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Effect of Various Forms of Training and Stimulation on the Incorporation of ^{32}P into Nuclear Phosphoproteins of the Rat Brain[1]

DIOGO O. SOUZA, ELAINE ELISABETSKY AND IVÁN IZQUIERDO

Departamento de Bioquímica, Instituto de Biociências
U.F.R.G.S. (centro), 90000 Pôrto Alegre, RS, Brasil

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SOUZA, D. O., E. ELISABETSKY AND I. IZQUIERDO. *Effect of various forms of training and stimulation on the incorporation of ^{32}P into nuclear phosphoproteins of the rat brain.* PHARMAC. BIOCHEM. BEHAV. 12(4) 481–486, 1980.—Incorporation of ^{32}P into acid-extractable nuclear proteins was measured in the hippocampus, caudate nucleus, rest of the brain, and liver of rats submitted to various different behavioral treatments in a shuttle-box. After 5 min of classical conditioning, of avoidance without CS-US pairing, and of avoidance with CS-US pairing (standard shuttle avoidance), there was an increased ^{32}P uptake by acid-extractable nuclear proteins in the hippocampus and caudate nucleus. The effect disappeared between 5 and 25 min of training. After 25 min of buzzers alone, or of footshocks alone, a similar ^{32}P uptake change was noted in the same brain structures, which raises doubts as to the specificity of the phenomenon in terms of learning mechanisms.

Classical conditioning Avoidance conditioning Sensory stimulation Nuclear phosphoproteins
Hippocampus Caudate nucleus Brain Liver

MACHLUS *et al.* [17,18] reported an increase of radioactive phosphate incorporation into non-histone acid extractable proteins of brain nuclei of mice and rats exposed to 5 min of one-way active avoidance training. This finding is potentially important, since phosphorylation of non-histone chromosomal proteins appears to be involved in gene transcription [14,29]. In fact, brief sessions of active avoidance training in rats or mice are followed by increased nucleoside incorporation into brain RNA [4, 10, 19, 30], by an increase of total hippocampal RNA concentration [10,20], and by an enhanced rate of brain protein synthesis [19, 22, 28]. Moreover, pharmacologic interference with brain RNA or protein synthesis at appropriate times after training results in an impaired memory for avoidance tasks (see [1, 8, 24] for reviews).

Due to a lack of adequate behavioral controls, however, most of the studies mentioned above are inconclusive as to whether the observed biochemical changes or pharmacological effects result from interactions with true learning factors, or with non-associative phenomena [2, 11, 12]. For example, several studies have made use of "yoked" controls, i.e., animals paired to the training record of other animals submitted to specific CS-US schedules which resulted in avoidance learning [17, 18, 30]. Yoked controls, by definition, involve a mixture of pseudoconditioning, plus Pavlovian conditioning with partial reinforcement, plus conflict behavior (animals may learn to avoid in some trials and then be punished for the same response in others) [23,24]. Therefore, they are inadequate controls for the presence of pseudoconditioning and Pavlovian elements in the training situation. In the experiments of Machlus *et al.* [18] on brain protein phosphorylation, however, Pavlovian elements in the one-way task seem to have been excluded by the observation that classical conditioning caused no biochemical changes. Other authors [10,19] have used pseudoconditioned controls. Pseudoconditioning is probably a component of all avoidance learning [11, 12, 13, 23], and it probably accounts for, or is a mixture of, a variety of non-associative elements, including influences of the US (footshocks) on general activity and reactivity, and preparedness phenomena [2]. However, pseudoconditioning may have biochemical effects of its own, indistinguishable from those of true learning, such as increased uridine uptake by hippocampal RNA [10], and depletions of brain catecholamines [26]. Indeed, even buzzers alone, or footshocks alone, such as are commonly used as CS and US, respectively, in avoidance learning paradigms,

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influence brain protein synthesis in the same general direction as active avoidance learning [9, 21, 28]. This appears not to be the case with brain nuclear protein phosphorylation, which is enhanced by avoidance training but not by handling or by footshocks [17,18]. This suggests a functional dissociation, or at least a low correlation, between this phenomenon and brain RNA or protein synthesis, at least with regard to active avoidance learning.

Another major drawback in the correlational and/or interventive studies mentioned is that, in most cases, the effects were measured either on the whole brain [17,18], or in ill-defined subdivisions of the brain (i.e., by perpendicular knife cuts) [30]. Different brain areas are well known to participate to different degrees in the regulation of learning and of non-associative events (see, for example [5, 6, 12, 25]), and presumably significant regional changes could go undetected, or become obscured, in observations carried out on the whole brain, or on anatomically complex brain fractions [10]. Because of this, it might be inappropriate to compare findings such as those of Machlus *et al.* [17,18] on whole brain nuclear protein phosphorylation, with data from other authors on hippocampal RNA [10, 19, 20], or on brain regional protein synthesis [11, 21, 24, 28].

The present study was undertaken in order to test the generality of the previous findings of Machlus *et al.* [17,18] with regard to other forms of learning, particularly classical conditioning in the shuttle-box [11, 12, 26]; to investigate the effect of longer periods of training [25], rather than just 5 or 10 min); to examine the effect of pseudoconditioning on brain nuclear protein phosphorylation; and to determine possible regional variations of this parameter following classical or avoidance learning and pseudoconditioning.

METHOD

Subjects and Behavioral Procedures

Sixty male Wistar rats from our own breeding stock were used (age, 3–4 months; weight, 170–260 g).

A 50×25×25 cm noncompartmentalized shuttle-box was used for all behavioral tests. The box was made of wood and painted grey, except for the front wall which was of glass. At the midline on the lid there was a 6 W light bulb hanging from the inside and a buzzer fixed to the outside. The light was on throughout the duration of each test. The floor of the box consisted of 2 mm bronze bars spaced 7 mm apart. A flat piece of wood, 5 mm wide, placed between the two centralmost bars, was the only marker between the right and left side of the box.

Animals were submitted to the 6 different behavioral situations, as follows:

Pseudoconditioning. The buzzer was set on for 5 sec every 10 to 40 sec. Footshocks (1.5 mA, 60 Hz, 2 sec) were delivered randomly interspersed among the buzzers, at randomly variable 5 to 35 sec buzzer-shock or shock-buzzer intervals, as follows: 8 shocks among the first 10 buzzers, 7 among the next 10; 5 among the following 10 buzzers; 3 among the next 10; and 2 among the last 10 buzzers. This decreasing footshock schedule was used in order to approximately match the incidence of shocks every 10 trials in the typical two-way avoidance situation (see below) [10,11]. Presentation of the shocks was independent of whatever responses were made to the buzzer. For further details on this, and on the next three behavioral tests, see [5, 6, 11, 12, 25, 26, 27].

Pavlovian conditioning. Buzzers were delivered as above, but each was immediately followed by a footshock (contiguity; CS-US interval=0 on all trials), irrespective of whether the animals shuttled to the buzzer or not [11,12].

Avoidance without CS-US pairing. Buzzers were presented at randomly variable 10 to 40 sec intervals, as in the two preceding tests; but each buzzer was followed, at an also randomly variable 5 to 35 sec interval, by a footshock, unless there was a shuttle response to the buzzer, in which case the next scheduled shock was cancelled. Therefore, the CS-US interval was unpredictable to the animals, and there was no stimulus pairing, in the sense of contiguity; however, there was an avoidance contingency by which each shuttle response to the buzzer cancelled one shock. For a review of differences and similarities between this test and others, particularly trace reflex techniques, see [11, 12, 25].

Avoidance with CS-US pairing, or standard two-way avoidance. Each buzzer was immediately followed by a foot-shock (contiguity, CS-US interval=0, as in the Pavlovian paradigm), but the shock was omitted in those trials in which the animals shuttled to the buzzer (avoidance contingency, as in the preceding test). Intertrial intervals were varied at random between 10 and 40 sec.

Buzzers alone. Buzzers presented at randomly variable 10 to 40 sec intervals; no footshocks.

Footshocks alone. Footshocks given at randomly variable 10 to 40 sec intervals; no buzzers.

The preceding tests were carried out for either 5 or 25 min. In the 5-min tests, 10 buzzers, or 10 buzzer-shock trials, or 10 footshocks were given, depending on the test. In the 25-min tests, the number of buzzers, buzzer-shock trials, or footshocks, was 50. Animals submitted to 5 min of pseudoconditioning received 10 buzzers and 8 shocks; and animals submitted to 25 min of pseudoconditioning received 50 buzzers and 25 shocks (see above).

Control groups. In all the behavioral situations described above, the animals were placed in the shuttle-box 5 min prior to the onset of stimulation. Therefore, three separate *box control* groups were used: one in which the animals were left undisturbed in the box for 5 min, another one in which they were left for 10 min, and a third group in which the animals remained in the box for 30 min. In addition, there was a group of *intact controls*, i.e., animals taken out of their home cages and sacrificed right away. Since no differences were observed between the intact controls and the box-5 min groups, the data from these two groups were pooled together.

Biomedical Procedures

Animals were sacrificed by decapitation immediately after the end of each of the 5- or 25 min behavioral treatments listed above. They received an intraperitoneal injection of 0.2 mCi of $H_3{}^{32}PO_4$, 2–3 hr before sacrifice. As can be seen in Fig. 1, at this time from injection radioactivity in the serum had fallen to asymptote levels, whereas liver radioactivity was still near maximum, and radioactivity in the brain did not vary significantly between 0.25 and well over 24 hr from injection of the labelled material.

Immediately after sacrifice, the brain (including midbrain and cerebellum), and a slice of liver, were withdrawn and kept below 4°C. All subsequent steps were at 4°C. The hippocampus and caudate nucleus were dissected out from the rest of the brain, and the four tissue samples (hippocampus, caudate, rest of the brain, and liver) were homogenized using a glass homogenizer and a Teflon pestle (approximate clear-

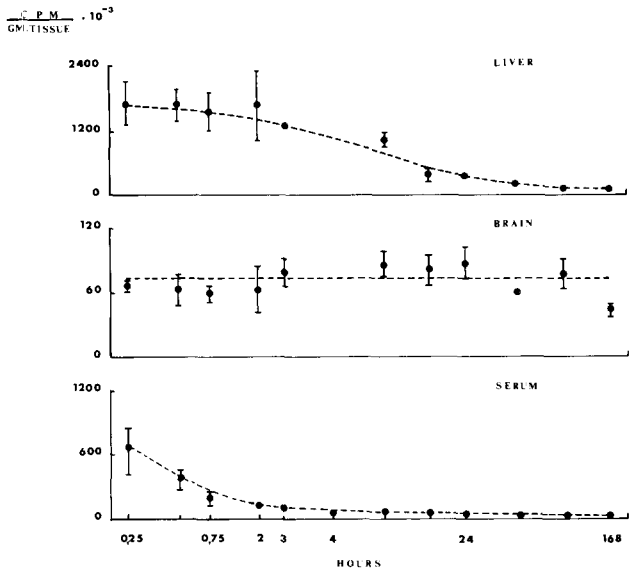

FIG. 1. Ordinates: ^{32}P radioactivity in liver brain and serum (cpm per g wet tissue$\times 10^{-3}$). Abscissae: time (hr) from intraperitoneal injection of $H_3\ ^{32}PO_4$. Data expressed as means ± SE.

TABLE 1

SHUTTLE RESPONSES TO THE BUZZER (MEANS ± SE) MADE BY RATS DURING 5 OR 25 MIN OF EXPOSURE TO BUZZERS ALONE, PSEUDOCONDITIONING, PAVLOVIAN CONDITIONING, AVOIDANCE WITHOUT CS-US PAIRING, AND STANDARD TWO-WAY AVOIDANCE

Behavioral treatment	Time of exposure to treatment 5 min	25 min
Buzzers alone	0.2 ± 0.2 (9)	0.4 ± 0.2† (5)
Pseudoconditioning	0.4 ± 0.2 (7)	3.4 ± 1.0 (5)
Pavlovian conditioning	0.7 ± 0.3 (9)	10.8 ± 2.0‡ (4)
Avoidance without CS-US pairing	1.5 ± 0.3* (8)	11.3 ± 2.6‡ (4)
Standard two-way avoidance	1.5 ± 0.4* (7)	17.0 ± 1.9‡ (4)

In this and following tables, sample size is shown in parentheses below the mean.

Significant differences from pseudoconditioned group in a Duncan multiple range test [4]: *at 5% level; †at 1% level; ‡at 0.5% level.

ance, 0.2 mm) in a solution containing 0.25 M sucrose, 0.025 M KCl, 0.005 M $MgCl_2$, and 0.005 M Tris, at pH 7.6. The hippocampus and caudate were homogenized in 4 ml of this solution, and the other two tissues in 10 ml. The homogenates were centrifuged at 1085×g for 10 min. This, and all subsequent centrifugations were in a Servall RC-2B centrifuge using a SS-34 rotor. The supernatant (a crude cytosol fraction) was saved for measurement of total ^{32}P radioactivity, and an aliquot of it was used for protein determination by the method of Lowry *et al.* [16]. The pellet was resuspended in 8 ml of 1.5 M sucrose, and centrifuged for 60 min at 27,000×g. The supernatant was discarded, and the remaining pellet (purified nulcear fraction, [18]) was resuspended in an 88% solution of ethanol in 0.01 N HCl. This was again centrifuged for 10 min at 27,000×g. No radioactivity was detected in the supernatant, which was discarded. The procedure (suspension in ethanol, centrifugation at 27,000×g) was repeated twice. Acid-soluble nuclear proteins [18] were extracted from the remaining pellet by resuspending it in 10 ml of 0.2 N HCl, and stirring for 30 min [17]. The suspension was again centrifuged for 10 min at 27,000×g, the HCl extraction was repeated twice, and the three extractions were combined. An aliquot of this material was used for protein determination by the method of Lowry *et al.* [16]. The remainder was pipetted onto filter paper slices, which were dried at room temperature, and then submerged in a scintillation mixture (4 g of 2,5-diphenyloxazole, and 50 mg of 1,4-bis[2-(5-phenyl-oxazolyl)]-benzene per liter of toluene). ^{32}P radioactivity was measured with a Beckman LS-100 scintillation counter (efficiency: 96%).

Some of the HCl extracts were dried in a vacuum rotator, resuspended in 2 ml of 0.2 N HCl, and passed through a G-75 Sephadex column (47 cm). Less than 3% of the samples corresponded to substances with a molecular weight lower than 3,000; the remainder corresponded to protein species with molecular weights above 10,000 (75% to proteins weighing more than 60,000).

Data were expressed as relative radioactivity [9, 17, 18, 21, 22]=cpm per mg protein in the acid extractable fraction/cpm per mg protein in the crude cytosol fraction.

Statistical

Comparisons among groups were by way of a randomized-group analysis of variance followed by a Duncan multiple range test [3].

RESULTS

Behavioral

Table 1 presents the performance of shuttle response to the buzzer in the five behavioral situations in which this was measured. The data agree closely with many others from this laboratory [5, 6, 11, 12, 25, 26, 28] and need no special comment here. In all tests in which shocks were used, animals responded to them with shuttling in less than 2 sec (100% escape reactions in all animals).

Biochemical

Biochemical findings are shown in Tables 2 to 5.

After 5 min of training in any of the three aversive learning paradigms (classical conditioning, avoidance without CS-US pairing, standard two-way avoidance), or after 25 min of either buzzers alone or footshocks alone, there was an increased ^{32}P incorporation into acid-extractable nuclear proteins of the hippocampus (Table 2) and caudate nucleus (Table 3). No changes were detected in the other groups in these two structures, or in any group in the rest of the brain (Table 4), or liver (Table 5).

TABLE 2

HIPPOCAMPUS: INCORPORATION OF ^{32}P INTO ACID-EXTRACTABLE NUCLEAR PROTEINS (CPM PER MG NUCLEAR PROTEINS/CPM PER MG PROTEIN IN CRUDE CYTOSOL FRACTION; MEANS ± SE) IN RATS SUBMITTED TO DIFFERENT BEHAVIORAL TREATMENTS FOR 5 OR 25 MIN

Behavioral treatment	Time of exposure to treatment 5 min	25 min
Control group	0.54 ± 0.03 (9)	
Box (no stimulation)	0.58 ± 0.03 (3)	0.45 ± 0.11 (2)
Pseudoconditioning	0.58 ± 0.07 (4)	0.42 ± 0.03 (3)
Pavlovian conditioning	0.86 ± 0.11† (4)	0.67 ± 0.06 (4)
Avoidance without CS-US pairing	0.97 ± 0.12† (3)	0.50 ± 0.01 (3)
Avoidance with CS-US pairing	1.11 ± 0.06‡ (4)	0.47 ± 0.07 (3)
Buzzers alone	0.42 ± 0.02 (4)	0.83 ± 0.08† (5)
Footshocks alone	0.53 ± 0.05 (5)	0.77 ± 0.20* (5)

Significant differences from control group and from both box groups in a Duncan multiple range test [3]: *at 5% level; †at 1% level; ‡at 0.5% level.

TABLE 3

CAUDATE NUCLEUS: INCORPORATION OF ^{32}P INTO ACID-EXTRACTABLE NUCLEAR PROTEINS (CPM PER MG NUCLEAR PROTEINS/CPM PER MG PROTEIN IN CRUDE CYTOSOL FRACTION; MEANS ± SE) IN RATS SUBMITTED TO DIFFERENT BEHAVIORAL TREATMENTS FOR 5 OR 25 MIN

Behavioral treatment	Time of exposure to treatment 5 min	25 min
Control group	0.52 ± 0.03 (9)	
Box (no stimulation)	0.52 ± 0.03 (4)	0.51 ± 0.08 (3)
Pseudoconditioning	0.47 ± 0.03 (4)	0.51 ± 0.11 (3)
Pavlovian conditioning	0.89 ± 0.07† (5)	0.61 ± 0.09 (3)
Avoidance without CS-US pairing	0.87 ± 0.08† (4)	0.46 ± 0.06 (4)
Avoidance with CS-US pairing	1.06 ± 0.04‡ (4)	0.45 ± 0.08 (3)
Buzzers alone	0.52 ± 0.04 (3)	0.70 ± 0.06* (4)
Footshocks alone	0.59 ± 0.05 (6)	0.81 ± 0.09† (5)

Significant differences from control group and from both box groups in a Duncan multiple range test [3]: *at 5% level; †at 1% level; ‡at 0.5% level.

TABLE 4

REST OF THE BRAIN: INCORPORATION OF ^{32}P INTO ACID-EXTRACTABLE NUCLEAR PROTEINS (CPM PER MG NUCLEAR PROTEINS/CPM PER MG PROTEIN IN CRUDE CYTOSOL FRACTION; MEANS ± SE) IN RATS SUBMITTED TO DIFFERENT BEHAVIORAL TREATMENTS FOR 5 OR 25 MIN

Behavioral treatment	Time of exposure to treatment 5 min	25 min
Control group	0.44 ± 0.03 (9)	
Box (no stimulation)	0.48 ± 0.02 (3)	0.41 ± 0.19 (2)
Pseudoconditioning	0.63 ± 0.13 (4)	0.41 ± 0.10 (2)
Pavlovian conditioning	0.58 ± 0.09 (5)	0.47 ± 0.09 (4)
Avoidance without CS-US pairing	0.35 ± 0.04 (3)	0.35 ± 0.06 (3)
Avoidance with CS-US pairing	0.46 ± 0.05 (4)	0.34 ± 0.09 (3)
Buzzers alone	0.39 ± 0.04 (4)	0.48 ± 0.02 (5)
Footshocks alone	0.41 ± 0.07 (5)	0.60 ± 0.15 (5)

TABLE 5

LIVER: INCORPORATION OF ^{32}P INTO ACID-EXTRACTABLE NUCLEAR PROTEINS (CPM PER MG NUCLEAR PROTEINS/CPM PER MG PROTEIN IN CRUDE CYTOSOL FRACTION; MEANS ± SE) IN RATS SUBMITTED TO DIFFERENT BEHAVIORAL TREATMENTS FOR 5 OR 25 MIN

Behavioral treatment	Time of exposure to treatment 5 min	25 min
Control group	0.63 ± 0.07 (8)	
Box (no stimulation)	0.68 ± 0.12 (3)	0.64 ± 0.08 (2)
Pseudoconditioning	0.56 ± 0.20 (3)	0.67 ± 0.10 (3)
Pavlovian conditioning	0.81 ± 0.09 (5)	0.64 ± 0.06 (4)
Avoidance without CS-US pairing	0.56 ± 0.01 (4)	0.65 ± 0.13 (4)
Avoidance with CS-US pairing	0.55 ± 0.13 (4)	0.52 ± 0.09 (4)
Buzzers alone	0.51 ± 0.10 (4)	0.77 ± 0.13 (5)
Footshocks alone	0.89 ± 0.17 (6)	0.61 ± 0.07 (5)

DISCUSSION

The present results confirm those of Machlus *et al.* [17,18], extend them to other forms of learning (classical conditioning, and two variants of avoidance conditioning in the shuttle-box), and show that they are specific to at least two brain structures: the hippocampus and the caudate nucleus (they do not occur in the rest of the brain, or in the liver). The hippocampus and the caudate nucleus are regions involved in the control of classical and avoidance shuttle-box learning [5, 6, 12, 25]. As was the case with the one-way avoidance task studied by Machlus *et al.* [18], the maximum increase of ^{32}P uptake by acid-extractable nuclear protein was noted at 5 min, and then the phenomenon declined with time, even if training was continued.

However, a similar increase of ^{32}P incorporation into hippocampal and caudate nucleus proteins occurred in the animals submitted to 25 min of buzzers alone, or of footshocks alone. Thus, the effect of the aversive training procedures on this biochemical parameter may be viewed as a simple acceleration of something that would have happened anyway, as a result of the mere presentation of the stimuli that were used as CS and US. Indeed, possibly the velocity with which this biochemical phenomenon occurs depends more on the mode of stimulation than on the performance of learned responses: animals trained for 5 min in the Pavlovian paradigm made no more shuttle responses to the buzzer than those in the pseudoconditioning or buzzer alone groups (Table 1), but they showed a much higher ^{32}P incorporation into hippocampal and caudate acid-extractable nuclear proteins than those two other group.

This raises an important doubt as to the specificity of the ^{32}P uptake phenomenon in relation to learning, as opposed to mere sensory stimulation, or other non-associative variables. In fact, whereas one definite form of learning (habituation) takes place in the animals submitted to 25 min of buzzers alone [27,28], it is questionable whether any learning at all occurs in the rats submitted to 25 min of footshocks. All animals escaped to all shocks in less than 2 sec, and escape latencies appeared to be unaffected by shock repetition (see also [27]). Of course, there may be slight behavioral changes during 25 min of repeated footshocks, which were not specifically measured, but which could represent some form of learning: subtle position changes, vocalization, increased or decreased jumping between shocks, etc. This raises the problem of whether a stimulated control group in which *no* learning of any kind takes place is at all possible in experiments such as the present one. Perhaps the group which comes closest to this desideratum is the one submitted to pseudoconditioning, in which the random presentation of buzzers and shocks might actually prevent the development of associative phenomena. However, there is a possibility that several forms of adventitious or unrecorded learning might occur in this group as well [11,12]. Anyway, pseudoconditioning for 5 or 25 min caused no detectable change of ^{32}P incorporation into acid-extractable nuclear proteins in any of the structures examined.

From a biochemical point of view, an important question is that of whether the changes observed were due to increased phosphorylation or to decreased dephosphorylation [17,18]. Neither the present data, nor those of Machlus *et al.* [18], provide any direct evidence in favor of either possibility. Elsewhere [27] we reported that after 5 or 25 min of training in the standard two-way avoidance situation there is no change in total 3′5′-cyclic adenosine monophosphate (cAMP) levels in rat hippocampus or caudate nucleus. If phosphorylation of acid-extractable nuclear proteins depends on activation of protein kinases by cAMP [7], those data would argue against the hypothesis of increased phosphorylation. However, it is possible that a cAMP increase took place in some compartment of brain tissue which we did not specifically measure [27], and there is no certainty that phosphorylation of brain nuclear proteins is mediated by cAMP-dependent, rather than by cGMP-dependent [15], or even cyclic nucleotide-independent, protein kinases. Until these matters are settled, it is probably wise to abstain from making correlations between cAMP levels, brain protein phosphorylation, and RNA and/or protein synthesis, as have been made by others in the past [24]. The decline of ^{32}P uptake by hippocampal and caudate nuclear proteins observed between 5 and 25 min of training in the present experiment, or between 5 and 15 min in the whole brain of the animals studied by Machlus *et al.* [18], can obviously be explained by an accelerated rate of dephosphorylation.

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