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Studies of Memory: A Reevaluation in Mice of the Effects of Inhibitors On The Rate of Synthesis of Cerebral Proteins As Related to Amnesia

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RAINBOW, T. C., P. L. HOFFMAN AND L. B. FLEXNER. *Studies on memory: A reevaluation in mice of the effects of inhibitors on the rate of synthesis of cerebral proteins as related to amnesia.* PHARMAC. BIOCHEM. BEHAV. **12**(1) 79–84, 1980.—Tests were made of the postulates stating that the degree of inhibition of protein synthesis either (a) at training or (b) following training is the critical variable that determines the degree of amnesia. As a first step it was found that the concentrations of numerous cerebral amino acids were substantially increased in 2 strains of mice 0.5 hr after treatment with amnesic doses of the inhibitors of protein synthesis, cycloheximide (CXM) and anisomycin. This observation led, in several different experiments, to a comparison of the apparent degree of inhibition of protein synthesis derived from the acid-soluble radioactivity with that derived from the specific radioactivity of tyrosine tagged with L(1-^{14}C)-tyrosine. In all instances the apparent degree of inhibition was decreased when based upon tyrosine's specific radioactivity. The effect of several treatments with CXM on memory of a 1-trial passive avoidance task provided data for analysis of the relationship between the degrees of amnesia and those of the more accurate estimates of inhibition of protein synthesis based upon the specific radioactivity of tyrosine. The results failed to support the views that the level of inhibition of protein synthesis at or after training are entirely sufficient to account for the behavioral results. The results also suggest that CXM produces with time some direct or indirect change in the brain that antagonizes the amnesic effects of the antibiotic and that consequently contributes to the survival of memory in mice trained 2 hr after a large amnesic dose of CXM.

Amnesia Memory Protein synthesis inhibition Cycloheximide Anisomycin

IN order to use a radioactively labeled amino acid's incorporation to determine the rate of synthesis of protein with maximal precision, it is essential to know the specific radioactivity of the immediate precursor pool. This is best approached by determination of the specific radioactivity of aminoacyl-t-RNA [1]. The difficulties of this determination, however, have usually led to substitution for it of the specific radioactivity of the precursor amino acid. In studies that correlate the amnesic effects of an antibiotic with its degree of inhibition of cerebral protein synthesis, the approach to characterization of the precursor pool has been further simplified. Measurements have been made, following treatment with the inhibitor, only of changes in the acid-soluble radioactivity of an homogenate; elevations in the endogenous level of an amino acid which may be important except at extreme levels of inhibition have not been included. On occasion, per cent inhibition of protein synthesis has been derived from the ratio of dmp/mg protein of antibiotic-treated mice to that of controls [17] thus again neglecting any differences between the 2 groups in the amounts of endogenous amino acid that may accompany the labeled precursor incorporated into protein.

These considerations have led us to investigate the effect of amnesic doses of 2 inhibitors of protein synthesis, cycloheximide (CXM) and anisomycin (Ani), on the cerebral levels of a series of amino acids. Since the concentration of many of these amino acids was increased by the antiobiotics, we then compared the apparent degree of inhibition of protein synthesis as derived from the radioactivity of a precursor amino acid with that derived from its specific radioactivity. A study, designed in part like earlier investigations [17], of the effect of several treatments with CXM on memory of a 1-trial passive avoidance task followed the chemical studies. These behavioral experiments provided observations for analysis of the relationship between the degree of amnesia and the degree of inhibition of protein synthesis. We were particularly interested in the finding of Squire and Barondes [23] that a dose of CXM which is amnesic when given 0.5 hr before training fails to cause amnesia when given 2 hr before training. Two important suggestions were based on this latter

finding; first, that at 2 hr there is sufficient synthesis of protein to sustain memory since inhibition of synthesis was found to be 20–30% less severe at 2 hr than at 0.5 hr after treatment; and, second, that several side-effects of CXM, which persist after the 2 hr interval, are ruled out as causative agents of the loss of memory. We have tested and found evidence for the possibility that the lack of amnesia following training 2 hr after treatment with CXM is due to the development of changes in the brain that antagonize amnesia rather than to a decrease in the severity of inhibition of protein synthesis.

METHOD

Young adult male C57B1/6J (Jackson Laboratories) and Swiss (West Jersey Biological) mice were housed 4 to a cage at room temperature with free access to food and water. CXM and Ani (gift of Pfizer, Inc.) were injected SC in 0.1 ml of saline. All mice were randomly assigned to groups and were sacrificed by cervical disolation between 1–3 p.m.

Biochemical

Amino acid analyses, with the exception of those for tyrosine and tryptophan, were carried out using a Durrum D-500 analyzer and the standard analysis method [21]. For this purpose, the supernatant obtained after homogenizing the cerebral hemispheres of a single mouse with 4 ml of 3% sulfosalicylic acid was applied directly to the column. In other samples, protein was precipitated with 6% TCA, and tryptophan in the supernatant was determined by the method of Denkla and Dewey [7] as modified by Bloxam and Warren [6]; tyrosine was measured by the method of Waalkes and Udenfriend [25], after passing the supernatant through a column of Dowex 50 W × 4 (H^+ form) [15].

Tyrosine was used to estimate the rate of synthesis of protein because of its ease of determination [25], the availability of carboxyl-labeled tyrosine, the radioactivity of which on degradation is lost as $^{14}CO_2$ and because, like leucine and valine, it is rapidly incorporated into cerebral proteins [4]. Mice were injected IP with 0.5 or, for low rates of synthesis, one μCi of L(1-^{14}C)-tyrosine (New England Nuclear) and sacrificed 10 min later. Protein of the cerebral hemispheres was precipitated by homogenization with 5 ml of cold 6% TCA followed by immersion in ice for 30 min. After centrifugation, 1 ml of the supernatant fluid was used for scintillation counting and the remainder applied to a Dowex column. Aliquots of the column eluate were taken for measurement of radioactivity and for determination of tyrosine. The TCA pellet, after washing with cold 12% TCA, 6% TCA and water, was dissolved overnight in NCS tissue solubilizer (Amersham) and added to OCS scintillation fluid (Amersham) after adjustment of the sample pH to 6–7 with glacial acetic acid. CPM were determined to a 5% standard error and were corrected for quenching. Small corrections due to chemoluminesce and contamination from the TCA supernatant fluid were applied to the TCA pellet. Degree of inhibition of protein synthesis was estimated (a) from the ratio of the radioactivity of the cold TCA in soluble fraction of brain to the radioactivity of the cold TCA soluble fraction and (b) from the quantity of tyrosine incorporated into the TCA insoluble fraction as derived from the product of its radioactivity and the reciprocal of the specific radioactivity of the TCA soluble fractions.

These estimates of inhibition of protein synthesis were made in C57B1/6J mice after treatment with 120, 30 and 15 mg/kg of CXM at times shown in Table 2. Analyses were also made on mice that had 2 sequential injections of CXM, the first of 120 mg/kg at 2 hr followed by the second of 30 mg/kg at 0.5 hr before sacrifice. Analyses in Swiss mice were limited to those treated with 120 mg/kg of CXM. The effect of 30 mg/kg of Ani on rate of protein synthesis was followed in both strains.

Behavioral

C57B1/6J mice were trained in a one-trial passive avoidance task using a two-compartment box identical, except for the absence of a light in the large compartment, to that described by Randt *et al.* [18]. A mouse was placed in the small compartment that was brightly illuminated from outside. Entry latencies into the large compartment were noted. Foot shock of 0.6 mA, the minimal shock level that produced consistent escape latencies in control and treated mice, was given immediately after a mouse entered the large compartment and was continued until it escaped back into the small compartment. Escape latencies were recorded. The mouse was then returned to its home cage. Retention tests duplicated the training procedure except that shock was omitted. Entry latencies in the retention tests were recorded up to 300 sec.

Behavioral experiments were limited to mice treated with CXM. Five groups of mice treated with CXM before training were retention-tested 24 hr after training. Three of these groups were treated, respectively, 0.5 hr before training with 120, 30 or 15 mg/kg. A fourth group received 120 mg/kg 2 hr before training. Subjects of the fifth group each received 2 injections of CXM, the first of 120 mg/kg 2 hr before training to be followed by the second of 30 mg/kg 0.5 hr before training (hereafter referred to as the 120/30 mg per kg group). Retention was tested 72 hr after training in one group trained 0.5 hr after 120 mg of CXM. Saline injections in controls matched the treatment-training intervals of the experimental groups. An additional control was added for the 120/30 mg per kg group. These control mice received noncontingent shock 0.5 hr after the second injection and 24 hr later were tested for their entry latencies in the passive avoidance apparatus.

Unless otherwise stated, statistical analyses were made with the Kruskal-Wallis one-way analysis of variance and *post hoc* comparisons with the Mann-Whitney U test.

RESULTS

The amino acid analyses (other than those of tyrosine and tryptophan) for each group of mice were made on samples from 2 different brains, thus providing two independent observations of the concentrations of each amino acid for each group. Of the 75 pairs of determinations, the members of the pairs in 45 instances agreed within 5%; in 26, within 6–11%; and in 4, within 12–17%. The averages of the concentrations of a pair have been used to derive the data of Table 1.

In all mice treated with Ani or CXM the concentrations of those amino acids of relatively small pool size (i.e., Thr, Pro, Val, Met, Ile, Leu, Phe, Lys, Arg, Tyr and Try) were increased to about 130–300% of control values. The concentrations of amino acids of larger pool size (i.e., Asp, Glu, Gly and Ala) showed little or no change in C57B1/6J mice treated with either of the two inhibitors or in Swiss mice treated with Ani. In Swiss mice treated with CXM, however, there was

TABLE 1

EFFECT OF ANISOMYCIN (ANI) AND CYCLOHEXIMIDE (CXM) ON CONCENTRATIONS OF CEREBRAL AMINO ACIDS IN C57B1/6J AND SWISS MICE

	C57B1/6J			Swiss		
Amino acid	Saline n mole/γ	ANI *% saline	CXM % saline	Saline n mole/γ	ANI % saline	CXM % saline
Asp	3204	102	108	2668	100	133
Thr	252	141	161			
Glu	10893	97	101	11571	80	111
Pro	70	189	231	103	176	202
Gly	924	109	117	755	100	160
Ala	529	88	101	691	80	97
Val	57	207	241	62	200	285
Met	42	170	177	32	191	253
Ile	33	148	170	28	189	232
Leu	60	152	188	54	181	261
Phe	43	160	167	42	136	267
Lys	164	158	182	225	131	154
Arg	116	176	209	185	143	157
Tyr	79	225	249	71	246	320
Try	6.0	200	148	3.9	192	203

Ani (30 mg/kg) and CXM (120 mg/kg) injected S. C. 30 min before sacrifice. With the exception of Tyr and Try (for each n=3–5 per group), values are averages of determinations made on 2 independent samples of cerebral hemispheres. The means ± S.E.M. (n mole/g) for Tyr and Try in C57B1/6J mice treated with saline were, respectively, 78.8 ± 8.0 and 6.0 ± 0.3; with Ani, 178 ± 18.2 and 12.0 ± 1.3; with CXM, 196 ± 14.6 and 8.9 ± 1.1. Corresponding values in Swiss mice treated with saline were 71.0 ± 3.9 ± and 3.9 ± 0.4; with Ani, 175 ± 6.9 and 7.5 ± 0.4; with CXM, 227 ± 10.1 and 7.9 ± 0.9.

*% saline=% of concentration in mice injected with saline.

evidence of an increase in concentration of both Asp and Gly. In both strains of mice, the combined amino acid concentrations of the Ani and CXM groups were significantly greater than that of the controls (t-test, $p<0.001$).

As judged by results with tyrosine, the duration of the effect of CXM (120 mg/kg) in C57B1/6J mice on the concentration of amino acids persisted for 4 hr; tyrosine's concentration was about 250% of the control value 0.5, 2 and 4 hr post-treatment and normal at 6 hr. In Swiss mice tyrosine levels were elevated approximately 3-fold 0.5 and 2 hr after treatment and were normal at 4 hr.

To calculate the rate of incorporation of tyrosine into protein, it was necessary to know the average specific radioactivity of the TCA-soluble fraction of the brain during the 10 min period after injection of ^{14}C-tyrosine. This average specific radioactivity was determined for control mice injected with saline, for mice injected with 120 mg/kg of CXM at 0.5, 2 and 6 hr before sacrifice and for mice injected with 30 mg/kg of Ani 0.5 hr before sacrifice. In these experiments, based on three determinations each at 2.5, 5.0 and 10 min after injection of 0.25 μCi of ^{14}C-tyrosine, the specific radioactivity of the TCA-soluble fraction increased linearily with time and its average value varied from 44–49% of that of the 10 min value. There was no significant difference in this average value among control and treated mice.

As shown in Table 2, the calculated degree of inhibition of protein synthesis in C57B1/6J and Swiss mice treated with CXM or Ani was greater in all instances when based on the radioactivity of the TCA-soluble fraction than when based on the average specific radioactivity of tyrosine, a result that reflects the increase in tyrosine concentration caused by the inhibitors [9]. In all cases, the differences between the results of the two methods of calculation were significant, p values varying from 0.029 to 0.004. These differences generally increased as inhibition of protein synthesis declined.

We were particularly interested in those levels of inhibition of protein synthesis that were related to the behavioral effects observed in mice trained 0.5 hr after treatment with 120, 30, 15 and 120/30 mg per kg of CXM (group A, Table 2). Analysis of variance of the degrees of inhibition found after these treatments was significant ($H=10.84$, $df=3$, $p<0.02$). *Post hoc* analyses showed there was no significant difference between the 120 and 120/30 mg per kg groups. Inhibition in both of these groups was, however, significantly greater than in the 30 and 15 mg/kg groups and that in the 30 mg/kg group was significantly greater than in 15 mg/kg groups, p values varying from 0.016 to 0.008.

In the behavioral experiments, entry and escape latencies at training were recorded for the subjects of group A of Table 2 and for their controls injected with saline. Median ± S.E. training entry latency of the pooled saline groups was 21 ± 5.3 sec, those of the CXM groups varying from 13 ± 6.4 to 28 ± 9.2 sec. Median ± S.E. escape latency in the pooled saline groups was 2.0 ± 0.3 sec while those in the CXM groups varied from 1.2 ± 0.3 to 1.9 ± 0.2 sec. One-way analysis of variance of these data showed that there was no significant difference among the groups in escape latencies or, as previously reported [17], in training entry latencies. It

TABLE 2

PERCENT INHIBITION OF PROTEIN SYNTHESIS BASED ON RADIOACTIVITY OR SPECIFIC RADIOACTIVITY OF "PRECURSOR" AND RELATION TO BEHAVIOR

Dose mg/kg	**Hr. after injection	Protein synthesis % inhibition TCA	S.A.Tyr	Test-trial entrance latency (sec)
A. C57B1/6J mice injected with CXM.				
120	0.5	98 ± 0.6	94 ± 1.5	86.5 ± 49.5
120	2	95 ± 1.0	85 ± 3.2	300 ± 39.2
120	4	85 ± 3.0	64 ± 6.3	
120	6	73 ± 4.1	61 ± 6.8	
30	0.5	96 ± 1.0	90 ± 2.5	28.0 ± 55.5
30	2	58 ± 6.8	19 ± 10	
15	0.5	89 ± 1.9	76 ± 4.8	203 ± 54.7
*120/30	2/0.5	98 ± 0.7	95 ± 1.6	300 ± 41.2
B. Swiss mice injected with CXM.				
120	0.5	97 ± 0.1	90 ± 3.5	
120	2	91 ± 4.3	62 ± 2.2	
120	4	45 ± 7.1	14 ± 7.4	
120	6	21 ± 10	7.0 ± 7.0	
C. C57B1/6J mice injected with Ani.				
30	0.5	91 ± 1.3	82 ± 0.3	
30	2	84 ± 1.3	57 ± 4.1	
D. Swiss mice injected with Ani.				
30	0.5	93 ± 2.4	84 ± 5.4	
30	2	87 ± 0.3	73 ± 1.0	
30	4	−17 ± 19	−38 ± 14	

Values are medians ± S.E. * injection of 120 mg/kg followed 1.5 hr later with 30 mg/kg; values obtained 0.5 hr later. See text for calculation of % inhibition. TCA=% inhibition using radioactivity of TCA soluble fraction. S.A. Tyr=% inhibition using specific radioactivity of tyrosine of TCA soluble fraction. C57B1/6J and Swiss controls incorporated, respectively, 30 ± 4.4 and 26 ± 5.3 n moles of tyrosine/10 min per g brain into protein. The test-trial entrance latency for C57B1/6J controls=300 ± 1.6 sec. 4–6 mice used for each time point of inhibition studies; 7–11 mice, for each time point of behavioral studies. **Hr after injection applies both to protein synthesis and time of training. Test-trials made 24 hr after training.

consequently appeared that all mice received essentially the same amount of shock and that CXM did not significantly alter their motor activity at training.

Test-trial entrance latencies were next examined statistically. Saline controls were pooled since all had median latencies of 300 sec. Analysis of variance of the test-trial entrance latencies of Table 2 plus those of the controls was significant ($H=44.58$, $df=4$, $p<0.001$). *Post hoc* analysis in which the latency of the control group was compared to that of mice treated with 120, 30, or 15 mg/kg of CXM 0.5 hr before training showed that the apparent amnesias of these groups were significant ($p=0.001$ or 0.002). Was there a significant difference among the degrees of amnesia of the 120, 30 and 15 mg/kg groups? Analysis of variance of these degrees of amnesia was not significant ($H=2.11$, $df=2$, $p>0.3$).

There was no significant difference between the test-trial entrance latencies of the controls and the mice of the following 2 groups: (a) those trained 2 hr after 120 mg/kg of CXM and (b) those trained 0.5 hr after 120/30 mg per kg of CXM. The long entrance latency of the latter group was not due to impaired performance. Its control group that was exposed to non-contingent shock 0.5 hr after the second injection of CXM had, 24 hr later, median ± S.E. entrance latencies of 24 ± 11 sec. It may be noted that there was a 7% mortality in mice treated with high doses of CXM; those mice that survived and that are represented in the behavioral data appeared normal in behavior.

The amnesia that appeared following training 0.5 hr after 120 mg/kg of CXM was transient. Twenty-four hr after training, the mice had median ± S.E. test latencies of 86.5 ± 49.5 sec. Another group tested 72 hr after training (n=8), had median ± S.E. test latency of 300 ± 33.9 sec, a value significantly different from that at 24 hr ($p=0.006$) but not significantly different from the controls.

Behavioral observations were limited to C57B1/6J mice since, with our procedure, we were unable to obtain a significant amnesia with CXM in our Swiss mice.

DISCUSSION

Our behavioral results are in part consistent with those reported by others. Thus, a high dose of CXM that was amnesic for an aversive object-discrinimation task 0.5 hr after its injection was not amnesic 2 hr after injection [23]. Additionally, in a one-trial passive avoidance task, such as we have used, amnesia followed training at 0.5 hr after treatment with 150, 75, 30, or 15 mg/kg of CXM [17]. These last results, however, differed from ours in that they were obtained using a lower shock (0.3 mA) and amnesia persisted up to 72 hr after training.

An initial objective of our studies was to test two postulates that relate the degree of inhibition of protein synthesis to the degree of amnesia. The first postulate identifies the critical variable as the level of inhibition of protein synthesis at the time of training [17,23]; the second states that the longer the post-training period of severe inhibition of protein synthesis the greater the degree of amnesia [13]. Our finding that Ani and CXM increase the concentrations of those amino acids that were used as precursors in the studies basic to those postulates led us to calculate the rate of protein synthesis from the specific radioactivity of the precursor amino acid. We avoided the use of tritiated or ^{14}C-uniformly labeled amino acids because of the retention of their radioactive products of degradation [4]. The following discussion is limited to our results derived from the specific radioactivity of (1-^{14}C)-tyrosine.

The data of Group A, Table 2 in which C57B1/6J mice were treated with a single dose of CXM fail to support the view that the degree of inhibition of protein synthesis at the time of training determines the degree of amnesia. As noted above, analysis of variance of the degrees of amnesia of the groups trained 0.5 hr after treatment with 120, 30 or 15 mg/kg was not significant; inter-group comparisons substantiated this conclusion. Yet the degrees of inhibition of protein synthesis in these 3 groups was significant in the order 120>30>15 mg/kg. In mice trained 2 hr after 120 mg/kg of CXM there was no impairment of memory although protein synthesis was inhibited 85% at the time of training. By contrast, mice trained 0.5 hr after 30 mg/kg of CXM developed amnesia; in these mice inhibition of protein synthesis at training (90%) was not significantly different from that 2 hr after 120 mg/kg of CXM. Additionally, mice trained 0.5 hr after 15 mg/kg of CXM were amnesic although the degree of inhibition of protein synthesis (76%)was significantly lower ($p=0.041$) than 2 hr after 120 mg/kg of CXM. Quinton and Kramarcy [17] in their extensive and well-planned study have also reported a lack of correlation between degrees of

inhibition of protein synthesis and those of amnesia in mice trained longer than 0.5 hr after CXM treatment. Finally, the mice with the greatest degree of inhibition of protein synthesis (95%), namely the 120/30 mg per kg group, were not amnesic when training followed the second treatment by 0.5 hr.

Nor do the data entirely support the view that the post-training duration of severe inhibition of protein synthesis is the critical variable that determines the degree of amnesia. It might be argued that the absence of CXM-induced amnesia in Swiss mice is related to the shorter duration of inhibition in this strain than in C57B1/6J mice (Table 2). However, in C57B1/6J mice trained 0.5 hr after 120 mg/kg of CXM severe inhibition of protein synthesis persisted for at least 1.5 hr after training. In mice trained 0.5 hr after 30 mg/kg of CXM, by contrast, the rate of protein synthesis 1.5 hr later (19% inhibition) was not significantly lower than the controls. There was, however, no significant difference in the test-trial latencies of the two groups. Again in contrast to the 30 mg/kg group, mice trained 2 hr after 120 mg/kg were not amnesic though post-training inhibition of protein synthesis was substantial. It may be noted that Squire and Barondes [23] found no impairment of memory in mice trained 2 hr after 120 mg/kg of CXM and injected 10 min later with a dose of Ani that caused severe inhibition of protein synthesis. This observation was an important part of the evidence leading them to suggest that the level of inhibition at the time of training is the critical variable in causing amnesia.

The absence of amnesia in mice trained 2 hr after 120 mg/kg of CXM was puzzling. These mice remembered in spite of evidence indicating the presence of numerous abnormalities at and after the time of training. Thus, protein and catecholamine [5, 10, 14, 16] synthesis was inhibited, the electroencephelogram was abnormal [19,26] and amino acid levels were increased with distortion of their ratios of concentrations. The lack of amnesia suggested either that some critical abnormality present early after treatment had disappeared at 2 hr or that some protective mechanism absent at 0.5 hr was present 2 hr after treatment. This latter possibility was supported by the finding that the amnesia produced by 30 mg/kg of CXM when given 0.5 hr before training was avoided if, 1.5 hr earlier, mice were treated with 120 mg/kg (Group A, Table 2). Following the two doses of CXM, inhibition of protein synthesis reached 95%. It consequently appears that in our mice CXM produces with time some direct or indirect change in the brain that antagonizes the amnesic effects of the antibiotic. This antagonism might, for example, be due to a degradation product of CXM, to the release of a protective peptide [12] or to some obscure neural compensatory process.

Our results do not exclude the possibility that inhibition of protein synthesis is an important causative factor in the development of amnesia. They do suggest, however, that other known or unknown effects of the inhibitors contribute to the loss of memory. These known effects, in addition to those noted above, include inhibition of synthesis of adrenal steroids [9] and inhibition of the activities of tissue proteases [27], of acetylcholinesterase [28,29] and as well of tyrosine [11,24] and tryptophan hydroxylase [3]. The effect of one or another of these actions has been separately investigated and when the isolated factor failed to affect memory it has, at times, been considered not to weaken the hypothesis that inhibition of protein synthesis alone is responsible for amnesia [8, 22, 24]. It appears reasonable, however, since the effects of the inhibitors occur together, that any one of them may contribute to the effects of the group and that consequently study of a single effect in isolation from others may lead to erroneous conclusions. Thus, for example, neither lesions of the adrenergic projection nor adrenalectomy alone caused amnesia in rats but both procedures together impaired memory [20]; again, CXM plus electroconvulsive shock produced amnesia when neither treatment alone was effective [2].

Our mice trained 0.5 hr after 120 mg/kg of CXM developed an amnesia that was transient. Perhaps the clearest thing we know about the relationship between memory and the antibiotics is that memory can survive, though with a transient failure of retrieval, in spite of the multiple abnormalities caused, for example, by CXM. The engram appears to be established and to persist independently of major changes in the environment and metabolism of neurones.

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