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Lordosis Facilitation in Estrogen Primed Rats by Intrabrain Injection of Pregnanes

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BEYER, C., G. GONZÁLEZ-MARISCAL, J. R. EGUÍBAR AND P. GÓMORA. *Lordosis facilitation in estrogen primed rats by intrabrain injection of pregnanes*. PHARMACOL BIOCHEM BEHAV **31**(4) 919–926, 1988.—Progesterone (P) and nine of its natural metabolites were bilaterally injected (5 μg in 0.5 μl oil) into either the ventromedial hypothalamus (VMH) or the medial preoptic area (MPOA) of estrogen primed rats to assess their relative potencies for stimulating lordosis. P, 5α-pregnanedione and 5β,3β-pregnanolone elicited lordosis when injected at either VMH or MPOA. By contrast, 5α,3β-pregnanolone as well as 20α-OH and 20β-OH-pregnenone were much more effective in stimulating lordosis when implanted in the MPOA. Finally, 5β-pregnanedione and 5β,3α-pregnanolone did not stimulate lordosis at neither VMH nor MPOA. The observation that lordosis was induced in estrogen primed rats both by pregnanes that bind to the P receptor (i.e., P; 5α-pregnanedione; 20α- and 20β-OH-pregnenone) and by pregnanes that do not (i.e., 5α,3β-; 5β,3β- and 5α,3α-pregnanolone) indicates that diverse cellular mechanisms are involved in the facilitation of lordosis by pregnanes.

Pregnanes Lordosis Progesterone metabolism Progesterone receptors GABAergic modulators

IN vitro studies indicate that pregnanes modify brain function through either a genomic mechanism involving binding to an intracellular receptor and induction of de novo protein synthesis (6, 21, 23, 52) or through the modulation of membrane processes modifying neuronal excitability (30, 35, 46, 67, 69, 92, 93) or the release or action of neurotransmitters or neuromodulators (9, 12, 14, 22, 26, 31, 32, 48, 54, 55, 76, 77, 96). Studies in structure-activity relationships indicate that particular conformations of the pregnane molecule are differentially involved in the genomic and in the membrane effects of pregnanes. Thus, binding to the intracellular receptor and stimulation of protein synthesis requires a delta-4-3-keto structure (15, 16, 27, 44, 83, 95), while membrane actions are mostly related to ring A-reduction at either the 5α- or the 5β-position (9, 32, 48, 55).

A well-known action of pregnanes is the facilitation of lordosis in estrogen primed rodents (23, 61, 103). Analysis of the literature on the effect of various pregnanes on lordosis suggests that they activate this behavior through more than one cellular mechanism. This idea is based on the fact that some pregnanes like 5α-pregnanedione, which bind weakly to the intracellular pregnane receptor (27, 28, 38, 44) are, nonetheless, capable of eliciting lordosis in estrogen primed rats (28, 29, 47, 58, 102). In general, delta-4-3-keto pregnanes like progesterone (P) or some synthetic pregnanes are more potent than ring A-reduced pregnanes to stimulate lordosis (28, 47, 58, 102), but bioavailability problems may account for this difference (8,11).

It has been proposed that the reflex arc for lordosis is regulated by two neural centers exerting opposite actions: the ventromedial hypothalamus (VMH), whose activation elicits lordosis, and the septal-medial preoptic area (MPOA) which tonically inhibits this behavior (65, 73, 74, 78, 101). The participation of pregnanes in the regulation of these two neural centers has been well documented for the VMH. Thus, the implantation of P into this area reliably facilitates lordosis in estrogen primed rats (28, 78, 88, 89, 100). By contrast, few works have explored the role of P in the MPOA, and the results obtained have been somewhat contradictory (51, 85, 87–89, 100). Recently, we found that 5β,3β-pregnanolone, but not P, induced intense lordosis when implanted as a crystal into the MPOA of estrogen primed rats (85). This result is of interest since 5β-3β-pregnanolone does not bind to the intracellular pregnane receptor (15, 27, 95), while exerting clear effects at the membrane (18, 45, 46, 69, 92). From these results, we believe lordosis can be activated by pregnanes through either of two mechanisms: 1) by increasing the excitability of VMH neurons, most likely through a genomic mechanism, or 2) by depressing the activity of MPOA neurons, most likely through membrane actions. We anticipated that delta-4-3-keto pregnanes would use the former mechanism while ring A-reduced pregnanes would use the latter. In order to test this model, we explored the ability of a series of natural delta-4-3-keto and ring A-redued pregnanes to induce lordosis when injected into either the MPOA or the VMH of estrogen primed rats.

METHOD

Subjects (Ss) were sexually inexperienced Wistar female rats raised in our colony under a controlled light:dark environment (14 hr light:10 hr dark) maintained at 23±2°C. They were housed in groups (3/cage) and fed Purina rat chow and water ad lib. When weighing 200–250 g they were ovariectomized under ether anesthesia, and two to three weeks later, they were bilaterally implanted into either the VMH or the MPOA. Ss anesthetized with pentobarbital (35 mg/kg, IP) were mounted onto a Kopf stereotaxic apparatus (David Kopf Instruments, Tujunga, CA). The skull was exposed, two small holes were drilled on the skull with a dental burr and 28 ga guide cannulae were lowered into either the MPOA or the VMH following coordinates provided by the De Groot atlas (13). A fine stainless steel mounting screw was positioned nearby to insure fastening onto the skull of the dental acrylic placed around the implants. The skin was sutured around them. After implantation, animals were housed individually. One week later, Ss received 4 μg estradiol benzoate (EB) and, 44 hr later, intracerebral injections were made through 30 ga insert cannulae introduced into the guide cannulae. The length of the insert cannulae was adjusted to end flush with the guide cannula. Insert cannulae were connected to a 5 μl Hamilton syringe that was driven by a micromanipulator. For intracerebral injections, lasting about 45 sec per side, Ss were lightly anesthetized with ether. They recovered from this procedure within 5 min. Pregnane dose was 5 μg per side. This dose was selected from a previous study showing that bilateral injections of 5 μg P into the VMH facilitate intense lordosis in nearly all Ss (19). Pregnanes were injected in 0.5 μl carthamus oil into either the VMH (Groups 2 to 10) or the MPOA (Groups 12 to 20), as follows:

Group 1:	oil	(n=19)
Group 2:	P	(n=7)
Group 3:	20α-hydroxy-4-pregnen 3-one (20α-OH-pregnenone)	(n=8)
Group 4:	20β-hydroxy-4-pregnen-3-one (20β-OH-pregnenone)	(n=7)
Group 5:	5α-pregnane-3,20-dione (5α-pregnanedione)	(n=5)
Group 6:	5α-pregnan-3α-ol-20-one (5α,3α-pregnanolone)	(n=6)
Group 7:	5α-pregnan-3β-ol-20-one (5α,3β-pregnanolone)	(n=8)
Group 8:	5β-pregnan-3,20-dione (5β-pregnanedione)	(n=8)
Group 9:	5β-pregnan-3α-ol-20-one (5β,3α-pregnanolone)	(n=5)
Group 10:	5β-pregnan-3β-ol-20-one (5β,3β-pregnanolone)	(n=10)
Group 11:	oil	(n=26)
Group 12:	P	(n=16)
Group 13:	20α-OH-pregnenone	(n=12)
Group 14:	20β-OH-pregnenone	(n=6)
Group 15:	5α-pregnanedione	(n=6)
Group 16:	5α,3α-pregnanolone	(n=10)
Group 17:	5α,3β-pregnanolone	(n=8)
Group 18:	5β-pregnanedione	(n=9)
Group 19:	5β,3α-pregnanolone	(n=9)
Group 20:	5β,3β-pregnanolone	(n=10)

Number of Ss in each group includes only those rats with adequate location of implants. All steroids were obtained from Sigma (St. Louis, MO). Tests for lordosis were conducted by placing Ss in a circular Plexiglas cage with a vigorous male. The lordosis quotient (LQ=number of lordosis/10 mounts × 100) was determined at 30, 120, 240 and 480 min after intracerebral injections. Following experiments, animals were anesthetized with ether and perfused through the heart with 10% formalin-0.9% saline. Brains were removed and stored in 10% formalin. The fixed brains were cut serially in the transverse plane into 7 μ sections, stained with hematoxilin-eosin and position of cannulae was then verified. Implants within 1.0 mm of the center of either VMH or MPOA were considered adequate and included in this study. The effect of pregnanes was assesssed by comparing LQs from Groups 2 to 20 against LQs from the corresponding control groups (i.e., Groups 1 or 11) using the Mann-Whitney U-test (94). Values of $p \leq 0.025$ were considered significant. In order to assess the Relative Potency of pregnanes, a lordosis response value (LR) was obtained for each animal by measuring the area under the curve generated from plotting its LQ values versus time. The LR, therefore, depends on three factors: latency, intensity and duration. LR ratios were then obtained by dividing LR of pregnane/LR of P and compared against control oil groups using Mann-Whitney's U-test (94).

RESULTS

Effect of Pregnane Injections into the VMH of Estrogen Primed Rats

As shown in Table 1, intrahypothalamic injections of carthamus oil in and around the VMH failed to induce significant lordosis, only three out of 19 Ss showing LQs above 50. P was highly effective in stimulating lordosis, significant values being obtained at two hr after injection, maximal ones seen at four hr after injection. Both α- and β-reduction of C20 resulted in pregnanes less potent than the original one (P), since neither 20α- nor 20β-OH-pregnanes elicited significant lordosis at any time. The effect of intrahypothalamic injections of 5α-reduced pregnanes varied considerably in relation to the functional group attached to C3. Thus, 5α-pregnanedione was effective in stimulating lordosis, significant levels being observed at two hr. Maximal values (i.e., LQ=100) were obtained in all five rats with adequate implants four hr after injections. Reduction of 5α-pregnanedione at either the 3α- or the 3β-position (yielding 5α,3α-pregnanolone and 5α,3β-pregnanolone, respectively), reduced the potency of the parent compound. The former pregnane was totally ineffective and the latter one showed a weak, though significant response eight hr after injection. Of the 5β-reduced pregnanes, only 5β,3β-pregnanolone was effective. This pregnane stimulated highly significant lordosis after only 30 min postinjection. At this time, 9 out of 10 Ss displayed lordosis, six of them with LQs of 50 or above. High levels of lordosis were still observed in these Ss eight hr after injection. Analysis of LR ratios, depicted in Fig. 1, showed that 5α-pregnanedione and P were almost equipotent, followed by 5β,3β-pregnanolone.

Effect of Pregnane Injections into the MPOA of Estrogen Primed Rats

Table 2 summarized the effects obtained with the various

TABLE 1

EFFECT OF VMH INJECTIONS OF VARIOUS PREGNANES ON THE LORDOSIS BEHAVIOR OF OVARIECTOMIZED RATS PRETREATED WITH 4 μg ESTRADIOL BENZOATE

Group	Treatment	No. of Ss	Mean LQ ± SD* (% Responsive Ss) 30′	120′	240′	480′
1	Oil	19	9 ± 27 (10%)	14 ± 32 (26%)	21 ± 38 (31%)	17 ± 33 (26%)
2	Progesterone	7	36 ± 43 (43%)	74 ± 31¶ (100%)	93 ± 17¶ (100%)	78 ± 34‡ (86%)
3	20α-OH-4-pregnen-3-one	8	17 ± 30 (25%)	41 ± 36 (62%)	41 ± 45 (50%)	29 ± 32 (62%)
4	20β-OH-4-pregnen-3-one	7	20 ± 35 (28%)	43 ± 45 (57%)	36 ± 43 (57%)	28 ± 46 (28%)
5	5α-pregnane-3,20-dione	5	0 ± 0 (0%)	78 ± 39† (80%)	100 ± 0¶ (100%)	80 ± 40‡ (80%)
6	5α-pregnan-3α-ol-20-one	6	7 ± 7 (50%)	18 ± 25 (50%)	33 ± 39 (50%)	3 ± 7 (17%)
7	5α-pregnan-3β-ol-20-one	8	25 ± 41 (37%)	25 ± 43 (25%)	40 ± 47 (62%)	59 ± 42† (75%)
8	5β-pregnane-3,20-dione	8	24 ± 37 (37%)	42 ± 45 (62%)	50 ± 50 (50%)	18 ± 32 (37%)
9	5β-pregnan-3α-ol-20-one	5	8 ± 18 (20%)	26 ± 36 (40%)	28 ± 44 (40%)	20 ± 45 (20%)
10	5β-pregnan-3β-ol-20-one	10	56 ± 33¶ (90%)	55 ± 40‡ (80%)	82 ± 36¶ (90%)	53 ± 37‡ (90%)

*Calculated from all Ss, i.e., responsive and nonresponsive; †$p \leq 0.025$, ‡$p \leq 0.01$, §$p \leq 0.005$, ¶$p \leq 0.001$.

pregnanes aimed at the MPOA. P was again effective in stimulating lordosis when injected in or around this area, significant responses seen at two hr postinjection, and maximal values being observed at four hr. In contrast to the VMH data, both 20α- and 20β-OH-pregnenones induced significant lordosis, Ss receiving the former pregnane showing this behavior already at 30 min after injection. 5α-pregnanedione stimulated significant lordosis only at eight hr postinjection, a latency suggesting a possible spread to the VMH. Surprisingly, 5α,3β-pregnanolone induced intense lordosis with a two hr latency, while its 3α-isomer was ineffective. Of the 5β-reduced pregnanes, 5β,3β-pregnanolone was the most effective, inducing intense lordosis in nearly all Ss within 30 min. This effect was maintained up to four hr after injection. By contrast, 5β-pregnanedione stimulated significant lordosis only at two hours after injection. Analysis of LR, depicted in Fig. 1, showed that 5α,3β-pregnanolone, which had a weak activity in the VMH, was the most effective of all pregnanes in the MPOA, followed closely by 5β,3β-pregnanolone. Almost equipotent to P were 5α-pregnanedione, 20α- and 20β-OH-pregnenone. Interestingly, the LR ratio shown by 5α,3α-pregnanolone suggested a slight potency for stimulating lordosis.

DISCUSSION

Contrary to our expectations, ring A-reduced pregnanes facilitated lordosis when injected into the VMH of estrogen primed rats. Thus, 5β,3β-pregnanolone, found ineffective when implanted as a crystal into the VMH in our previous study (85), induced intense lordosis with a short latency when injected in oil. Similarly, confirming the report of Glaser *et al.* (28), 5α-pregnanedione also elicited lordosis when injected into this area. Surprisingly, some pregnanes, including both ring A-reduced and delta-4-3-keto, induced lordosis when injected into the MPOA. Thus, 5β-3β-preganolone and 5α,3β-pregnanolone elicited lordosis in nearly all Ss, the former one with a very short latency. P was also effective in stimulating lordosis, a finding confirming an early report (100) Previous failures to induce lordosis with crystal implants of P in the MPOA (51, 87, 88, 89), including work from our laboratory (85), may be due to differences in the rate and extent of pregnane diffusion from crystals versus from oil solutions. Steroids diffuse more rapidly from oil solutions than from crystals, and the semispherical shape of the oil depot provides a much larger absorption area than the basically flat surface of crystalline implants (33). These factors may be of critical importance at the MPOA, where

TABLE 2

EFFECT OF MPOA INJECTIONS OF VARIOUS PREGNANES ON THE LORDOSIS BEHAVIOR OF OVARIECTOMIZED RATS PRETREATED WITH 4 μg ESTRADIOL BENZOATE

Group	Treatment	No. of Ss	Mean LQ ± SD* (% Responsive Ss) 30′	120′	240′	480′
11	Oil	26	12 ± 26 (31%)	29 ± 42 (35%)	29 ± 41 (42%)	19 ± 34 (31%)
12	Progesterone	16	19 ± 29 (37%)	64 ± 43‡ (75%)	72 ± 42‡ (75%)	17 ± 24 (62%)
13	20α-OH-4-pregnen-3-one	12	46 ± 38§ (83%)	62 ± 43† (75%)	57 ± 49 (80%)	28 ± 35 (50%)
14	20β-OH-4-pregnen-3-one	6	2 ± 4 (17%)	55 ± 46 (67%)	68 ± 39 (83%)	47 ± 33‡ (67%)
15	5α-pregnane-3,20-dione	6	3 ± 7 (17%)	38 ± 42 (50%)	53 ± 44 (87%)	68 ± 37‡ (83%)
16	5α-pregnan-3α-ol-20-one	10	19 ± 36 (40%)	53 ± 47 (70%)	52 ± 51 (70%)	32 ± 40 (50%)
17	5α-pregnan-3β-ol-20-one	8	41 ± 46 (50%)	84 ± 33§ (87%)	86 ± 19§ (100%)	51 ± 41† (75%)
18	5β-pregnane-3,20-dione	9	20 ± 35 (33%)	62 ± 45† (78%)	40 ± 45 (55%)	53 ± 45 (67%)
19	5β-pregnan-3α-ol-20-one	9	13 ± 33 (33%)	12 ± 22 (33%)	16 ± 33 (44%)	25 ± 43 (55%)
20	5β-pregnan-3β-ol-20-one	10	63 ± 46¶ (90%)	74 ± 43§ (80%)	72 ± 45‡ (80%)	56 ± 50 (60%)

*Calculated from all Ss, i.e., responsive and nonresponsive; †$p \leq 0.025$, ‡$p \leq 0.01$, §$p \leq 0.005$, ¶$p \leq 0.001$.

neurons related to the expression of lordosis have a scattered distribution (62, 72, 101). Consequently, the activation of a number of neurons sufficient to elicit lordosis by P would require the diffusion of the steroid to a much larger area, a requirement probably not met by crystal implants. On the other hand, this greater diffusion of steroids may have allowed P injected at the MPOA to diffuse into the VMH to stimulate lordosis. This interpretation is supported by our recent finding that dosages as low as 200 nanograms of P bilaterally implanted as oil microdepots into the VMH stimulate intense lordois in estrogen primed rats, while similar levels of lordosis are observed following MPOA implants only when dosages 10-fold greater are used (Beyer *et al.*, unpublished observations). This explanation, however, cannot be valid for all pregnanes, since: a) 5β,3β-pregnanolone has been reported to facilitate lordosis even when unilaterally implanted as a crystal into the MPOA (85) and b) 5α,3β-pregnanolone, along with 20α- and 20β OH-pregnenone, which induced intense lordosis in the MPOA, produced only marginal or null responses in the VMH.

It is generally considered that the stimulation of lordosis by P is mediated by its binding to the intracellular pregnane receptor (7, 60, 71, 84). Support for this interpretation comes from the observation that P binds to this receptor (28,40) and that a clear correlation exists between the appearance of lordosis and the concentration of the pregnane receptor in

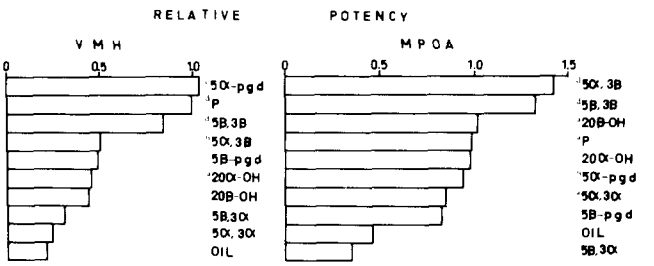

FIG. 1. Relative potencies of pregnanes to induce lordosis when implanted as oil solutions into either the ventromedial hypothalamus (VMH) or the medial preoptic area (MPOA) of ovariectomized estrogen primed rats. The lordosis response (LR) of individual animals was calculated by measuring the area under the curve generated from plotting LQ values versus time. The mean LR was calculated for all pregnanes and the one obtained by progesterone was equal to 1. Relative potencies of remaining pregnanes were thus calculated from the ratio: LR of pregnane/LR or progesterone. 5α-pgd=5α-pregnanedione; P=progesterone; 5β,3β=,5β3β-pregnanolone; 5β-pgd=5β-pregnanedione; 20α-OH=20α-OH-pregnenone; 20β-OH=20β-OH-pregnenone; 5β,3α=5β,3α-pregnanolone; 5α,3β=5α,3β-pregnanolone; 5α,3α=5α,3α-pregnanolone. [a]$p \leq 0.05$; [b]$p \leq 0.025$; [c]$p \leq 0.01$; [d]$p \leq 0.001$ versus oil.

the hypothalamus (7, 53, 60, 71, 84). This mechanism may mediate the facilitation of lordosis by 20α- and 20β-OH-pregnenone and even by 5α-pregnanedione, though the affinity of this last pregnane for the intracellular receptor is about one order of magnitude lower than that of P (27, 28, 39, 44, 95). However, the remaining three effective pregnanes used bind weakly, if at all, to the intracellular pregnane receptor (15, 16, 27, 44, 83, 95). These facts strongly suggest that alternative mechanisms mediate the facilitation of lordosis by pregnanes.

It is only possible to speculate on the nature of these alternative cellular mechanisms. As we recently proposed (85), the facilitation of lordosis by crystalline implants of 5β,3β-pregnanolone into the MPOA may involve a decrease in the excitability of preoptic neurons, tonically inhibiting lower neural structures which favors lordosis (74, 75, 79, 105). The possibility that other effective ring A-reduced pregnanes facilitated lordosis through a similar mechanism is supported by the fact that some of them powerfully depress neuronal firing and EEG activity (30, 46, 49, 67, 97). This inhibitory action of ring A-reduced pregnanes has been recently proposed to be mediated by their capacity to enhance GABAergic activity through their interaction with the barbiturate site of the GABA receptor (9, 26, 32, 48, 55). This mechanism of action fits well with the high concentration of GABA fibers existing in the MPOA (20, 25, 99) and with the observation that iontophoretic administration of GABA consistently inhibits MPOA neuronal firing (56). However, the fact that the most potent pregnanes to inhibit neuronal firing and to enhance GABAergic activity, i.e., 5α,3α- and 5β,3α-pregnanolone, exerted negligible effects on lordosis, militates against this interpretation.

An alternative mechanism through which pregnanes could facilitate lordosis involves their capacity to release LHRH. Ramírez and his group have recently explored the effect of several pregnanes on the release of LHRH by hypothalami of estrogen primed rats (42, 43, 70, 82). As in the case of lordosis, a wide spectrum of pregnanes with distinct conformations (P; 20α-OH-pregnenone; 5α-pregnanedione and 5β,3β-pregnanolone) elicited LHRH release. Also in parallel to the stimulation of lordosis, 5β,3β-pregnanolone was an extremely effective pregnane to elicit LHRH release, while its 3α-isomer was ineffective (70). Therefore, it is tempting to propose that 5β,3β-pregnanolone, and perhaps other ring A-reduced pregnanes, stimulated lordosis by releasing LHRH from both MPOA and VMH. Important concentrations of this peptide exist in these regions (1, 57, 104) and injections of LHRH into these structures induce lordosis in estrogen primed rats (17, 63, 64, 73, 86). The cellular processes facilitating LHRH release by pregnanes are not clear, but they appear to involve the release of noradrenaline and a raise in hypothalamic cyclic AMP (68,81). This mechanism would also explain the prolongation of P-induced lordosis by phosphodiesterase inhibitors (3) and the stimulation of this behavior by cyclic nucleotides (4, 24, 59) in estrogen primed rats.

A pertinent question arising from pharmacological studies relates to the functional meaning of the reported findings. Several facts suggest, though not prove, that the effects observed in this study may be of physiological relevance. Some ring A-reduced pregnanes, i.e., 5α,3α-pregnanolone; 5α,3β-pregnanolone; 5α-pregnanedione, are secreted in large amounts by the rat ovaries and adrenals into the peripheral circulation (34, 36, 37, 90). However, their role in the regulation of brain functions is probably limited by bioavailability problems arising from their rapid sulfoconjugation in the liver (8, 11, 80). A more plausible mechanism for the participation of pregnanes in the regulation of brain activity would arise from P neuronal metabolism (39). P is transformed to ring A-reduced pregnanes, the favored metabolic pathways being reductions in the 5α- and in the 3β-positions (2, 10, 39). These reactions yield 5α-pregnanedione and 5α,3β-pregnanolone, both of them effective pregnanes to elicit lordosis. P is also transformed to 5β-reduced pregnanes (41,80), but the importance of this metabolic pathway in the regulation of brain function is unclear since it occurs in a smaller proportion than 5α-reduction (2, 10, 39). Nonetheless, Rasinghani *et al.* found that the low concentration of 5β-pregnanes resulting from the metabolism of injected P were sufficient to induce clear alterations in vigilance (80).

In summary, the present results indicate that, irrespective of their site or mechanism of action, many P metabolites can induce significant lordosis behavior when infused into the brain. The elucidation of the functional significance of these findings, as well as the precise cellular mechanisms involved in this effect will require further studies.

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