Differential involvement of amygdala corticosteroid receptors in visceral hyperalgesia following acute or repeated stress

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Myers B, Greenwood-Van Meerveld B. Differential involvement of amygdala corticosteroid receptors in visceral hyperalgesia following acute or repeated stress. Am J Physiol Gastrointest Liver Physiol 302: G260–G266, 2012. First published November 3, 2011; doi:10.1152/ajpgi.00353.2011.—Symptoms of irritable bowel syndrome (IBS) are exacerbated by stress. Previously, we demonstrated that the stress hormone corticosterone applied directly to the amygdala induced visceral hypersensitivity through the actions of glucocorticoid receptor (GR) and mineralocorticoid receptor (MR). However, the involvement of amygdaloid GR and MR in the regulation of visceral sensitivity following psychological stress is unknown; therefore, the goal of the present study was to determine the relative importance of amygdaloid GR and MR in the regulation of visceral sensitivity in a rodent model of behavioral stress. Male F-344 rats were stereotaxically implanted with micropellets bilaterally on the dorsal margin of the amygdala containing the GR antagonist mifepristone, the MR antagonist spironolactone, or cholesterol as a control. Animals were then exposed to 1 h of water-avoidance stress (WAS) or sham stress for 1 day (acute) or 7 days (repeated). Visceral sensitivity was assessed either 1 h or 24 h after the final session of WAS and quantified as the number of contractions of the external abdominal oblique, a visceromotor response, in response to colorectal distension at pressures of 0–60 mmHg. Acute stress induced transient visceral hyperalgesia, which was absent 24 h after WAS and independent of GR and MR. Conversely, repeated WAS induced sustained visceral hyperalgesia that was abolished by specifically targeting the amygdala with GR and MR antagonists. These results demonstrate that the amygdala corticosteroid system plays an essential role in mediating the effects of repeated WAS on visceral sensitivity. Furthermore, our findings suggest that amygdaloid GR and MR may be involved in IBS symptomatology.

CHRONIC ABDOMINAL PAIN attributable to altered visceral perception and abnormal bowel patterns including diarrhea, constipation, or alternating episodes of both is the characteristic symptom of irritable bowel syndrome (IBS). IBS affects up to 10–15% of the population with an estimated 1.7 billion dollars in annual direct costs (13, 31), yet the etiology of the disorder remains unknown. Stress has been implicated in the pathophysiology of IBS, as stress levels correlate with the subsequent intensity of bowel symptoms as well as indices of anxiety and depression (1, 12, 35). The principal neuroendocrine stress system is the hypothalamic-pituitary-adrenal (HPA) axis, which regulates the production of adrenal corticosteroids, cortisol in humans and corticosterone (CORT) in rodents, following corticotropin-releasing factor (CRF) synthesis in the hypothalamus. Dysregulation of the HPA axis has been demonstrated in patients with IBS on the basis of multiple reports of elevated cortisol levels in response to stress (3, 7). Additionally, CRF stimulation elicits exaggerated adrenocorticotropic hormone, cortisol, and colonic motility responses in patients with IBS compared with healthy controls (9). Studies of brain activation in response to visceral stimulation have shown that limbic regions regulating sensory processing and emotion, including the amygdala, show greater responsiveness in patients with IBS than controls (20, 36).

The amygdala is involved in affective processing of sensory information, including nociception, and is a critical site for the generation of anxiety and fear (6, 23, 27). In addition, the amygdala is sensitive to corticosteroids and increases the excitability of central nervous system sites regulating behavioral, neuroendocrine, and autonomic responses to stress (6, 15). Specifically, the central nucleus of the amygdala (CeA) provides both direct and indirect innervation of the nucleus of the solitary tract, locus coeruleus, and hypothalamic nuclei, allowing for modulation of HPA responses, affective processes, and autonomic functions including gastric emptying and colonic motility (6, 10, 26).

Previous studies in rodents found that local exposure of the amygdala to CORT increases the sensitivity to visceral and somatic stimuli as well as inducing anxiety-like behavior through glucocorticoid receptors (GR) and mineralocorticoid receptors (MR) (11, 17–19). Furthermore, pharmacological activation of either GR or MR in the amygdala is sufficient to induce visceral hyperalgesia and anxiety-like behavior (18); however, the necessity of amygdaloid GR and MR for visceral responses to psychological stress is unknown. We hypothesized that the increased activity of the amygdala and elevated levels of CORT in response to psychological stress lead to alterations in visceral pain processing through GR- and MR-dependent mechanisms. To address this hypothesis, we employed a model of behavioral stress-induced visceral hyperalgesia (2) in rats with micropellets of the GR antagonist mifepristone, the MR antagonist spironolactone, or cholesterol as a control on the amygdala. These animals were exposed to 1 h of water-avoidance stress (WAS) for either 1 day or 7 days with assessment of visceral sensitivity occurring either 1 h or 24 h poststress.

MATERIAL AND METHODS

Animals. Experiments were performed on male Fischer 344 rats (250–325 g) purchased from Charles River Laboratories (Wilmington, MA) and housed under standard conditions with a 12-h:12-h light/dark cycle and unrestricted access to standard rat chow and water. To reduce stress associated with shipping and the laboratory environment, rats were acclimated to the animal facility for at least 7
days followed by a second 7-day period of acclimatization to the experimental environment. During this acclimatization period, rats were brought into the laboratory between the hours of 10:00 AM and 2:00 PM, weighed, and handled by the investigator. All experiments were performed at the same time each day and were in compliance with the National Institutes of Health Guide for Care and Use of Laboratory Animals and the International Association for the Study of Pain Research Guidelines. The experimental procedures were approved by the VA Animal Care and Use Subcommittee at the Oklahoma City VA Medical Center.

**Stereotaxic implantation of micropellets.** Rats were anesthetized with a combination of ketamine (100 mg/kg, ip) and xylazine (10 mg/kg, ip) with body temperature maintained at 37°C using a homeothermic heating blanket (Harvard, Ealing, UK). Rats were placed in a digital stereotaxic frame (Kopf, Tujunga, CA), and a midline incision was started at the frontal region and extended to the occipital region of the skull. Vertical measurements were made at the positions of lambda and bregma to ensure that the skull was level. Small holes were made in the skull at the coordinates 2.5 mm posterior to bregma and 4.2 mm to the right and left of midline. The ventral coordinate was 7.0 mm below the dura, and this placement delivers hormones to the amygdala without damaging the structure and avoids contact with the hippocampus (18). Importantly, previous studies by Shepard and colleagues (30) demonstrated that a single delivery of steroid micropellets to the dorsal amygdala leads to elevated levels of steroids in the area of the amygdala 7 days postinjection. Micropellets were created by tamping steroid into a 25-gauge stainless steel cannula, and the crystalline pellet was extruded using a stylet cut to the length of the cannula. The empty cannula was removed, and gel foam was placed in the holes in the skull. The skin was closed, and antibiotic/analgesic cream was applied to the wound.

**Stress protocol.** Rats in the WAS group were placed on a square platform (8 × 8 × 8 cm) mounted in the center of a white semitransparent plastic container (50 × 35 × 33 cm) filled with fresh, room temperature water to 1 cm below the surface of the platform. Animals in the sham stress group were placed in containers without water. All animals were left undisturbed for 60 min, and the number of fecal pellets produced during the WAS or sham stress was recorded to evaluate stress-induced colonic motility. An additional group of rats remained in the animal facility during the stressor and served as home cage controls.

**Assessment of visceral sensitivity.** The level of visceral sensitivity was determined by recording the visceromotor response (VMR) to colorectal distension (CRD) in rats that were unrestrained and freely moving in their home cages. The VMR is a pseudoffective reflex contraction of the abdominal musculature induced by CRD (22). In preparation for visceral sensitivity assessment, animals were placed under isoflurane anesthesia (1.5–2.5%) while a strain gauge force transducer (R.B. Products, Stillwater, MI) was placed on the external abdominal oblique muscle and a 5-cm colorectal latex balloon catheter was inserted via the anal canal. Briefly, the strain gauge was sutured in place, and the skin was closed over the strain gauge force transducer while connecting the strain gauge to a computer with Polyview Data Acquisition Software (Grass Technologies, West Warwick, RI). CRD was induced by inflating the balloon using a constant pressure barostat (G&J Electronics, Toronto, Canada). CRD was performed at graded pressures of 20, 40, and 60 mmHg for 10 min each with a 10-min recovery period between distensions.

**Histological localization of stereotaxic implants.** Animals were anesthetized with isoflurane (5%) and decapitated, and brains were rapidly removed and frozen in chilled 2-methylbutane (Fisher Scientific, Fair Lawn, NJ). Brains were then stored at −80°C until cryosectioning at −20°C (Bright OTF, Fairfield, NJ). Serial coronal sections (40 μm) were mounted onto slides followed by verification of micropellet placement by light microscopy.

**Collection of organs.** Adrenal and thymus glands were collected and weighed as indirect measures of HPA activation. To account for differences in body weight, thymus and adrenal gland indices were calculated as organ weight divided by body weight multiplied by 100.

**Determination of plasma CORT.** A tail nick was employed to collect blood for the CORT assay (8, 32). Blood samples were taken within 3 min of the tail nick under light manual restraint to prevent activation of the HPA axis (34). Plasma CORT was measured in blood samples (30–50 μl) collected in heparinized glass containers on ice. Basal blood samples were collected, and rats were subjected to 1-h WAS with a second blood sample collected immediately following the termination of the stressor. A final blood sample was taken 90 min after WAS. A commercial enzyme immunoassay kit (Immunodiagnostic Systems, Fountain Hills, AZ) was used to determine plasma CORT concentration according to the manufacturer’s instructions. Plasma samples were processed in duplicate, and the interassay and intra-assay coefficients of variability were 8.6% and 6.6%, respectively.

**Experimental protocol.** Experiment 1 consisted of nine groups (n = 6 each) in which all animals received implants of cholesterol (30 μg) on the amygdala. Animals in the acute WAS groups received 1 h of stress for 1 day with visceral sensitivity assessed 1 h or 24 h poststress. Rats in the repeated WAS groups were exposed to 1 h of stress for 7 days with assessment of visceral sensitivity 1 h or 24 h after the final stress session. Control animals were exposed to sham stress (container without water) for 1 day or 7 days with VMR measured either 1 h or 24 h later. An additional group of rats remained unhandled in the animal facility during stress sessions and served as home cage controls.

**Experiment 2** featured six groups of rats (n = 6 each) receiving implants of either the GR antagonist mifepristone or the MR antagonist spironolactone (15 μg each) on the amygdala. This concentration of antagonists was based on concentration-response studies showing that 15 μg of mifepristone or spironolactone inhibited the effects of amygdaloid CORT on visceral hypersensitivity and anxiety-like behavior, additionally, the effects of selective GR and MR agonists on pain sensitivity and anxiety-like behavior were blocked by 15 μg of theses antagonists (17, 18). Animals with GR or MR antagonists were subject to 1 day of WAS with VMR 1 h afterward or 7 days of WAS with VMR 1 h or 24 h poststress. To investigate the role of amygdaloid GR and MR in the effects of WAS, the cholesterol-implanted WAS rats from experiment 1 were compared with antagonist-implanted WAS animals in experiment 2. Experiment 3 employed four groups (n = 7 each) to identify the potential neuroendocrine mechanisms mediating the effects of GR and MR. WAS and sham animals received cholesterol implants, while two additional groups of WAS animals received either mifepristone or spironolactone on the amygdala. These four groups went through the repeated WAS paradigm with blood sampling pre- and poststress on day 7 followed by organ collection.

**Data analysis.** Data are presented as the means ± SE. Sample sizes were determined by power analysis (nQuery Advisor) based on preliminary data for the maximal difference in VMR to CRD in rats. One-way ANOVA and Bonferroni posttests were performed on the acute stress fecal pellet output (FPO) and organ weights, whereas two-way repeated-measures ANOVA and Bonferroni posttests were performed on the repeated stress FPO and plasma CORT to determine the effect of treatment over time. Changes in the VMR attributable to CRD were also analyzed by two-way repeated-measures ANOVA followed by Bonferroni posttests with both treatment and distension pressure as factors. In all cases, posttests were only conducted when there was a significant main effect of treatment, pressure, or time.
showed an increased number of abdominal contractions to higher pressures of CRD, or visceral hyperalgesia, compared with rats receiving sham stress or home cage controls ($n = 6$/group). However, this effect was transient, as animals assessed 24 h following acute WAS (Fig. 2B) showed no significant differences in the number of abdominal contractions during CRD ($n = 6$/group). The colonic motility response to acute WAS was determined by the number of fecal pellets expelled during the 60-min stress (Fig. 2C). Animals subject to WAS exhibited a significantly ($P < 0.01$) higher number of fecal pellets compared with animals receiving sham stress ($n = 12$ each).

Effect of repeated stress on visceral sensitivity and FPO. Animals subject to 7 days of WAS displayed visceral hyperalgesia when assessed 1 h poststress compared with animals receiving repeated sham stress or home cage controls ($n = 6$/group; Fig. 3A). Furthermore, the repeated WAS-induced visceral hyperalgesia was persistent, as animals assessed 24 h after the final session of WAS also showed significant ($P < 0.05$) elevations in visceral sensitivity compared with controls ($n = 6$/group; Fig. 3B). WAS-induced colonic motility, quantified by FPO, was significantly ($P < 0.05$) greater across all 7 days of stress in animals receiving repeated WAS compared with sham-stressed animals ($n = 12$/group), suggesting a lack of habituation to the stress (Fig. 3C). Although there were no differences in FPO over time within the WAS group, sham-stressed animals demonstrated a significant ($P < 0.05$) reduction in FPO on days 4, 5, and 7 compared with day 1.

Lack of involvement of amygdaloid GR or MR in acute stress-induced visceral hyperalgesia and FPO. The increased VMR to CRD present 1 h after acute stress was not inhibited by GR or MR antagonism with mifepristone or spironolactone implantation on the amygdala ($n = 6$/group; Fig. 4A). The presence of visceral hyperalgesia in these animals suggests that acute stress increases visceral sensitivity independent of GR and MR in the amygdala. The increased FPO induced by acute WAS ($n = 12$) was not affected by implantation of the GR antagonist mifepristone ($n = 6$) or the MR antagonist spironolactone ($n = 6$) on the amygdala (Fig. 4B). The lack of significant differences in FPO based on treatment suggests that GR and MR are not responsible for colonic motility responses during acute WAS.

Importance of amygdaloid GR and MR for repeated stress-induced visceral hyperalgesia and FPO. Visceral hypersensitivity observed 1 h following repeated WAS was significantly
(P < 0.05) inhibited in animals receiving a GR or MR antagonist (n = 6 each) on the amygdala (Fig. 5A). The presence of GR or MR antagonists also significantly (P < 0.01) inhibited the repeated WAS-induced visceral hyperalgesia when assessed 24 h poststress (Fig. 5B), illustrating the necessity of both GR and MR for repeated stress-induced visceral hyperalgesia. During 7 days of WAS, there were no significant differences in FPO between animals receiving implants of mifepristone, spironolactone, or cholesterol (n = 12 each; Fig. 5C). However, analysis of FPO over time revealed that animals treated with the GR antagonist produced significantly (P < 0.01) fewer pellets on days 6 and 7 than day 1; additionally, rats receiving the MR antagonist showed significantly (P < 0.05) decreased FPO on days 4–7 compared with day 1.

Involvement of amygdaloid GR and MR in repeated stress-induced adrenal hypertrophy and CORT secretion. Repeated WAS did not significantly affect thymus mass (n = 7/group; Fig. 6A); however, repeated WAS significantly (P < 0.05) increased the mass of the adrenal glands relative to body weight, an effect that was inhibited by GR or MR antagonism in the amygdala (n = 7/group; Fig. 6B). On the final day of WAS, animals undergoing stress showed significantly (P < 0.001) greater plasma CORT than sham-stressed animals (n = 5–7/group; Fig. 7). The peak levels of CORT following WAS were significantly (P < 0.05) decreased by the presence of GR or MR antagonists in the amygdala (n = 5–7/group), indicating that corticosteroid receptors in the amygdala are necessary for adrenocortical responses to repeated WAS.

DISCUSSION

In the current study, we examined the relative contribution of corticosteroid receptors within the amygdala to the development of stress-induced colonic hypersensitivity through the use of selective GR or MR antagonists. The major findings from our study are that acute exposure to WAS induces transient visceral hyperalgesia immediately following the stressor that is independent of GR and MR at the level of the amygdala. However, repeated WAS over multiple days led to increased visceral sensitivity immediately following the stressor that was also present 24 h poststress. These findings extend our earlier studies in which we investigated the effects of elevated amygdala CORT on colonic function. In these earlier studies, pharmacological activation of the amygdala, produced by stereotoxic implantation of micropellets containing CORT on the dorsal margin of the amygdala, produced colonic hypersensitivity and heightened levels of anxiety-like behavior (11, 17–19). In these same studies, we investigated the relative contribution of the two types of corticosteroid receptors using selective antagonists to GR or MR. We found that a combination of either mifepristone, a GR antagonist, or spironolactone, a MR antagonist, with CORT produced a significant inhibition of colonic hypersensitivity and anxiety. However, in the present study, we aimed to advance these earlier observations by investigating the importance of steroid-receptor-mediated
mechanisms in the amygdala on colonic sensitivity following a repetitive exposure to a psychological stressor.

Imaging studies employing rectosigmoid balloon distension in patients with IBS have shown abnormal activity in a network of brain structures involved in both sensory-discriminative functions as well as emotional-motivational processes. Areas such as the amygdala, insula, cingulate, and prefrontal cortex showed greater activation in patients with IBS than controls (20, 21, 36). In our own work, we found significant differences in brain activation in response to luminal distension of the colon in response to elevated amygdala CORT (14). Although the amygdala expresses both GR and MR (25), the involvement of amygdaloid GR and MR in the regulation of visceral hypersensitivity and abdominal pain following psychological stress remains to be investigated. The stressor used in our study was that of repeated water avoidance, which in our hands caused a significant increase in plasma CORT as well as higher adrenal weights compared with sham stress controls, which, taken together, are suggestive of increased HPA axis tone. Further substantiating abnormal glucocorticoid feedback at the level of the amygdala, we found that the elevated systemic CORT and higher adrenal weights induced by WAS were prevented by either GR or MR antagonism in the amygdala. Collectively our data support the hypothesis that amygdaloid CORT has excitatory effects on physiological responses to stress. In particular, CORT increases CRF mRNA expression in the CeA (28, 29), and the release of CRF in the CeA following stress can be prevented by corticosteroid receptor antagonism (4). Furthermore, CORT appears to produce feed-forward effects in the amygdala that can be linked to enhanced stress excitability, and recruitment of the amygdala CRF system has been proposed as a consequence of elevated CORT release during chronic or repeated stress (5). Thus the present

Fig. 5. Importance of amygdaloid GR and MR for repeated stress-induced visceral hyperalgesia and fecal pellet output. Increased visceral sensitivity induced by repeated stress was significantly \( P < 0.05 \) inhibited by GR or MR antagonism in the amygdala both 1 h (A) and 24 h (B) poststress \((n = 6/\text{group})\). *\( P < 0.05 \), **\( P < 0.01 \), ***\( P < 0.001 \) compared with WAS + mifepristone; ††\( P < 0.01 \), †††\( P < 0.001 \) compared with WAS + spironolactone. C: on each day of testing there was no significant between-group difference in stress-induced fecal pellet output between animals receiving implants of mifepristone, spironolactone, or cholesterol \((n = 12/\text{group})\). However, over the 7 days of repeated WAS, rats with implants of mifepristone or spironolactone showed significant \((P < 0.05)\) within-group reductions in WAS-induced fecal pellet output compared with their initial response on day 1. **\( P < 0.01 \) compared with WAS + mifepristone day 1; †\( P < 0.05 \), ††\( P < 0.01 \), †††\( P < 0.001 \) compared with WAS + spironolactone day 1.

Fig. 6. Involvement of amygdaloid GR and MR in repeated stress-induced adrenal hypertrophy. A: there was no significant effect of repeated stress or drug treatment on thymus mass \((n = 7/\text{group})\). B: repeated WAS significantly \((P < 0.05)\) increased mass of the adrenal glands, an effect that was inhibited by GR or MR antagonism in the amygdala \((n = 7/\text{group})\). *\( P < 0.05 \) compared with all other groups.

Fig. 7. Importance of amygdaloid GR and MR for repeated stress-induced corticosterone secretion. On day 7 of WAS, animals undergoing stress displayed significantly \((P < 0.001)\) greater plasma corticosterone than sham-stressed animals. The presence of GR or MR antagonists in the amygdala during WAS significantly \((P < 0.05)\) decreased the corticosterone response to stress \((n = 5–7/\text{group})\). †††\( P < 0.001 \) compared with sham: *\( P < 0.05 \) compared with WAS + mifepristone and WAS + spironolactone; solid black line represents WAS.
study supports the proposal that different circuits are recruited after repeated stress than those that regulate acute responses. We have previously demonstrated that the long-term effects of CORT in amygdala on pain and anxiety are dependent on CRF₁ receptors (19), and it is likely that the inhibitory effects observed in the present study are mediated by preventing the upregulation of CRF signaling by corticosteroid receptors.

In the rodent model, WAS led to exaggerated pain responses or hyperalgesia, and, although patients with IBS exhibit hyperalgesia, painful responses to innocuous stimuli or allodynia are also a common clinical complaint but were not observed in the current model. This issue has particular relevance when considering the translational importance of this work, as different neurobiological mechanisms are involved in allodynia and hyperalgesia; however, we have previously demonstrated that activation of MR in the amygdala leads to visceral hyper-sensitivity in response to all levels of CRD, indicating that the effects of amygdala corticosteroids are not limited to hyperalgesia (18). Furthermore, the results of the present study provide a potential neurobiological mechanism for the effects of psychological stress on visceral pain perception. Specifically, the altered balance in stress modulation induced by both amygdala hyperactivity and corticosteroids acting through GR and MR may represent an essential aspect of IBS.

In the present study, we assessed the effect of WAS on fecal pellet production and found that WAS increased fecal pellet output. However, the increased FPO observed during an acute session of WAS could not be blocked by pretreatment with a GR or MR antagonist, suggesting that this effect of acute stress is not dependent on the amygdala corticosteroid system. Although other investigators have demonstrated a role for CRF₁R in mediating the increased FPO during acute WAS (16), our group has demonstrated a role for amygdaloid GR and MR in poststress alterations in colonic motility. Specifically, we found that exposure of the amygdala to elevated levels of CORT did not affect spontaneous fecal output and did not influence the ability of an acute psychological stressor to increase fecal output. However, there was a reduction in colonic transit measured by glass bead expulsion in the period immediately following WAS that was reversed by antagonism of GR or MR in the amygdala (32).

An important observation from the present study comes from the significant elevations in FPO displayed on all 7 days of stress, illustrating that repeated presentation of WAS did not lead to acclimatization of the autonomic response to stress. The continued response to repeated homotypic stressors is a characteristic of Fischer 344 rats (33) and implies that this rodent strain may be a relevant model for human conditions associated with stress hyperresponsiveness such as IBS. Having demonstrated that daily repeated WAS for 7 days led to a significant increase in FPO over sham control rats, we next investigated whether the effects of repeated WAS on FPO were altered by pretreatment with a GR or MR antagonist. On each day of testing there was no significant between-group difference in WAS-induced FPO between animals receiving implants of mifepristone, spironolactone, or cholesterol. However, over the 7 days of repeated WAS, rats with implants of mifepristone or spironolactone showed significant within-group reductions in WAS-induced FPO compared with their initial response on day 1. These results implicate amygdaloid GR and MR in blocking the habituation of motility responses to repeated stress.

In conclusion, this study provides new evidence supporting a role for GR and MR at the level of the amygdala in visceral pain induced by repetitive exposure to a psychological stressor. The enhanced visceral pain reporting produced by repeated stress was abolished by the presence of GR or MR antagonist on the amygdala, demonstrating a critical role for these receptors in mediating repeated psychological stress-induced visceral hyperalgesia. These findings also suggest that the increased amygdala activation and elevated cortisol levels observed in patients with IBS may be particularly relevant to the pathophysiology of the disorder (3, 7, 20, 36). Taken together, our findings suggest that activation of the amygdala under the influence of chronic stress dysregulates the HPA axis by affecting the negative feedback mechanisms that terminate the HPA response to an acute psychological stressor. Furthermore, this steroid-specific modulation of the amygdala by stress may be relevant to the etiology of IBS and offer a novel therapeutic approach to the treatment of patients with abdominal pain that is exacerbated during periods of chronic stress.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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