reviews see [17, 18, 30, 39]). Fucose has to be phosphorylated [9,21] and transformed into GDP-fucose [20], before finally coupling to a previously formed glycan chain of a glycoprotein [5,25]. But the significance of the relatively small pool of free L-fucose in brain tissue (we determined only 0.02 μmoles/g in rat hippocampus in comparison to 0.8 μmoles/g bound fucose in the same brain structure [33]) for the rate of glycoprotein synthesis is not easily assessed, since the kinetics of the three enzymatic reactions is not yet definitely known, which is also true of rate-limiting step(s) and regulating factors.

In order to gather more information on the metabolic and functional importance of fucose and its incorporation into glycoproteins, we investigated the effects of fucose application on brain protein synthesis as well as on acquisition and retention of two different learning tasks in rats.

METHOD

Biochemical Experiments

Male Wistar rats weighing approximately 180–190 g were used. The animals were provided with food and water ad lib.

L-(1-^{3}H)fucose (specific radioactivity 1 Ci=37 GBq/mmole) and L-(4,5-^{3}H)leucine (specific radioactivity 58 Ci=2.146 TBq/mmole) were purchased from The Radiochemical Centre, Amersham, Great Britain.

The hippocampus was homogenized in a 20-fold volume of ice-cold Tris-HCl buffer, pH 7.4, and centrifuged in a refrigerated ultracentrifuge at 100,000×g for 60 min. The resulting supernatant, designated as the soluble proteins, contained approximately 35–40% and 15–20% of the initial homogenate proteins and fucose-containing glycoproteins, respectively. The insoluble pellet was solubilized in a mixture of 8 M urea, 10% β-mercaptoethanol, 5% Triton-X-100 and 0.05 M K_2CO_3 and designated as the solubilized insoluble proteins, containing the rest of the 60–65% proteins and 80–85% fucose-containing glycoproteins [33].

The protein content in homogenates was determined by the method of Lowry *et al.* [26] with bovine serum albumin as the reference substance, and by using an amido black technique [35] elaborated for protein content measurements of solubilized material, since constituents of the solubilizer used interfered with the Folin phenol reagent employed by Lowry *et al.* [26]; therefore, the protein content in solubilized fractions was determined by the amido black proce-

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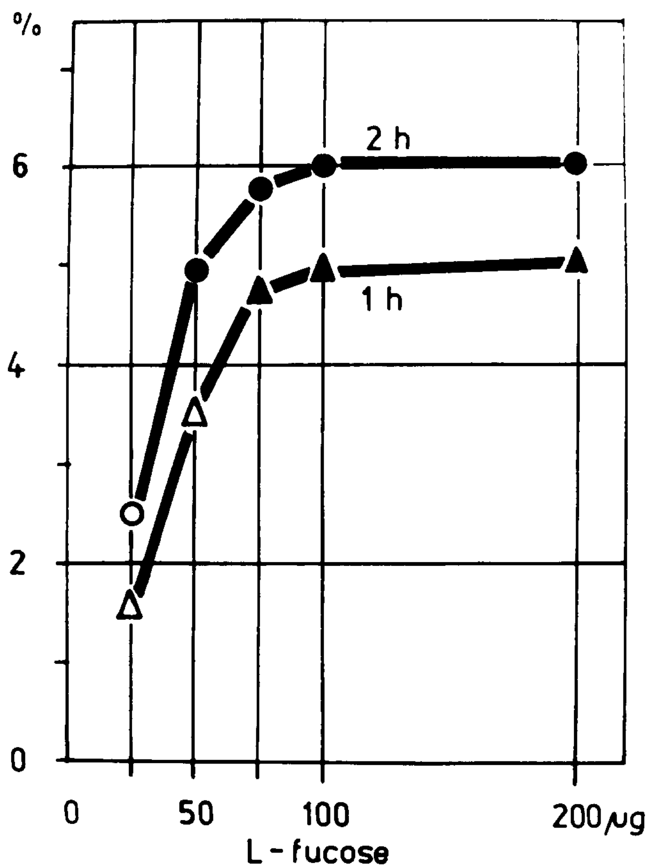

FIG. 1. Percentage increase in total protein content of rat hippocampus vs controls 1 and 2 hr after intraventricular application of 25–200 μg L-fucose dissolved in 10 μl artificial cerebrospinal fluid. The calculation was based on measurements of mg protein per g wet tissue, while the DNA content was not altered by fucose application. Each point represents the mean of eight determinations in duplicate. The filled circles and triangles denote statistically significant values ($p<0.05$).

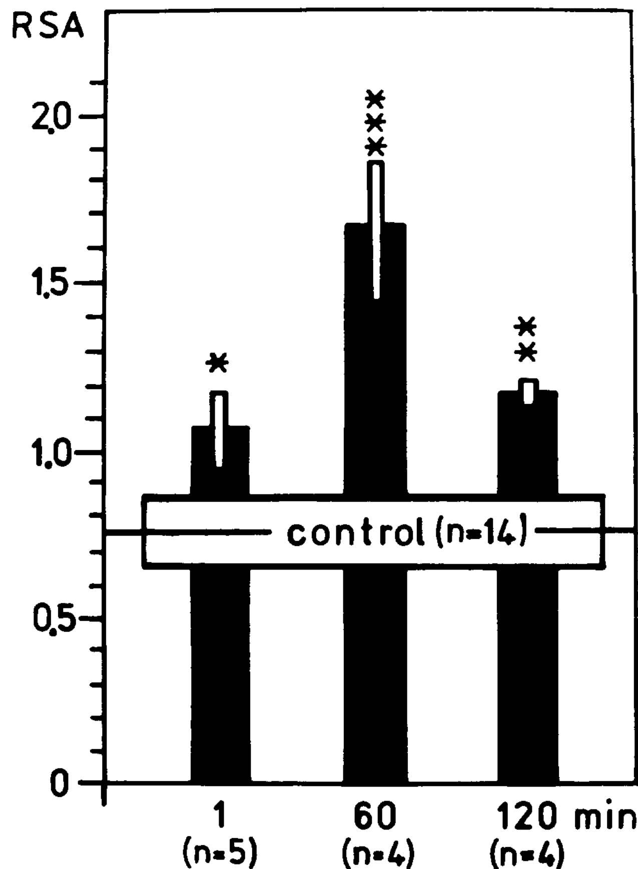

FIG. 2. Influence of L-fucose on the incorporation of tritiated L-leucine into TCA-insoluble proteins of rat hippocampus. The incorporation of labeled leucine is expressed as relative specific activity (RSA, ratio of specific radioactivity of proteins to radioactivity of free, non-incorporated leucine). Tritium-labeled leucine was intraventricularly injected 1, 60 or 120 min after intraventricular application of 75 μg L-fucose. The controls received artificial cerebrospinal fluid instead of L-fucose. The incorporation time was 15 min. Number of animals is given in parentheses. Data are presented as means ± SEM. *=$p<0.05$; **=$p<0.02$; ***=$p<0.01$, fucose-treated animals vs controls.

dure only. DNA content was determined by the method of Burton [8].

In a first series of experiments, rats were intraventricularly injected with 25, 50, 75, 100 and 200 μg (0.152, 0.305, 0.457, 0.610 and 1.220 μmoles) L-fucose as well as 100 μg (0.610 μmoles) D-fucose dissolved in 10 μl artificial cerebrospinal fluid (ACF) [29]. The controls received ACF only. The rats were decapitated 1 and 2 hr after injection.

In a second series, 100 μCi (=3.7 MBq) (^{3}H)leucine was intraventricularly injected 1, 60 and 120 min after application of 75 μg L-fucose. Controls received 10 μl ACF before injection of labeled leucine. The leucine incorporation into hippocampal proteins was expressed as relative specific activity (RSA), i.e. the ratio of specific radioactivity of proteins to the radioactivity of free, non-incorporated precursor [22,24].

In a third series, it was tested if under the present experimental conditions in vivo the L-fucose incorporation system was specific for L-fucose or could be influenced by the unnatural for mammalian tissue D-isomer as well as by the presumed L-fucose precursor D-mannose [9, 14, 15] and the unnatural L-mannose. Thirty rats were divided into 5 groups of 6 animals each: (a) *Controls:* Intraventricular injection of 100 μCi (=3.7 MBq) tritium-labeled L-fucose (containing 16 μg=0.100 μmoles L-fucose) dissolved in 10 μl ACF. (b) *L-Fucose-group:* 100 μCi labeled L-fucose plus 0.510 μmoles L-fucose (a total of 0.610 μmoles L-fucose) in 10 μl ACF. (c) *D-Fucose-group:* 100 μCi labeled L-fucose plus 0.610 μmoles D-fucose in 10 μl ACF. (d) *D-Mannose-group:* 100 μCi labeled L-fucose plus 110 μg (=0.610 μmoles) D-mannose in 10 μl ACF. (e) *L-Mannose-group:* 100 μCi labeled fucose plus 110 μg L-mannose in 10 μl ACF. The incorporation time was 2 hr in all cases of this experiment. The technique concerning sample preparation for radioactivity measurements is described elsewhere [33].

Student's t-test was used to evaluate the statistical significance of differences between means.

Behavioral Experiments

In the first experiment, 18 adult male Wistar rats weighing 150–200 g and housed with 10 animals per cage were used. Food and water were available ad lib.

An automatic shuttle-box (25×25×60 cm) with a grid floor and buzzer located at the midline was used. The box was divided into two equal compartments by a 5 cm hurdle. Each compartment could be illuminated by 40 W bulbs mounted in the lids. Prior to the training session the animals

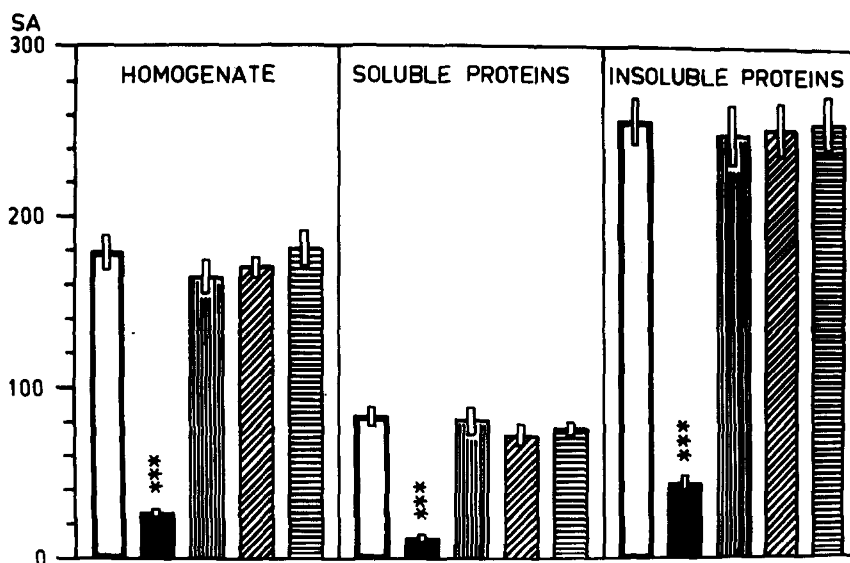

FIG. 3. Effects of intraventricularly injected L-fucose (black columns), D-fucose (vertically shaded columns), D-mannose (diagonally shaded columns) and L-mannose (horizontally shaded columns) in equimolar amounts (0.610 μmoles) on the incorporation of L-(1-^{3}H)fucose (100 μCi, 0.100 μmoles L-fucose) into rat hippocampus 2 hr after combined application. The controls (white columns) received tritiated fucose in cerebrospinal fluid only. Specific activity is expressed in disint/min/μg protein ± SEM (six determinations in duplicate in each case). ***=p<0.01, experimental animals vs controls. For further details see the section 'Methods'.

were allowed to explore the box for 5 min. The conditioned stimulus (buzzer plus light) lasted 4.4 sec and was followed by a 0.4 mA foot-shock if the animal did not avoid. The intertrial interval was 30 sec. The training session was continued until the animal reached a criterion of 5 avoids. Animals not reaching the criterion during 70 trials were eliminated. Retention was tested 24 hr after training in a relearning session using the same criterion. One hundred mg/kg L-fucose was injected intraperitoneally 30–40 min before the training session. Controls received saline injections (1.0 ml/100 g body weight).

In the second experiment, we used 20 adult male Wistar rats weighing 190–250 g. They were housed with 10 animals per cage and provided with food and water ad lib. The rats were trained to learn a foot-shock motivated brightness discrimination in a semiautomatic Y-maze [31]. Runs into the dark alley were punished by application of 1 mA foot-shocks, whereas the illuminated alley was the safe area. The mean intertrial interval was 1 min. The training session was completed after 22 trials. Retention of the brightness discrimination task was tested in a relearning session 24 hr after training. For further details see [31]. One hundred mg/kg L-fucose was injected intraperitoneally 30 min before the training session. Control animals received saline injections (1.0 ml/100 g body weight). Evaluation of results: Running into the dark alley of the Y-maze was counted as an error. Number of training errors and number of relearning errors were used to calculate the % savings:

$$\% \text{ savings} = \frac{\text{training errors - relearning errors}}{\text{training errors}} \times 100\%$$

In the third experiment, we used 157 adult male Wistar rats weighing 180–220 g. They were housed in single cages and provided with food and water ad lib.

One week prior to the experiments, a chronic microcannula was implanted into the right lateral ventricle under light hexobarbital-urethane anesthesia. Experimental animals received intraventricularly L-fucose or D-fucose dissolved in 10 μl ACF injected through the implanted cannulas. Controls received 10 μl ACF only.

The rats were trained to learn a foot-shock motivated brightness discrimination as described for the second experiment. The training session was completed after 40 trials (in the experiments related to different injection-training intervals) or 31 trials (in all other experiments). In the first series, 75 μg L-fucose was injected 4 hr or 30 min before or 1 min after the training session. In the second series, 75 μg or 150 μg L-fucose was injected 30 min before training. In the third series, 30 min pre-training injections of either 75 μg L-fucose or D-fucose were given. In all experiments, retention of the brightness discrimination task was tested in a relearning session 24 hr after training.

For statistical evaluation of behavioral results, the Mann-Whitney U-test was used.

RESULTS

Biochemical Experiments

As depicted in Fig. 1, intraventricularly applied L-fucose, at doses exceeding 75 μg/10 μl ACF, caused a small but statistically significant increase in total proteins of rat hippocampus, whereas D-fucose exhibited no effect at the same dosage. As shown in Table 1, this increase was mainly attributed to the Tris-insoluble fraction (involving predominantly membrane constituents).

The results obtained with labeled leucine after L-fucose premedication (Fig. 2) confirm the above findings in terms that a true increase in protein synthesis may have become evident.

As demonstrated in Fig. 3, within 2 hr after fucose application there were no signs of influence of L-fucose due to exogenous applied D-fucose, D-mannose and L-mannose, since the dilution degree of labeled L-fucose was influenced

TABLE 1

EFFECT OF INTRAVENTRICULARLY APPLIED L-FUCOSE OR D-FUCOSE (100 μg/10 μl ARTIFICIAL CEREBROSPINAL FLUID) ON PROTEIN CONTENT OF RAT HIPPOCAMPUS FRACTIONS*

	Controls	L-fucose treatment	D-fucose treatment
Homogenate	92.4 ± 1.8	99.1 ± 1.3†	91.7 ± 1.4
Soluble proteins	38.8 ± 2.7	41.7 ± 1.9	40.0 ± 2.4
Insoluble proteins	50.7 ± 2.6	59.8 ± 3.2†	52.8 ± 3.0

*Means of 6 determinations in duplicate of protein content using an amido black technique [35], expressed as mg/g wet tissue ± SEM.

†$p<0.05$, 2 hr L-fucose treatment vs controls receiving 2 hr before death artificial cerebrospinal fluid only.

TABLE 2

EFFECT OF 100 mg/kg L-FUCOSE INJECTED INTRAPERITONEALLY 30 MIN BEFORE TRAINING ON RETENTION OF BRIGHTNESS DISCRIMINATION*

Group	n	Training errors	Relearning errors	% savings
Controls	10	7.7 ± 0.6	4.6 ± 0.6	38.4 ± 8.3
L-fucose-treated animals	10	7.8 ± 0.5	3.3 ± 0.4	58.8 ± 3.0†

*Means ± SEM.

†$p<0.05$.

(in a proportional manner) by addition of L-fucose only, i.e. it is most likely that L-fucose remained as unchanged substrate during the 2 hr experiments.

Behavioral Experiments

In the first experiment using a shuttle-box procedure, the number of training trials was not influenced by the L-fucose pretreatment (Fig. 4). However, the number of relearning trials to reach the criterion was significantly reduced in fucose-treated animals. Escape latencies and intertrial crossings were not changed by L-fucose.

Table 2 shows the results of the second experiment. While the number of training errors was not influenced by the intraperitoneal injection of 100 mg/kg L-fucose, the number of relearning errors in the fucose group was reduced, thus resulting in significantly higher % savings.

The results of the third experiment using different injection-training intervals are summarized in Fig. 5. A significant improvement of the 24 hr retention was found only if L-fucose was injected 30 min before the training session. Therefore, this interval between intraventricular injection and training was used in the further experimental series.

Table 3 shows the influence of 75 and 150 μg L-fucose on retention of the brightness discrimination task. Both doses of the substance exhibited a retention-enhancing effect; the more pronounced effect induced by application of 75 μg L-fucose was statistically significant.

In the third series of this experiment, it was found that after application of 75 μg L-fucose, but not after 75 μg D-fucose, retention was improved (Table 4). Animals in-

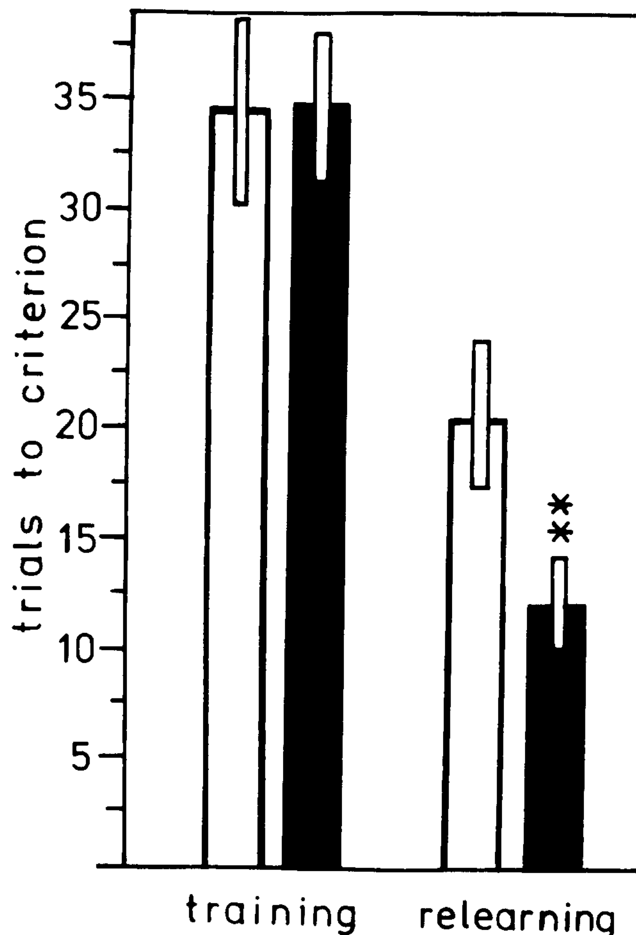

FIG. 4. Effect of 100 mg/kg L-fucose injected intraperitoneally 30–40 min before training on retention of shuttle-box avoidance (means of seven animals in each group ± SEM). **=$p<0.02$, L-fucose-treated animals (black columns) vs controls (white columns).

jected with D-fucose showed relearning values similar to those of the controls. The relearning differences between L-fucose and D-fucose were statistically significant. In Fig. 6, mean values of all rats receiving 75 μg L-fucose intraventricularly 30 min before training (resulting from the second and third experimental series) are summarized. The difference between L-fucose-treated animals and related controls in relearning errors is clearly shown.

DISCUSSION

Previous investigations in our laboratory demonstrated that after acquisition of a brightness discrimination in rats, the incorporation of L-fucose into hippocampal proteins was significantly increased, indicating an augmentation of the glycoprotein formation in this brain structure during consolidation of a learned behavior [32,36]. The different parts of the hippocampus formation showed differences with regard to the groups of proteins so far involved as well as to the time course of their synthesis [32,36]. These results support the findings of other laboratories on the increased glycoprotein formation in connection with the formation of memory traces as well as correlates of responses to environmental changes [3, 7, 11, 19].

We also observed that stimulation of dopaminergic recep-

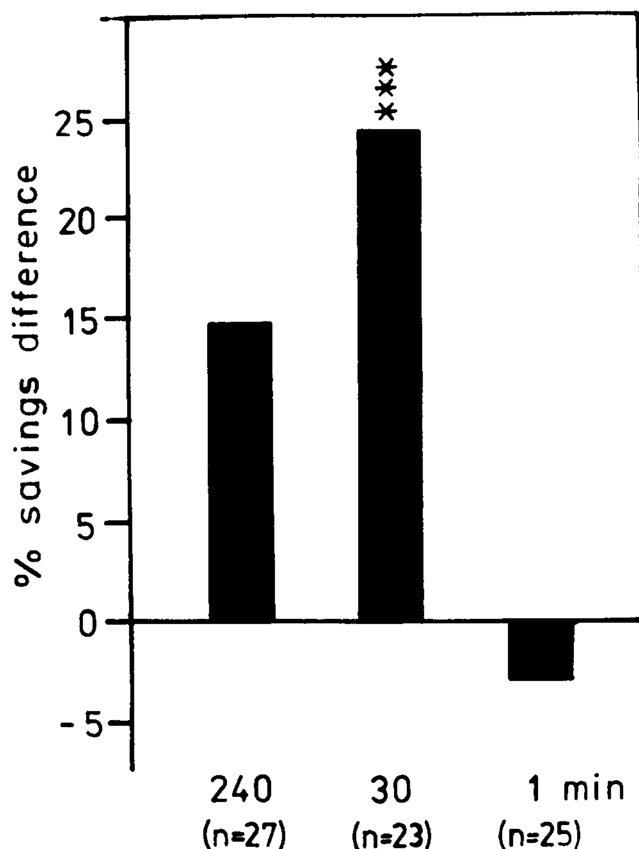

FIG. 5. Effect of 75 μg L-fucose injected intraventricularly 4 hr or 30 min before training or 1 min upon completion of training on retention of brightness discrimination. Ordinate: % savings of L-fucose-treated animals minus % savings of controls. Number of animals is given in parentheses. ***=$p<0.01$.

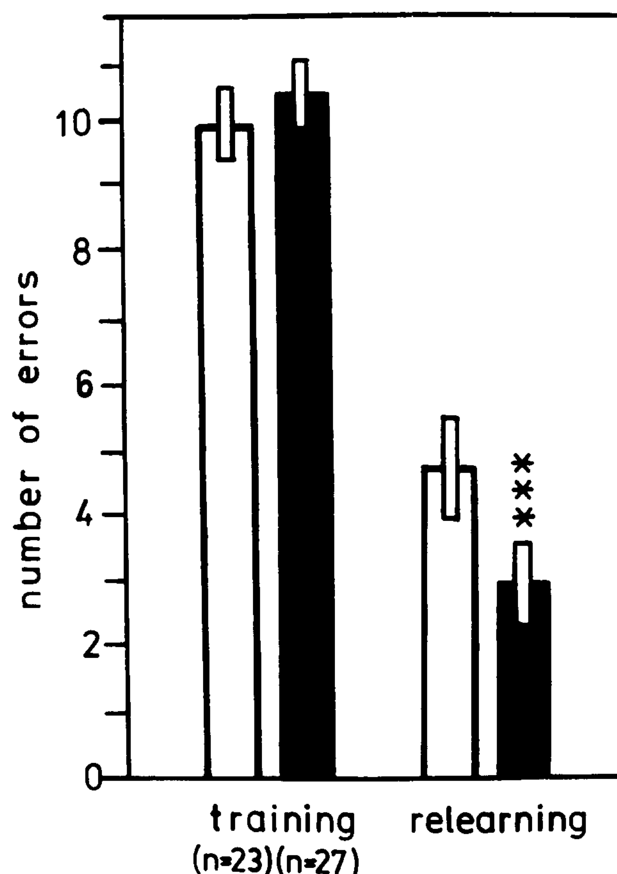

FIG. 6. Effect of 75 μg L-fucose injected intraventricularly 30 min before training on retention of brightness discrimination (means ± SEM). Number of animals in parentheses. ***=$p<0.01$. L-fucose-treated animals (black columns) vs controls (white columns).

TABLE 3

EFFECT OF TWO DIFFERENT DOSES OF L-FUCOSE INJECTED INTRAVENTRICULARLY 30 MIN BEFORE TRAINING ON RETENTION OF BRIGHTNESS DISCRIMINATION*

Group	n	Training errors	Relearning errors	% savings
Controls	16	9.4 ± 0.8	4.5 ± 0.7	53.6 ± 6.4
75 μg L-fucose	19	10.7 ± 0.8	2.9 ± 0.3	71.1 ± 3.7†
150 μg L-fucose	21	10.7 ± 0.7	3.3 ± 0.4	64.8 ± 4.7

*Means ± SEM.
†$p<0.05$, L-fucose-treated animals vs controls.

TABLE 4

EFFECT OF L-FUCOSE OR D-FUCOSE INJECTED INTRAVENTRICULARLY 30 MIN BEFORE TRAINING ON RETENTION OF BRIGHTNESS DISCRIMINATION*

Group	n	Training errors	Relearning errors	% savings
Controls	7	11.1 ± 1.0	5.1 ± 1.5	55.7 ± 10.3
75 μg L-fucose	8	9.6 ± 1.0	2.5 ± 0.9†	74.1 ± 8.3
75 μg D-fucose	11	11.6 ± 0.8	4.3 ± 0.5	61.8 ± 5.0

*Means ± SEM.
†$p<0.05$, L-fucose-treated animals vs D-fucose-treated animals.

tors in the hippocampus by post-trial microinjection of apomorphine did improve the retention of a brightness discrimination [28] as well as increase the incorporation of labeled L-fucose into hippocampal proteins in vivo and in vitro [23], thus indicating a possible role of a dopaminergic system operating in the consolidation of a long-term trace.

The present results obviously confirm the assumption that glycoproteins with fucosyl endgroups in their glycan chains seem to play an important functional role in the consolidation of a long-term trace, as the retention of the acquired behavior can be significantly improved in two different learning tasks, in the brightness discrimination as well as in the conditioned avoidance of a shuttle-box paradigm, by application of L-fucose. The effect is specific to L-fucose, the physiological substrate participating in the glycosylation of proteins, while D-fucose is ineffective.

The application of L-fucose 30 min before training is much more effective than 4 hr before training, but ineffective

after training. This would not indicate a direct effect on acquisition, since the training parameters do not differ in controls and treated animals. Rather we have to consider that L-fucose obviously needs some time for distribution in the brain tissue after intraventricular injection. Nevertheless, a maximal effect seems to depend also on the occurrence of an increased fucose concentration at a relatively early stage of consolidation, which already arises during the training period and shortly thereafter. During this time, the availability of free fucose probably determines the rate of the last step of fucosyl glycoprotein formation and the resulting changes. It is difficult, however, to explain at present the effects of L-fucose on the synthesis of peptide chains, as expressed by the increased RSA values of hippocampal proteins after labeling with tritiated leucine as well as by the increase in the amounts of proteins. Two mechanisms can be mainly discussed in this connection: (a) A negative feedback control of not yet fucosylated glycoproteins on the synthesis of their corresponding polypeptides or on the polypeptide synthesis in general, or (b) the formation of fucose-containing glycoproteins functioning as activators at the translational level of protein synthesis. Which of these alternatives were relevant, in any case the increased formation of glycoproteins by the terminating step of fucosylation would induce the supply of newly synthesised polypeptide chains for its further formation. Future investigations may elucidate the validity of this assumption.

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