Proceedings of the National Academy of Sciences
Vol. 67, No. 4, pp. 1959–1966, December 1970

S100 Brain Protein: Correlation with Behavior

Holger Hydén and Paul W. Lange

INSTITUTE OF NEUROBIOLOGY, FACULTY OF MEDICINE, UNIVERSITY OF GÖTEBORG, GÖTEBORG, SWEDEN

Communicated by Alfred E. Mirsky, September 29, 1970

Abstract. The brain-specific acidic protein, S100, in the pyramidal nerve cells of the hippocampus was investigated as a possible correlate to learning during transfer of handedness in rats. The amount of S100 increased during training. Intraventricular injection of antiserum against the S100 protein during the course of training prevented the rats from further increases in learned behavior but did not affect motor function in the animals. Antibodies against the S100 protein could be localized after injection by immunofluorescence, in hippocampal structures, penetrating presumably through slight ependymal lesions caused by the injection. By contrast, control animals subjected to the same training and injected with S100 antiserum that had been absorbed with S100 protein or with other antisera against γ-globulins showed no decrease in their ability to learn. The conclusion is that the brain-specific protein, S100, is linked to the learning process, at least within the training used.

We have investigated the acidic brain protein, S100, in hippocampal nerve cells. We wish to report that the amount of nerve-cell S100 protein increases in trained animals and that the S100 protein is specifically correlated to learning. This linkage was demonstrated by the use of antiserum against the S100 protein that was injected intraventricularly during the course of training and could be localized in the hippocampus by specific fluorescence. The presence of antiserum against the S100 protein in the hippocampus prevents further learning during continued training.

The S100 protein is a defined and specific protein of brain and its correlation to learning seems important since brain-specific protein can be supposed to mediate neural functions. This protein, described in 1965 by Moore *et al.*,[1] has a molecular weight of 21,000, a high content of glutamic and aspartic acid, and, therefore, moves close to the anodal front upon electrophoresis at pH > 8. The S100 protein is mainly a glial protein but occurs also in the nerve cells,[2] and constitutes about 0.2% of the total brain proteins. The anodal band that contains S100 can be separated into at least three components, two of which precipitate with antiserum against S100 and have a high turnover.[3] The S100 protein seems to be composed of three subunits, of molecular weight 7000 (ref. 4). Its appearance in the human cortex parallels the onset of neurophysiological function.[5]

Moore and Perez have described another acidic, brain-specific protein (14-3-2) localized in nerve cells.[6] Still another acidic protein ("antigen α"), unique to the brain, has been characterized by Bennett and Edelman.[7] In addition, evidence

for the existence of other brain-specific soluble proteins has been presented by Bogoch,[8] MacPherson,[9] Kosinski,[10] and Warecka and Bauer.[11]

The training of animals involves a number of variables, such as motor and sensory activity, motivation, orientation reflexes, stress, and the learning processes *per se*. Active controls, in which these factors (except learning) are held constant are, therefore, essential.

In a well-planned behavioral test, surgical, mechanical, or electrical measures to the body should be avoided and the stress factor should be small. We have chosen reversal of handedness in rats as a behavior experiment.[12] The active controls act similarly to the experimental animals.

81 Sprague-Dawley rats, 150–175 g, were used. The experimental set-up has been described in detail.[12] The rat retrieved one food pill at a time by reaching into the glass tube housing the pills. The rats were induced to use the nonpreferred paw by arranging a wall parallel and close to the glass tube on the opposite side of the preferred paw. The controls used the preferred paw and received the same amount of reward as the experimental animals. The rats were trained during two sessions of 25 min per day. The performance, defined as number of reaches per day, was linear up to the 8th day (Fig. 1). All rats showed performance curves similar to that in Fig. 1. Once learned, this new behavior will remain for a long time.[13]

Training of rats, measurement of performance, injections of sera, and chemical analysis were performed by persons who did not know the history of the individual rat treated.

Fresh pyramidal nerve cells of the CA3 region of the hippocampus were used. The method for dissection has been described elsewhere.[14] The cell sample, which weighed about 1 μg, was homogenized in a microhomogenizer in the following solution: 20 μmol sodium thioglycolate–0.25 M sucrose with 0.1% Triton X-100 solution, buffered at pH 6.7 by the addition of 0.5 M Tris phosphate to a final concentration of 0.06 M. After centrifugation, the protein sample was separated by electrophoresis on a 400-μm diameter polyacrylamide gel in glass capillaries.[15]

Increase of the S100 protein. When the electrophoretic pattern of the samples was studied, we observed the presence of a double anodal protein band in *trained*

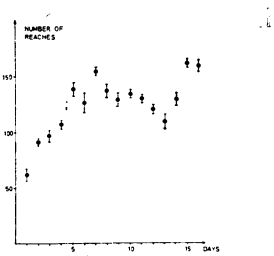

FIG. 1. Performance curve of a group of twelve rats, given as the average number of reaches as a function of number of training sessions (2 × 25 min per day).

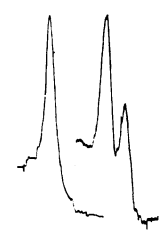

FIG. 2. Two recordings of the front anodal protein band from the electrophoretic pattern of the soluble hippocampal protein from a control rat (*left*) and a trained rat (*right*).

rats. The electrophoretic pattern from the samples of the *control* rats, however, showed only one anodal protein band.[16] Densitometric recordings were made of 75 electrophoretic patterns from 23 rats (Fig. 2). Table 1 shows the presence of

TABLE 1. *Frequency of single- and double-frontal anodal proteins in the electrophoretic pattern of 75 polyacrylamide gels from 23 rats (7 controls, 4 resumed training on the 14th day, 12 resumed training on the 14th day and on the 30th day).*

Controls		Resumed training on day 14		Resumed training on day 30	
1 fraction	2 fractions	1 fraction	2 fractions	1 fraction	2 fractions
20	0	5	10	20	20

two frontal protein bands in 30 recordings out of 55 (16 rats) from trained animals. Only frontal band was observed in all 20 recordings from the controls (7 rats). Note that the observation of double or single frontal bands only applies to protein separation on 200–400 μm diameter gels. Double frontal bands have sometimes been observed in control material separated on 1–4 mm gels.

The following two tests were performed to see whether both, or only one, of these frontal bands contained S100 protein. Protein extracted from pyramidal nerve cells of the CA3 hippocampal region from five trained rats was separated. After the separation, the gel cylinders were placed for 15 min in saturated ammonium sulphate solution (to dissolve the S100 protein) and briefly rinsed. The protein was fixed in sulfosalicylic acid and stained with brilliant blue. The band immediately behind the S100 diminished in size but did not disappear completely. Hippocampal nerve cell protein from another group of five trained rats was electrophoresed on 400-μm diameter gels and precipitated with 80% alcohol for 3 min. The gels were placed in fluorescein-conjugated antiserum against S100 (dilution 1:4) for 24 hr, then examined in a fluorescence microscope and photographed. Both frontal protein bands showed specific fluorescence and thus had reacted positively with the anti-S100 antiserum.

We conclude that a second protein band, migrating in front of the S100 protein complex, had emerged in the nerve-cell protein from the trained rats. This band contains S100 protein. The results also indicate that the original S100 band contains proteins other than the S100 component.

The amount of S100 protein increased in the nerve cells of the trained animals. The absorbance of the single protein band from controls, and the two bands of the trained animals, were measured and compared with an integrating microphotometer. The same amount of protein from trained and control animals was electrophoresed and the procedure was identical in all experiments. The protein was stained with brilliant blue. The electrophoretic patterns were photographed together with a step wedge, and the areas under the curves were calculated. It was found that the amount of protein in the two anodal bands of the trained rats was 10% greater than the amount of protein contained in the one band of the controls. Fig. 2 gives an example of the recordings of the S100 bands. The integrated value of the largest peak of the bands from the trained rats (*right*, Fig. 2) did not differ from that of the single protein band of the controls (*left*, Fig. 2). In addition, there is the new band containing exclusively S100 protein in the samples from the trained rats (*right*, Fig. 2).

The effect of antiserum against S100 protein. The next question was whether the increase of the S100 protein during reversal of handedness specifically relates the S100 protein to learning processes occurring in the hippocampal nerve cells. As we pointed out above, training involves several factors not related to learning *per se.* In the reversal of handedness experiments, such unspecific factors have been reduced to a minimum. The motor and sensory activity, attention, motivation, and reward are equated between the experimental and control animals, and the stress involved in reversal of handedness is minimal. In the following experiments, designed to test the specificity of the S100 protein increase, even the stress factors have been equated between controls and experimental rats. A group of six rats were trained twice, 25 min each training period per day for 3 days. Between the first and second training session on the fourth day, the rats were injected intraventricularly on both sides with 2 × 30 μg (in 30 μl) of antiserum against S100. During further training for 3 days after the injection, the rats did not increase in performance, i.e. the number of reaches per day remained at the same values as those immediately before the injection (Fig. 3). The rats were not affected by the S100 antiserum with respect to motor function and sensory responses.

The specific effect of the antiserum against the S100 protein was demonstrated by the following experiment.

The mixture of S100 antiserum and the S100 protein was left for 6 hr at room temperature and transferred to +4°C for a further 65 hr.[17] Three times a day the mixture was agitated for 30 min with a magnetic stirrer; it was finally centrifuged. The supernatant was tested for the effect of antibodies against the S100 protein by Coons' double-layer method using sheep anti-rabbit γ-globulin conjugated with fluorescein (Flow Lab., Irvine, Scotland). The test material was cryostat sections through the Deiters' nucleus from rats that were fixed in cold acetone. Both absorbed and unabsorbed antiserum against S100 were applied to the sections, which were finally treated with the fluorescein-conjugated anti-rabbit γ-globulin. When the sections were observed in the fluorescence microscope, a bright specific fluorescence was found in glial cell bodies in the sections treated with unabsorbed antiserum. On the other hand, using the absorbed antiserum, only a weak unspecific fluorescence was found in the glial cells, not stronger than that in the nerve cell cytoplasm. Since the S100 protein is mainly a glial protein, localized to glial cell bodies and to nuclei of nerve cells, the result

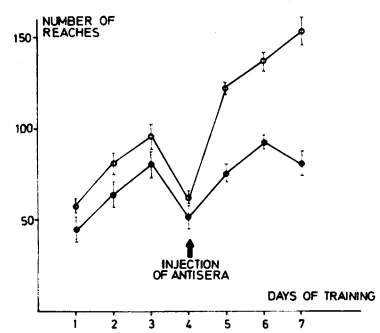

FIG. 3. Performance curves of 6 rats injected intraventricularly on day 4 with 2 × 30 μg of antiserum against S100 protein ●——● and of 8 rats injected with 2 × 30 μg of antiserum against S100 protein that had been absorbed with S100 protein O——O.

showed that the absorption with glial and nerve-cell homogenate had removed the antibodies against the S100 protein in the antiserum.

8 rats were trained for 4 days, and injected on day 4 with 2 × 30 μg of S100 antiserum absorbed with S100. The rats were then trained for a further 3 days. The performance of the rats (number of reaches per training day; Fig. 3) increased in the same way as did the performance of the uninjected control rats shown in Fig. 1.

Since the active molecules of an antiserum have a large molecular weight, it is important to know if the antibodies injected intraventricularly reached the hippocampal structures. Therefore, rats were injected intraventricularly with 2 × 30 μg of antiserum against S100, or with 2 × 30 μg S100 antiserum absorbed with S100 protein. 1 hr (2 rats) and 18 hr (4 rats) after the injection, the rats were decapitated and cryostat sections were made of the hippocampus. Coons' double-layer method was applied to demonstrate the possible localization of antibodies to cell structures, using the sheep anti-rabbit-γ-globulin conjugated with fluorescein isothiocyanate. Fig. 4 demonstrates specific fluorescence localized to nerve cells in the hippocampus of rats injected with antiserum against S100 (4*a*). Evidently, the hippocampus differs from the brain stem, insofar as the astro- and oligo-dendroglia do not contain the S100 protein in amounts sufficient to give an immunofluorescent reaction. No such nerve cell fluorescence can be observed in the material from rats injected with S100 antiserum absorbed with S100 protein (4*b*).

A pertinent question is whether the effect of the S100 antiserum on behavior is due to an S100-antibody–antigen reaction in limbic structures since Klatzo *et al.*[18] reported that fluorescein-labeled globulin does not penetrate through the ependyma into the brain tissue. However, when a small, local, cold lesion was produced on the surface of the brain cortex,[19] fluorescein-labeled globulin entered through the minute surface lesion and spread rapidly through the underlying

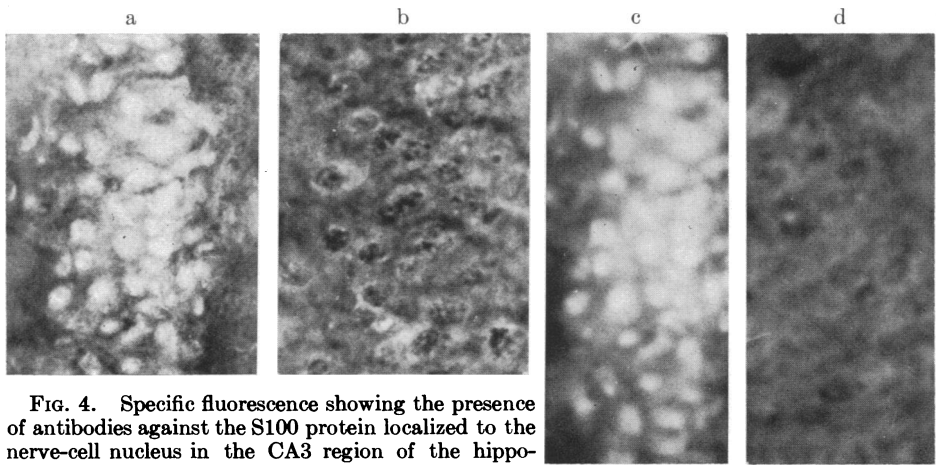

FIG. 4. Specific fluorescence showing the presence of antibodies against the S100 protein localized to the nerve-cell nucleus in the CA3 region of the hippocampus (*a*) and in granular cells of gyrus dentatus of rats injected with 60 μg of antiserum against S100 protein (*c*). There is no specific fluorescence to be seen in the corresponding structures of rats injected with antiserum against S100 protein absorbed with S100 protein (*b* and *d*).

subcortical area. In our present experiments, the antisera are injected into the narrow lateral ventricles. The thin needle (gauge 22) may slightly damage the walls of the lateral ventricles when inserted and thus give free passage to globulins to enter. Sham-injections were therefore made with NaCl intraventricularly in rats in our standard way. The ventricles were then carefully exposed, flooded for 5 sec with a 0.1% erythrocin solution in 0.9% NaCl and, after washing examined under low power magnification in UV-light. From the remaining stain, it was seen that the walls of the lateral ventricles were superficially damaged where the needle had been touching the walls.

Thus, we can conclude that the S100 protein is specifically correlated with learning processes within training. However, injection of S100 protein need not necessarily give rise to a spontaneous change of handedness.

To study a possible effect of injected antiserum that contained antibodies not directed against the S100 protein, 6 rats were injected with 2×25 μg of antiserum against rat γ-globulin from goat, 4 rats with the same amount of antiserum against rat γ-globulin from rabbit, and 4 rats with rabbit anti-γ-globulin from goat. As is seen from the curves in Fig. 5, this did not impede their performance. Before injection of antisera, all rats followed an identical performance curve. After the injection of antisera, these rats followed a performance curve that was an extrapolation of the performance curve before the injection, as did a control rat injected with the same volume of physiological NaCl solution.

Another way to present the results is the following. For each rat, the sum of reaches for the first three training days is calculated, as is also the sum of reaches for the last three training days. The number of reaches during the day of injection is thus not included in these sums. The difference between the second and first sum is calculated. The averages of this difference are 60 ± 14 for the rats injected with S100 antiserum and 178 ± 14 for the control rats. The difference between these numbers is highly significant ($P < 0.001$, 12 degrees of freedom). It is clear that the experimental rats show a decrease in learning capacity.

Discussion. In the present experiments, we have used a uniform nerve cell population of the hippocampus in rats. This brain region was chosen because of its importance for learning.[16] In an ideal learning experiment, all factors involved in the training should be identical for experimental and control animals with the exception of learning. The above experiments with four types of antisera fulfill these requirements. The increase of the S100 protein in hippo-

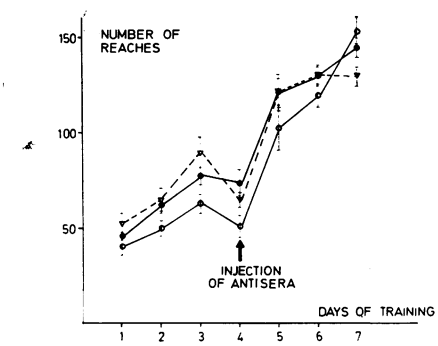

FIG. 5. Performance curves of rats injected intraventricularly on day 4 with 2×30 μg of antiserum against rat gamma-globulin from rabbit (4 rats ●——●), and from goat (4 rats ○——○), and against rabbit gamma-globulin from goat (4 rats ▽——▽).

campal nerve cells, its alteration in physical properties, and the blocking effect of the S100 antiserum on behavior point to the S100 protein as a true biochemical correlate to behavior. This conclusion is strengthened by the localization of the antibodies against the S100 by specific immunofluorescence and by the lack of effect of antisera not specifically directed to the S100 protein.

Presumably, the S100 protein is not the only protein of importance for behavior, and hence, the role of other brain proteins in this experiment may be discussed. A young adult rat's brain contains about 200 mg of protein of which S100 constitutes about 0.4 mg. Antisera were administered in amounts of 0.05 mg per rat, i.e. 0.025% of the total protein. The amount of antiserum against S100 constituted 12.5% of the total amount of the S100 protein of the whole brain. It is to be noted, though, that the antisera were injected intraventricularly and thus in the vicinity of the hippocampus.

Even so, it must be realized that the amount of anti-γ-globulin serum is not only exceedingly small relative to the total amount of brain protein, but also relative to the total hippocampal protein. It is therefore questionable that the antiserum would influence total brain-protein synthesis in a measurable way. The antiserum against the S100 protein is selective for the 0.2% of the total brain proteins that is S100. The antiserum does not affect total protein synthesis of brain. To confirm this assumption, the incorporation of [^{3}H]leucine into hippocampal nerve-cell protein was studied after the last training session on the seventh day. No difference in incorporation of [^{3}H]leucine into hippocampal protein was found between rats receiving antiserum against S100 and rats receiving rat γ-globulin antiserum intraventricularly. Thus, the effect of the S100-protein antiserum on behavior cannot be due to changes in total protein synthesis in the hippocampus.

In a recent paper,[16] we studied the response during reversal of handedness of two protein fractions of hippocampal nerve cell (CA3).* These proteins move as acids on electrophoresis at pH 8.3. During 1 month of intermittent training (day 1–4, 14, 30–33), the incorporation of [^{3}H]leucine into protein was increased after the first and second training period, but not after the last training. The rats performed well at each training period. This temporal link between behavior and protein synthesis indicated that protein synthesis was linked to the learning processes of the neurons, and was not an expression of increased neural function in general. The two acidic protein fractions are most probably not pure (single) proteins. It is therefore of special interest that in the experiments presented in this paper we have been able to correlate a change in behavior to a single, defined, nerve-cell protein which is, furthermore, brain specific.

Jankovic *et al.*[20] reported the *in vivo* effect of anti-brain protein antibodies on defensive conditioned reflexes in the cat. Their results show that the intraventricular injection of these antibodies produces significant changes in conditioned responses immediately after administration, and on subsequent days. Injection of anti-liver antibody, normal γ-globulin, and saline produced no effect.

Mihailovic *et al.*[21] studied the effects of intraventricularly-injected anti-brain antibodies on visual discrimination test performance in Rhesus monkeys. The animals were injected with anti-caudate nucleus, antihippocampal, and normal

γ-globulin, respectively. The anti-caudate- and anti-hippocampal-treated animals were significantly impaired in performance as compared to the normal, γ-globulin-treated animals. The impairment was temporary.

It is also interesting to note that a high level of environmental stimulation leads to a thicker hippocampus, with a higher density, in both oligo- and astroglia as compared to hippocampi of control rats living in isolation.[22]

***Note in Proof.** After submission of this article, we obtained results indicating that both these proteins contain the brain-specific protein 14-3-2, as defined by Moore and Perez (ref. 6.)

We thank Dr. L. Levine, Brandeis University, Waltham, Mass., who kindly provided the antiserum against the S100 protein, and Dr. Blake Moore, Department of Psychiatry, Washington University, School of Medicine, St. Louis, Mo., who generously gave us S100 protein.

This study has been supported by the Swedish Medical Research Council, grant B69-11X-86-05B, and a grant from Riksbankens Jubileumsfond.

[1] Moore, B. W., and D. McGregor, *J. Biol. Chem.*, **240**, 1647 (1965).
[2] Hydén, H., and B. McEwen, *Proc. Nat. Acad. Sci. USA*, **55**, 354 (1966).
[3] McEwen, B. S., and H. Hydén, *J. Neurochem.*, **13**, 823 (1966).
[4] Dannies, P. S., and L. Levine, *Biochem. Biophys. Res. Commun.*, **37**, 587 (1969).
[5] Zuckerman, J., H. Herschman, and L. Levine, *J. Neurochem.*, **17**, 247 (1970).
[6] Moore, B. W., and V. J. Perez, in *Physiological and Biochemical Aspects of Nervous Integration*, ed. F. D. Carlson (Englewood Cliffs, N.J.: Prentice-Hall, Inc., 1968), p. 343.
[7] Bennett, G. S., and G. M. Edelman, *J. Biol. Chem.*, **243**, 6234 (1968).
[8] Bogoch, S., *The Biochemistry of Memory* (London: Oxford University Press, 1968).
[9] MacPherson, C. F. C., and A. Liakopolou, *Fed. Proc.*, **24**, Abstr. 272 (1965).
[10] Kosinski, E., and P. Grabar, *J. Neurochem.*, **14**, 273 (1967).
[11] Warecka, K., and H. Bauer, *J. Neurochem.*, **14**, 783 (1967).
[12] Hydén, H., and E. Egyhazi, *Proc. Nat. Acad. Sci. USA*, **52**, 1030 (1964).
[13] Wentworth, K. L., *Genet. Psychol. Monogr.*, **26**, 55 (1942).
[14] Hydén, H., *Nature*, **184**, 433 (1959).
[15] Hydén, H., and P. W. Lange, *J. Chromatog.*, **35**, 336 (1968).
[16] Hydén, H., and P. W. Lange, *Brain Res.*, **22**, 423 (1970).
[17] Mihailovic, L., and H. Hydén, *Brain Res.*, **16**, 243 (1969).
[18] Klatzo, I., J. Miquel, P. J. Ferris, J. D. Prokop, and D. E. Smith, *J. Neuropathol., Exp. Neurol.*, **23**, 18 (1964).
[19] Steinwall, O., and I. Klatzo, *Acta Neurol. Scand.*, **41**, Suppl. 13 (1964).
[20] Jankovic, B. D., L. Rakic, R. Veskov, and J. Horvat, *Nature*, **218**, 270 (1968).
[21] Mihailovic, L., I. Divac, K. Mitrovic, D. Milosevic, and B. D. Jankovic, *Exp. Neurol.*, **24**, 325 (1969).
[22] Walsh, R. N., O. E. Budtz-Olsen, J. E. Penny, and R. A. Cummins, *J. Comp. Neurol.*, **137**, 361 (1969).