Interactions between N-methyl-D-aspartate, nitric oxide and serotonin in the control of prolactin secretion in prepubertal male rats

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Abstract

The role of N-methyl-D-aspartate (NMDA) in the control of prolactin (PRL) secretion was analysed in prepubertal male rats. In experiment 1, males of different ages were decapitated after administration of NMDA or vehicle. In experiment 2, 30-day-old males were killed at different times after administration of vehicle, NMDA, MK-801 (a non-competitive NMDA antagonist) or NMDA plus MK-801. In experiment 3, 23-day-old males were sham-orchidectomized or orchidectomized. Orchidectomized males were or were not implanted with Silastic capsules containing different amounts of testosterone. On day 30, the animals were decapitated after administration of vehicle, NMDA or MK-801. In experiment 4, 30-day-old male rats were decapitated after being injected with vehicle, NMDA, Nω-nitro-L-arginine methyl ester (NAME) (an inhibitor of nitric oxide (NO) synthase), or NMDA plus NAME. Serum PRL concentrations, and dopamine pituitary and hypothalamic content were measured. In experiment 5, males pretreated with vehicle or NAME were killed after administration of the precursor of serotonin synthesis 5-hydroxytryptophan (5-HTP), the 5-HT1 receptor agonist 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) or the 5-HT2 agonist (±)2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI). Finally, the effects of NMDA, NAME and sodium nitroprusside (SNP) were tested in dispersed adenohypophyseal cells. We found that: (i) antagonism of NMDA receptors with MK-801 decreased PRL secretion in intact, orchidectomized and testosterone-treated orchidectomized male rats; (ii) NMDA inhibited PRL release in vivo through an increase in dopamine release and this effect was potentiated by NAME and prevented by testosterone; (iii) NMDA inhibited PRL secretion in vitro and this effect was observed in presence of both SNP and NAME; (iv) NAME blocked the stimulatory effects of 5-HTP and DOI on PRL secretion. We conclude that endogenous glutamate stimulates PRL release and that NO might have a pivotal role in the mechanisms involved in the control of PRL release, inhibiting the release of dopamine and modulating the effects of NMDA and 5-HT.

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Introduction

Prolactin (PRL) release is controlled by multiple hypothalamic and peripheral signals. Dopamine is the principal central inhibitory signal (1), while serotonin, vasoactive intestinal peptide, thyrotrophin-releasing hormone and other neuropeptides increase PRL release (2, 3). Recently, it has been shown that systemic administration of N-methyl-D-aspartic acid (NMDA), an agonist of NMDA receptors, increases the secretion of PRL in cyclic female rats (4–6). In adult male rats, NMDA stimulated (7) or had no effects (8–10) on PRL secretion. The effects of NMDA on PRL secretion seem to change according to the age of the animal and the endocrine milieu, as indicated by the following findings: NMDA inhibits PRL secretion in prepubertal but not in adult females (11); the stimulatory effect of NMDA on PRL secretion is observed in adult males only after orchidectomy (9); inhibitory effects of NMDA on PRL secretion are observed in hyperprolactinaemic conditions such as lactation (4, 5) or after neonatal administration of oestrogens (10). Because the effects of NMDA on PRL secretion have not been analysed in prepubertal male rats, the first aim of the present experiments was to analyse the role of NMDA receptors in the secretion of PRL by prepubertal intact, orchidectomized and testosterone-treated orchidectomized males.

A second major aim of the present experiments was to analyse the relationship between nitric oxide (NO), NMDA and serotoninergic drugs in the control of PRL secretion. NO production has been shown to increase after activation of NMDA receptors (12) and NO was an important intra- or intercellular messenger involved in the control of the hypothalamic–pituitary axis (13–16) and in the actions of NMDA on luteinizing hormone...
(LH) release (17). Sodium nitroprusside (SNP), a donor of NO, inhibited PRL secretion in vitro (18), while administration of blockers of NO synthase (NOS) inhibited basal and ovulatory secretion of PRL (11, 19). The inhibitory actions of NOS blockers seemed to be mediated by an increase in hypothalamic dopamine release (11).

**Material and methods**

**Animals and drugs**

Wistar rats born in our laboratory were maintained under controlled conditions of light (12 h light:12 h darkness, lights on at 0700 h) and temperature (22 °C) with free access to pelleted food (Pacsa Sanders, Seville, Spain) and tap water. On day 1 of life, each dam was left with eight pups. Testosterone, NMDA, Nω-nitro-L-arginine methyl ester (NAME) and 5-hydroxytryptophan (5-HTP) were from Sigma (Barcelona, Spain). MK-801 (an NMDA antagonist), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT, a 5-HT1 agonist) and (±)2,5-dimethoxy-4-iodoamphetamine hydrochloride (DOI) (a 5-HT2 agonist) were from Research Biochemical International (RBI, Natick, MA, USA). Experiments were carried out between 1000 and 1200 h. Special precautions were taken to avoid any stressing influences (all the animals were handled daily for a week before the experiment and killed by the same person, and the different drugs were injected at random). The number of animals per group is provided in the tables and figure legends.

**In vivo experiments**

**Experiment 1** Fifteen minutes after the i.p. injection of vehicle or NMDA (15 mg/kg), 4-, 8-, 12-, 16-, 20-, 30-, 60- and 90-day-old male rats were decapitated and trunk blood was collected.

**Experiment 2** Thirty-day-old males were injected with vehicle or MK-801 (1 mg/kg, i.p.) and 45 min later with vehicle or NMDA (15 mg/kg). The animals were killed 5 and 15 min after the last injection.

**Experiment 3** Animals were sham-orchidectomized or orchidectomized on day 23. Orchidectomized males were implanted with Silastic capsules (length 1.5 cm; inside diameter 1.519 mm, outside diameter 3.060 mm) that were either empty or contained testosterone. On day 30, animals were decapitated 15 min after administration of vehicle or NMDA (15 mg/kg), and 60 min after vehicle or MK-801 (1 mg/kg) treatment. Additional groups implanted with Silastic capsules of different lengths (0.5, 1.0 and 1.5 cm) were killed 15 min after NMDA or vehicle injection.

**Experiment 4** Thirty-day-old males were injected with vehicle or NAME (40 mg/kg, i.p.) at −60 min and with vehicle or NMDA (15 mg/kg) at −15 min and killed at 0 min. Trunk blood was collected. The hypothalamus and pituitary were dissected and frozen immediately in liquid nitrogen.

**Experiment 5** Thirty-day-old males were injected i.p. with vehicle or NAME (40 mg/kg) at −60 min and with 5-HTP (100 mg/kg), 8-OH-DPAT (1 mg/kg) or DOI (10 mg/kg) at −30 min and killed at 0 min.

**In vitro experiments**

The effects of NMDA, NAME, SNP, NAME plus NMDA and NMDA plus SNP were tested in dispersed adenohypophyseal cells obtained from 30-day-old male rats. The rats’ pituitaries were dissociated into individual cells according to the method described by Smith and Vale (20) with minor modifications. Thirty-day-old male rats were decapitated and the pituitaries removed immediately. After the posterior lobes were discarded, the anterior lobes were washed in Heps dissociating buffer (137 mmol/l NaCl, 5 mmol/l KC1, 0.1 mmol/l Na2HPO4, 25 mmol/l Heps and 50 μg/ml gentamicin sulphate, adjusted to pH 7.3) and cut into 1 mm pieces. Fragments from five anterior pituitary glands were transferred to a 25 ml flask previously siliconized and incubated in Heps dissociating buffer (5 ml) containing 0.1% trypsin, 0.2% collagenase type II, 0.2% glucose and 0.2% bovine serum albumin (BSA). The pituitaries were agitated mechanically for 2 h in a Dubnoff metabolic shaker (60 cycles/min) at 37°C in an atmosphere of 95% CO2–5% O2. When necessary, an aliquot of DNase type II (4 μg/ml) was added to the mixture. The fragments were dispersed into individual cells by gentle trituration through a siliconized Pasteur pipette. The cell suspension from all pituitaries (six flasks/30 ml) was mixed and then filtered through a fine nylon mesh, and the cells were washed twice with medium 199 to separate cells from viscous material, or debris from connective tissues freed during enzyme digestion. The pelleted cells were obtained by centrifugation at 120 g for 10 min at room temperature. The cells were washed three times in sterile culture medium. After the last wash, the cells were suspended in an appropriate volume of medium before being counted in a haemocytometer using trypan blue dye exclusion. The yield for one anterior pituitary was approximately 1.2 × 106 cells, and cell viability was greater than 90%. Growing medium consisted of medium 199, supplemented with 10% horse serum, 2.5% foetal calf serum and 50 μg/ml gentamicin sulphate. Cells were plated in 24 multiwell culture dishes at a density of 0.3 × 104 per well/ml medium. The culture dishes were then placed in a water-jacketed incubator at 37°C under a water-saturated atmosphere of 5% CO2–95% air. After a 3-day culture, the cells were washed twice with medium 199 to remove all traces of serum. Short-term incubation was performed in 250 μl medium 199 containing 0.1% BSA.
60 µmol/l ascorbic acid and either free medium or several pharmacological agents. The incubation period was 4 h. At the end of the test period, the medium was removed and stored at −20°C until analysed by radioimmunoassay.

**PRL determinations**

After centrifugation, serum was collected, frozen and stored at −20°C until use. The concentrations of PRL were determined by a double RIA method using kits supplied by the NIDDK (Bethesda, MD). Rat PRL I-6 were labelled with 125I by the Chloramine T method (21). The PRL concentrations are expressed using rat PRL RP-3 as standards. All samples were measured in duplicate. The intra- and interassay variations were 9% and 11% respectively and the sensitivity was 10 pg/tube. In some experiments, serum LH levels were measured as described previously (10).

**Dopamine determinations**

Pituitaries were sonicated in 1 ml mobile phase (aqueous solution of 100 mmol/l formic acid, 0.33 mmol/l octane sulphonic acid, 1 mmol/l citric acid, 0.10 mmol/l EDTA, 5% methanol and 0.25% diethylamine, adjusted to pH 3.1 with KOH). After centrifugation at 75 000 g for 20 min at 4°C, the supernatants were filtered through 0.22 µm pore filters and then centrifuged at 54°C for 20 min at 4°C. The HPLC system consisted of a Beckman System Gold Series with a 116 pump, a 7215 S injection valve equipped with a 20 µl sample loop and an NEC system controller (Beckman Instruments Inc., San Ramon, CA, USA), a BAS LC-4B amperometric detector with a glassy carbon transducer kit (Bioanalytical Systems Inc., West Lafayette, IN, USA) and a Merck LC-C-18 reverse phase, 5 µm, 125 × 4 mm column (Merck, Darmstadt, Germany). The flow rate was 1 ml/min. The concentration of dopamine in the samples was determined by measuring peak areas and comparing them with known amounts of the standard (Sigma). The retention time and sensitivity were 8.5 min and 50 pg, respectively.

**Statistics**

Data are expressed as the mean ± S.E.M. Differences between groups were analysed by one- or two-way analysis of variance (ANOVA) followed by Tukey’s test.

**Results**

**Effects of NMDA on PRL secretion at different ages (experiment 1)**

Serum PRL levels increased significantly after day 16 and an inhibitory effect of NMDA on PRL secretion which was observed on days 4, 20 and 30 disappeared in adulthood (Table 1).

**Effects of NMDA and MK-801 on PRL secretion in 30-day-old male rats (experiment 2)**

Serum PRL levels decreased significantly 5 and 15 min after NMDA administration and 50 and 60 min after treatment with MK-801. The inhibitory effect of NMDA on PRL secretion was not blocked by pretreatment with MK-801 (Fig. 1). This reduction in the basal secretion of PRL in males injected with MK-801 (alone or combined with NMDA) was not anticipated. To exclude any experimental artefact, we measured the serum LH samples in this experiment. As expected, NMDA increased LH secretion, which decreased after administration of MK-801. At the doses used, MK-801 blocked the LH release induced by NMDA (Fig. 1).

**Effects of orchidectomy and testosterone replacement in the NMDA and MK-801 inhibition of PRL secretion (experiment 3)**

Serum PRL levels were unaffected by orchidectomy or testosterone replacement (Table 2). The principal finding was the significant decrease induced by MK-801 treatment (F=42.91, P < 0.001, two-way ANOVA). The inhibitory effect was significant in intact, orchidectomized and testosterone-treated orchidectomized males. In this experiment, NMDA induced a significant decrease (F=8.33, P ≤ 0.05, two-way ANOVA) in PRL levels in intact and orchidectomized males, but not in the orchidectomized group treated with testosterone. To check the possibility that the effect of NMDA on PRL secretion was dependent on gonadal secretion (9), orchidectomized males implanted with different amounts of testosterone were killed 15 min after administration of vehicle or NMDA. The ventral prostate weight, reduced after orchidectomy, was normalized with implantation of Silastic capsules of 0.5 cm length.

**Table 1 Effect of NMDA (15 mg/kg) on serum PRL levels in rats of different ages. Values are means ± S.E.M. of 9–12 animals/group.**

<table>
<thead>
<tr>
<th>Age (days)</th>
<th>Vehicle (ng/ml)</th>
<th>NMDA (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>3.62 ± 0.40</td>
<td>1.41 ± 0.06*</td>
</tr>
<tr>
<td>8</td>
<td>1.58 ± 0.10</td>
<td>1.47 ± 0.12</td>
</tr>
<tr>
<td>12</td>
<td>1.88 ± 0.22</td>
<td>1.97 ± 0.17</td>
</tr>
<tr>
<td>16</td>
<td>3.30 ± 0.36</td>
<td>1.85 ± 0.21</td>
</tr>
<tr>
<td>20</td>
<td>12.48 ± 3.32</td>
<td>4.66 ± 1.19**</td>
</tr>
<tr>
<td>30</td>
<td>10.42 ± 3.32</td>
<td>5.06 ± 1.13**</td>
</tr>
<tr>
<td>60</td>
<td>13.91 ± 3.74</td>
<td>16.96 ± 2.61</td>
</tr>
<tr>
<td>90</td>
<td>10.40 ± 4.12</td>
<td>11.05 ± 3.49</td>
</tr>
</tbody>
</table>

*P ≤ 0.05, **P ≤ 0.01 compared with corresponding vehicle-injected group (ANOVA followed by Tukey’s test).
Implantation of longer capsules increased ventral prostate weight to higher values than in intact males and abolished the inhibitory effect of NMDA on PRL secretion (Fig. 2).

Effects of NMDA and NAME on PRL secretion in 30-day-old male rats (experiment 4)

Taking into account that we have previously described how NMDA and NAME inhibited PRL secretion in prepubertal female rats through an increase in the hypothalamic release of dopamine (10), we simultaneously measured PRL in serum and dopamine in hypothalamus and pituitary after NMDA and NAME administration. Serum PRL levels decreased after NMDA and NAME administration. Values are given as means ± S.E.M. of 9–12 animals/group.

Table 2 Effects of NMDA (15 mg/kg) and MK-801 (1 mg/kg) on serum PRL levels in 30-day-old intact, orchidectomized (ORCH) and testosterone-treated orchidectomized male rats (ORCH + T). The animals were orchidectomized on day 23 and implanted or not with Silastic capsules (1.5 cm length) containing testosterone. Blood samples were obtained 15 and 60 min after administration of NMDA or MK-801 respectively. Values from animals killed 15 and 60 min after vehicle injection were similar and were pooled. Values are means ± S.E.M. of 9–12 animals/group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Intact PRL (ng/ml)</th>
<th>ORCH PRL (ng/ml)</th>
<th>ORCH + T PRL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>3.66 ± 0.65</td>
<td>6.50 ± 1.0</td>
<td>4.13 ± 0.54</td>
</tr>
<tr>
<td>NMDA</td>
<td>2.28 ± 0.48*</td>
<td>4.01 ± 0.52*</td>
<td>6.62 ± 1.55</td>
</tr>
<tr>
<td>MK-801</td>
<td>1.25 ± 0.22**</td>
<td>2.24 ± 0.23**</td>
<td>1.51 ± 0.10**</td>
</tr>
</tbody>
</table>

* P ≤ 0.05, ** P ≤ 0.01 compared with corresponding vehicle-injected group (ANOVA followed by Tukey’s test).
administration and the effect was reinforced by combined administration of NAME (Table 3). Pituitary dopamine concentrations increased slightly after NAME administration and clearly after NMDA or NMDA plus NAME administration. The hypothalamic dopamine concentrations were reduced in males treated with NMDA, NAME or NMDA plus NAME (Fig. 3).

**Effects of NAME on PRL secretion induced by serotoninergic drugs (experiment 5)**

Serum PRL levels increased significantly 30 min after 5-HTP and DOI administration and remained unchanged after 8-OH-DPAT treatment. The stimulatory effect of 5-HTP and DOI was abolished by pretreatment with NAME (Fig. 4).

**Effects of NMDA, NAME and SNP in dispersed adenohypophyseal cells**

NMDA in vitro was a weak inhibitor of PRL release and the higher dose (20 mmol/l) decreased PRL secretion by a 30%, while dopamine at a much lower dose (100 mmol/l) inhibited PRL release by 80%. SNP did not affect PRL secretion and a significant increase was observed in the presence of NAME (100 μmol/l) (Fig. 5A). The effect of NMDA on PRL secretion

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**Table 3** Effects of NMDA (15 mg/kg), NAME (40 mg/kg) and NMDA plus NAME on serum PRL levels in 30-day-old intact male rats. Values are means ± S.E.M. of 9–12 animals/group.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>15 min</th>
<th>PRL (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh + Veh</td>
<td>10.16 ± 1.90</td>
<td></td>
</tr>
<tr>
<td>Veh + NMDA</td>
<td>6.10 ± 0.90*</td>
<td></td>
</tr>
<tr>
<td>NAME + Veh</td>
<td>8.04 ± 0.88</td>
<td></td>
</tr>
<tr>
<td>NAME + NMDA</td>
<td>4.60 ± 0.64**</td>
<td></td>
</tr>
</tbody>
</table>

* P ≤ 0.05, ** P ≤ 0.01 compared with corresponding vehicle-injected group (ANOVA followed by Tukey’s test).
Discussion

From the present results, the following conclusions may be drawn: 1) NMDA given systemically inhibits PRL release only in prepubertal males. This effect is mediated, at least partially, through an increase in the hypothalamic release of dopamine and by direct action at the pituitary level. 2) Antagonist of NMDA receptors with MK-801 decreased PRL secretion in intact, orchidectomized and testosterone-treated orchidectomized males. 3) Blockade of NO generation with NAME slightly inhibited PRL secretion, reinforced the inhibitory effect of NMDA and blocked the stimulatory effects of 5-HTP and DOI on PRL secretion.

In adult male rats, PRL secretion seems unchanged after NMDA administration (8–10), and a significant increase in serum PRL levels has been described only after orchidectomy (9). The present results show a significant inhibitory effect of NMDA on PRL secretion in prepubertal males, which agrees with data obtained in prepubertal female rats (11). The NMDA receptor mRNA is expressed in the pituitary (22) and the NMDA receptors were localized in lactotrophs (23), which explains the NMDA inhibition of PRL secretion by dispersed pituitary cells. This effect was repeated in three experiments, was dose-dependent and persisted in the presence both of SNP and NAME. Our data also suggest that, at least in part, the inhibitory action of NMDA on PRL secretion is mediated by an increase in hypothalamic dopamine release, as indicated by the increase in pituitary dopamine concentrations induced by NMDA.

The inhibitory effect of NMDA on PRL secretion depends on gonadal function, because it disappeared both in adult males and in prepubertal groups treated with supraphysiological doses of testosterone. Given that orchidectomy or testosterone replacement alter neither NMDA receptor concentrations or affinity nor NMDA receptor mRNA in the hypothalamus (24), the dependence of the different effects of NMDA on testosterone levels may derive, at least in part, from a change in the action of NMDA at the pituitary level, where the mRNA for NMDA receptors is regulated by steroids (22). Testosterone may have decreased the number of pituitary NMDA receptors, which may explain both the lack of NMDA effect in adult males and the abolition of the inhibitory effect in prepubertal rats treated with high doses of testosterone. In contrast with present experiments developed in males, glutamate stimulated PRL release by pituitary cells from adult but not from prepubertal female rats (25), which confirmed the role of gonadal secretion in the NMDA effects in PRL secretion.

Administration of MK-801 to intact, orchidectomized and testosterone-treated orchidectomized males inhibited PRL release, which suggests that tonic activation of NMDA receptors stimulated PRL release. This seems to contradict the decrease in PRL secretion after systemic administration of NMDA. At the moment, we do not have a clear explanation of this apparent contradiction, which was previously revealed by the observation that systemic administration of both NMDA and L,2-amino-5-phosphonopentanoic acid, an NMDA receptor antagonist, elicited PRL discharges in adult males (7). If one assumes that NMDA modulates PRL secretion by increasing the release of dopamine and prolactin releasing factors (PRFs) (4, 5) and through a direct action on lactotrophs, the inhibition observed after systemic administration of NMDA might be a consequence of an increase in dopamine release, pituitary action, or both. In contrast, the inhibition of PRL secretion after MK-801 administration might be due to a possible blockade of the action of endogenous aspartate on neurones releasing PRFs.
The present findings showed that pretreatment with MK-801 did not block the inhibitory action of NMDA on PRL secretion. It is unlikely that the dose of MK-801 used was inadequate to antagonize NMDA actions, as the LH discharge induced by this agonist was completely blocked by MK-801. The persistent decrease in PRL secretion after combined administration of MK-801 and NMDA may be explained assuming that NMDA given systemically exerted its action predominantly on pituitary lactotrophs and dopaminergic terminals, while MK-801 inhibits the release of PRFs. If the activation of PRF neurones predominates over activation of dopaminergic neurones, which is probably what occurs when basal PRL secretion is increased in prepubertal males, the PRL secretion would be inhibited after administration of MK-801 alone or combined with NMDA.

Despite the fact that NAME itself increased dopamine pituitary concentrations, the decrease in serum PRL levels was not significant. This suggests that NO does not have a role in basal prolactin secretion and that the increase in dopamine release was counteracted by other actions, perhaps the release of some PRFs. Another possibility is that the increase in pituitary dopamine concentrations was not sufficient to modify PRL release. The NAME-stimulated release of dopamine, a finding also observed in female rats (11), would explain the suppression of the PRL surges in pro-oestrous and in steroid-primed ovariectomized females (19). The potentiation of the inhibitory effects of NMDA on PRL secretion by NAME was probably due to their additive effects increasing dopamine pituitary concentrations because, at the pituitary level, NAME stimulated PRL release weakly. Absence of an effect of SNP on in vitro PRL secretion contrasts with the inhibitory action observed when incubated hemipituitaries were used (18). Intercellular connections (present in hemipituitary incubations but not in cell cultures) would be necessary for the detection of an inhibitory effect of SNP on PRL release.

Serotonin synthesized after 5-HTP administration exerted its actions through different postsynaptic receptor subtypes (26). Activation of 5-HT1, 5-HT2, and 5-HT3 receptors increased PRL release (27–31). In our experiments, we have tested the possible role of NO in mediating the effects of 5-HTP, 8-OH-DPAT and DOI. 5-HTP and DOI elicited clear increases in serum PRL levels. Lack of an effect of 8-OH-DPAT might be attributable to the dose used, as higher doses seemed to be necessary to elicit a clear PRL response in prepubertal rats (32, 33). The increase in PRL secretion after administration of 5-HTP and DOI was blocked by NAME, probably through the increased release of dopamine. Alternatively, it is possible that the effects of serotonin on PRFs require the generation of NO; further experimental approaches are needed to clarify the different possibilities.

In conclusion, the present findings indicate that, in prepubertal male rats, endogenous aspartate stimulates PRL release and exogenous NMDA administration decreases PRL secretion by increasing the release of dopamine and by a direct inhibition at the pituitary level. Blockade of NOS with NAME potentiates the inhibitory effect of NMDA and counteracts the increase in PRL release after serotonergic activation.

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