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Long-Term Treatment of Rats with Haloperidol: Lack of an Effect on Brain *N*-Acetyl Aspartate Levels

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Proton magnetic resonance spectroscopy (^{1}H-MRS) studies of schizophrenia suggest an effect of the disease or of antipsychotic medications on brain *N*-acetyl aspartate (NAA), a marker of neuronal viability. We studied in rat the effect of haloperidol on NAA, glutamate, and glutamine in several brain regions where metabolite reductions have been reported in chronically medicated patients with schizophrenia. Two groups of 16 rats each were treated with haloperidol depo (38 mg/kg/month) and vehicle for 6 months and were killed. Concentrations of metabolites were determined by high-resolution magic angle proton magnetic resonance spectroscopy (HR-MAS ^{1}H-MRS) at 11.7 T in *ex-vivo* punch biopsies from the following brain regions: medial frontal and cingulate cortex, striatum, nucleus accumbens, dorsal and ventral hippocampus, amygdala, and temporal cortex. Factorial ANOVA of NAA concentrations revealed no significant effect of drug group ($F(1,212) = 1.5$; $p = 0.22$) or a group by brain region interaction ($F(7,212) = 1.0$; $p = 0.43$). There was a significant main effect of region ($F(7,212) = 17.8$; $p < 0.001$) with lower NAA in the striatum. A prolonged exposure to the dopamine D2 receptor blockade effects of haloperidol does not result in changes in NAA, glutamate, glutamine, and other metabolites in the proton spectrum. These results are consistent with the only other two studies of the effect of antipsychotic drugs on NAA in the rat brain. The documented lower NAA in chronically treated schizophrenia patients is most likely not a simple effect of antipsychotic medications.
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INTRODUCTION

Proton magnetic resonance spectroscopy (^{1}H-MRS) studies of schizophrenia have reported reduced *N*-acetyl aspartate (NAA) mainly in frontal and mesial temporal regions, mostly in chronically treated patients (for a review, see Rowland *et al*, 2001). NAA is produced in the neuronal mitochondria and is found almost exclusively in the neuron (Tsai and Coyle, 1995; Moffet and Namdoori, 1995). Although initially considered a relatively stable marker of neuronal density, NAA is now also viewed as a sensitive marker of neuronal viability and function (Moore and Galloway, 2002). We have presented preliminary longitudinal data in schizophrenia patients with history of minimal previous treatment that documents reductions in frontal NAA during the first year of treatment with antipsychotic medications (Bustillo *et al*, 2002). Antipsychotic drugs exert both their therapeutic and deleterious extrapyramidal side effects by blocking dopamine D2 receptors and have been shown to lead to both structural and metabolic changes in the brain (Keshavan *et al*, 1994; Chakos *et al*, 1995; Holcomb *et al*, 1996). Hence, it is possible that NAA reductions in schizophrenia are the result of antipsychotic medications, but separating the impact of the disease from the treatment is problematic in clinical populations. The two studies that assessed the effect of exposure to antipsychotic agents on NAA measures in rat brain found negative results (Lindquist *et al* (2000), 1 week and Bustillo *et al* (2004), 6 week exposure). However, a more prolonged exposure that better corresponds to the longer term treatments typical in clinical populations may still induce reductions in NAA. The current study investigated the effects of 6 months of haloperidol, an

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antipsychotic with high D2 affinity, on NAA and other metabolite levels on various brain regions in rats, using high-resolution magic angle spin (HR-MAS) ^{1}H-MRS.

METHODS

Drug Exposure

All animal experiments were approved by the Institutional Animal Care and Use Committee of the University of New Mexico (UNM) Health Sciences Center and were performed according to the guidelines of the National Institutes of Health. Male Sprague–Dawley rats weighing about 300 g each were purchased from Harlan (Indianapolis, IN), housed in pairs and allowed to acclimatize for 2 weeks before the start of the study. Rats were housed at the UNM animal facility under a 12 : 12 light : dark cycle (lights on at 7 am) and received Purina rat chow and tap water *ad libitum*. After a handling period of about 1 week, rats were administered by intramuscular (i.m.) injection 38 mg/kg/month of haloperidol-depo ($n = 16$; Novaplus). This dose is in the high range of similar rat studies of chronic exposure (about 6 months) of haloperidol-depo (Andreassen and Jorgensen (1995) and Meshul *et al* (1996) used 38 mg/kg/month; See *et al* (1992) and Grimm *et al* (1998), used 21 mg/kg/Q3weeks). Rats were lightly anesthetized with isoflurane and half of the calculated drug volume was injected in each hind leg. Dosages were given once per month (between 1 and 4 pm) for 6 months. Control rats ($n = 16$) received equivalent volumes of purified sesame oil i.m. following the same procedures used with the haloperidol-depo group.

At 1 month after administration of the sixth injection, rats were exposed briefly to isoflurane and then killed by decapitation. Brains were rapidly removed, placed into rat brain matrix and 2 mm coronal slices obtained on an ice-chilled stage. Four slices were selected which approximately corresponded to the following sections of a standard rat brain atlas (Paxinos and Watson (1998); + or − refers to anterior or posterior from Bregma, respectively): slice 1, +3.7 to +1.7 mm; slice 2, +1.7 to −0.3 mm; slice 3, −2.3 to −4.3 mm; and slice 4 from −4.3 to −6.3. Regions of interest were identified visually in each slice (Galloway *et al*, 1986): medial prefrontal cortex-MF (slice 1), cingulate cortex-Ci, anterior striatum-Str and accumbens nuclei-Acc (slice 2), dorsal hippocampus-HiD and amygdale-Amy (slice 3), and ventral hippocampus-HiV and auditory cortex-Au (slice 4). These regions were selected because of their distinct dopamine projection fields and/or their homologous overlap with areas studied with clinical ^{1}H-MRS in chronically treated schizophrenia subjects (Rowland *et al*, 2001). Two millimiter circular punches were obtained from appropriate regions and immediately placed in precooled plastic centrifuge tubes and frozen on dry ice. Samples were stored at −80°C until HR-MAS ^{1}H-MRS analysis.

HR-MAS ^{1}H-MRS

Samples (~4 mg) were weighed and then inserted into a 10 µl zirconium rotor (Bruker) containing 4 µl PO_4 buffer (pH 7.4) containing 50% D_2O, Na-formate for autophasing (8.44 ppm), and trimethylsilane as reference (0 ppm). Rotors, maintained at 4°C, were spun at 4.2 kHz at 54.7° relative to Bo (static magnetic field) in a Bruker Avance 11.7 T 500 MHz magnet using a rotor-synchronized CPMG (Cheng *et al*, 1997) pulse sequence with TR = 3500 ms, bandwidth 8 kHz, 16 k complex points analyzed, 256 averages, and Taq ~ 15 min. Spectra were analyzed with a custom LCModel (Provencher, 1993) and absolute quantities of each neurochemical determined, weight corrected, and expressed as nmol/mg wet weight (see Figure 1). The precision of the LCModel fit to the spectral data was estimated with Cramer Rao lower bounds, which were typically <10%; values >20% were not considered for further analysis. The LC model was constructed with a basis set of 27 neurochemicals.

Based on the clinical schizophrenia literature (Rowland *et al*, 2001), we hypothesized that NAA would be reduced in frontal and temporal regions with exposure to haloperidol. An ancillary hypothesis was that glutamine in the cingulate cortex would be decreased by treatment with haloperidol (based on Theberge *et al*, 2003). Each metabolite level was analyzed with a 2 (treatment group) by 8 (region) factorial ANOVA using SAS software. Significant effects were followed up with Fisher's least significant difference method of *post hoc* comparisons. Owing to potential correlations of regional metabolite values within subjects, results were confirmed with mixed model ANOVA. Data are presented as mean ± SD in all cases.

RESULTS

The quantification of NAA, Glu, Gln, Cho, and Cre total concentrations in the eight regions of interest from haloperidol- and vehicle-treated rat groups is shown in the Table 1. For NAA, the principal metabolite of interest, factorial ANOVA revealed no significant group by region interaction ($F(7,212) = 1.0$, $p = 0.43$) and no main effect of group ($F(1,212) = 1.5$, $p = 0.22$). These results do not support an effect of antipsychotic drug exposure on NAA levels in any of the regions studied. However, there was a significant main effect of region ($F(7,212) = 17.8$, $p < 0.001$). *Post hoc* pairwise comparisons by *t*-test revealed significantly different NAA ($p < 0.05$) in the following areas: auditory cortex higher than all others; dorsal hippocampus, cingulate and mediofrontal cortex higher than the remaining regions; and ventral hippocampus, amygdala, and accumbens higher than striatum, which was the region with the lowest NAA level. These higher concentrations in cortex and lowest in striatum are consistent with the previous rat studies (Koller *et al*, 1984; Florian *et al*, 1996, Bustillo *et al*, 2004). Factorial ANOVAs also failed to document a drug main effect or interaction ($p > 0.05$) for the other metabolites measured: alanine, aspartate, betaine, PEA, choline, glutamine, GPC, lactate, NAAG, succinate, taurine, GABA, glycine, glutamate, GSH, inositol, phospho choline, and creatinine total. Finally, the results did not significantly change with mixed model ANOVA reanalyses.

DISCUSSION

In rats exposed to haloperidol for 6 months we found no differences in NAA, Glu, Gln, Cho, or Cre concentrations in medial frontal, cingulate and auditory cortices, striatum,

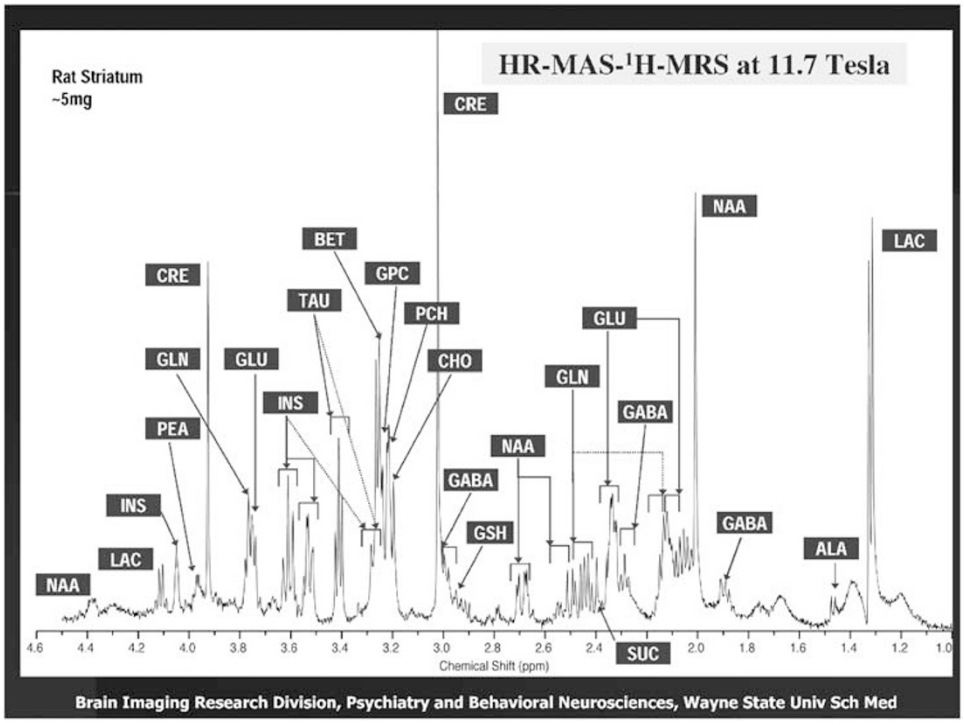

Figure 1 Example of a representative ^{1}H-MRS spectrum from an intact 5 mg sample of rat striatum acquired with HR-MAS at 11.7 T. NAA, *N*-acetyl aspartate; LAC, lactate; INS, inositol; PEA, phosphorylethanolamine; CRE, creatine; GLU, glucose; TAU, taurine; BET, betaine; GPC, glycerophosphorylcholine; PHC, phosphocholine; CHO, choline; GABA, gamma amino butyric acid; GSH, gluthathione; GLN, glutamine; SUC, succinate, and ALA, alanine.

accumbens, amygdala, dorsal or ventral hippocampus. These neurometabolites are of interest because they are often reported in clinical ^{1}H-MRS studies of schizophrenia. We are aware of only two previous studies assessing the impact of antipsychotic drugs on NAA brain levels in animals. Lindquist *et al* (2000) treated rats with intraperitoneal daily injections of olanzapine (1 mg/kg/day; $n=8$), clozapine (10 mg/kg/day; $n=8$), haloperidol (0.2 mg/kg/day; $n=8$), and vehicle ($n=8$) for 1 week. Ratios of NAA to creatine (NAA/Cre) were calculated from spectra measured *in vivo* at 4.7 T with single voxel ^{1}H-MRS, before, after 1 day, and at the end of treatment. The voxel studied was 0.216 cc and located '…in front of the cerebellum, and within the volume defined by the left and right ventricle' (Lindquist *et al*, 2000). There were no differences in NAA/Cre between the four groups at the end of treatment. There was a small reduction in NAA/Cre between baseline and 1 week values only in the olanzapine-treated group (1.16 *vs* 1.12; $p<0.04$). However, this was not a predicted result, uncorrected for multiple comparisons and the authors interpreted the overall findings as not supportive of a medication effect on NAA/Cre ratios.

We studied three groups of eight rats treated with haloperidol (6 mg/kg/day), clozapine (70 mg/kg/day), and vehicle for 6 weeks using gastric gavage twice daily (Bustillo *et al*, 2004). Animals were killed and concentrations of NAA were determined by high-performance liquid chromatography (HPLC) from the following brain regions: cortex, striatum, thalamus, hippocampus, and cerebellum. There were no differences in any brain region between the three groups ($F(2, 24)=0.034$; $p=0.966$). These three studies, examining acute, intermediate, and long-term antipsychotic (typical and atypical) exposure, with different methods (HPLC, *in vivo* and *ex vivo* ^{1}H-MRS) consistently suggest an absence of drug effect on NAA in rats.

Most studies using ^{1}H-MRS to study NAA in schizophrenia involve cross-sectional assessments of patients chronically treated with mainly typical antipsychotics. These studies report lower NAA or NAA/Cre in several brain structures like mesial temporal lobe, anterior cingulate cortex; dorsolateral prefrontal cortex (DLPFC), larger (8–12 cc) prefrontal locations; thalamus, and the cerebellum, but there are inconsistencies and several negative studies (for a review, see Rowland *et al*, 2001).

Few longitudinal studies of schizophrenia have assessed NAA in the context of antipsychotic drug treatment. Choe *et al* (1996) found no changes in left frontal NAA/Cre during naturalistic treatment with typical and atypical agents. Bertolino *et al* (2001), in a retrospective study, reported higher NAA/Cre in the DLPFC in patients while treated with antipsychotic medication compared to when they were medication free. We found reductions in left frontal NAA in patients with minimal prior lifetime antipsychotic exposure (less than 3 weeks) during the first year of randomized treatment with either haloperidol or quetiapine (Bustillo *et al*, 2002). Inconsistent findings in these longitudinal studies likely reflect methodological differences, but our preliminary results (Bustillo *et al*, 2002) suggest frontal NAA reductions early in schizophrenia in the context of initial antipsychotic exposure. Results from the present study do not support a simple medication effect. Progression of the disease or an interaction between disease and antipsychotic medications are alternative explanations.

Table 1 Metabolic Profile of MR-Visible Compounds (nmol/mg Wet Weight) from Eight Brain Regions in Rats Exposed to Haloperidol and Vehicle Treatments for 6 Months (Means ± Standard Deviations)

	Medial frontal	Cingulate cortex	Striatum	Accumbens	Hippocampus dorsal	Amygdala	Hippocampus ventral	Auditory cortex
NAA								
Haloperidol	5.68 ± 1.13 (*N* = 13)	6.07 ± 0.98 (*N* = 15)	4.37 ± 0.96 (*N* = 16)	5.09 ± 0.60 (*N* = 15)	6.10 ± 0.55 (*N* = 15)	5.05 ± 1.00 (*N* = 16)	5.05 ± 1.11 (*N* = 11)	6.10 ± 1.36 (*N* = 12)
Vehicle	6.01 ± 0.77 (*N* = 13)	5.88 ± 0.81 (*N* = 16)	4.45 ± 0.79 (*N* = 16)	4.95 ± 0.44 (*N* = 14)	5.91 ± 1.00 (*N* = 13)	5.20 ± 0.64 (*N* = 15)	5.33 ± 0.64 (*N* = 15)	6.90 ± 0.89 (*N* = 14)
Cho								
Haloperidol	0.21 ± 0.30 (*N* = 16)	0.24 ± 0.16 (*N* = 15)	0.36 ± 0.27 (*N* = 16)	0.32 ± 0.28 (*N* = 15)	0.28 ± 0.18 (*N* = 15)	0.33 ± 0.34 (*N* = 16)	0.26 ± 0.30 (*N* = 13)	0.29 ± 0.31 (*N* = 13)
Vehicle	0.22 ± 0.22 (*N* = 15)	0.24 ± 0.15 (*N* = 16)	0.27 ± 0.09 (*N* = 15)	0.36 ± 0.27 (*N* = 14)	0.41 ± 0.38 (*N* = 13)	0.31 ± 0.40 (*N* = 15)	0.31 ± 0.25 (*N* = 15)	0.40 ± 0.44 (*N* = 15)
Gln								
Haloperidol	1.55 ± 1.10 (*N* = 16)	1.39 ± 0.33 (*N* = 15)	2.09 ± 0.59 (*N* = 16)	2.27 ± 0.53 (*N* = 15)	1.68 ± 0.31 (*N* = 15)	2.13 ± 0.36 (*N* = 16)	1.41 ± 0.74 (*N* = 13)	2.05 ± 0.31 (*N* = 12)
Vehicle	1.51 ± 0.67 (*N* = 15)	1.41 ± 0.61 (*N* = 16)	2.18 ± 0.34 (*N* = 15)	2.37 ± 0.44 (*N* = 14)	1.74 ± 0.38 (*N* = 13)	2.05 ± 0.58 (*N* = 15)	1.74 ± 0.52 (*N* = 15)	1.98 ± 0.72 (*N* = 15)
Glu								
Haloperidol	6.42 ± 3.54 (*N* = 16)	7.39 ± 1.13 (*N* = 14)	6.13 ± 1.12 (*N* = 16)	6.01 ± 1.06 (*N* = 14)	7.34 ± 0.60 (*N* = 15)	6.76 ± 1.35 (*N* = 16)	5.81 ± 2.89 (*N* = 13)	7.30 ± 2.64 (*N* = 13)
Vehicle	7.51 ± 2.86 (*N* = 15)	7.45 ± 1.09 (*N* = 16)	6.27 ± 0.82 (*N* = 14)	6.07 ± 0.74 (*N* = 14)	7.41 ± 1.17 (*N* = 13)	6.85 ± 0.93 (*N* = 15)	7.11 ± 0.81 (*N* = 15)	7.86 ± 2.52 (*N* = 15)
Cre								
Haloperidol	3.93 ± 2.47 (*N* = 16)	5.03 ± 0.83 (*N* = 15)	4.60 ± 0.84 (*N* = 16)	4.82 ± 0.70 (*N* = 15)	5.87 ± 0.51 (*N* = 15)	4.62 ± 1.06 (*N* = 13)	4.52 ± 2.28 (*N* = 15)	4.87 ± 1.28 (*N* = 13)
Vehicle	4.55 ± 1.87 (*N* = 15)	4.90 ± 0.81 (*N* = 16)	4.73 ± 0.78 (*N* = 15)	4.56 ± 0.64 (*N* = 15)	5.64 ± 0.92 (*N* = 13)	4.77 ± 0.72 (*N* = 13)	5.30 ± 0.71 (*N* = 15)	5.15 ± 1.61 (*N* = 15)

NAA, *N*-acetyl aspartate; Cho, choline; Gln, glutamine; Glu, glutamate; Cre, creatine total.

More recent H-MRS studies at higher field (4T) suggest elevations of glutamine, a marker of glutamatergic neurotransmission, in anterior cingulate of never-medicated schizophrenia (Theberge *et al*, 2002). Furthermore, reductions of this metabolite in chronically treated patients (Theberge *et al*, 2003) suggest glutamatergic-related disease progression. The absence of an effect of haloperidol on cingulate glutamine supports the interpretation that these clinical findings are related to the disease and not exclusively to antipsychotic treatment.

Several limitations of the present study should be considered when interpreting our results. First, animals were exposed to a fixed dose of haloperidol which may not be equivalent to dosages of antipsychotics used in clinical populations. Since we did not measure haloperidol serum levels, drug underexposure cannot be excluded. However, drug delivery was assured by the use of long acting injections and the higher end (38 mg/kg/month) of the dosages used in studies of tardive dyskinesia in rats were selected. With the same drug dosage and delivery protocol in rats, Andreassen *et al* (1996) found haloperidol serum levels of 16.5(SD = 2.25) nmol/l, commensurate with clinical studies (Palao *et al*, 1994).

Second, the brain regions studied were rather large and a putative drug effect on NAA may be limited to more discrete areas or different regions. However, we specifically selected regions in which NAA reductions have been repeatedly described in chronically treated patients (frontal, cingulate, temporal cortex, and hippocampus).

Third, the animals studied were healthy and not representative of the fundamental neurobiological substrate that underlies schizophrenia, raising the possibility of an interaction between the disease and the therapeutic or deleterious effects of the medications.

Fourth, the negative findings could be due to a Type II error since the study had 80% power to detect an effect size of 1.0 for NAA in frontal and temporal regions. The one study we are aware off that specifically compared schizophrenics chronically treated with haloperidol ($n = 16$) and healthy controls ($n = 18$), reported a significant difference, with lower NAA in frontal lobe of patients and an effect size of 0.88 (Bustillo *et al*, 2001). Hence, it is possible that such an effect was missed in the present study and future investigations in animals with larger samples are warranted.

Fifth, the comparability of HR-MAS *ex vivo* ^{1}H-MRS in rats and *in vivo* ^{1}H-MRS in humans is an important issue. Enhanced spectral resolution, line widths, and signal-to-noise afforded by the high magnetic field and magic angle spinning provide information not currently available with *in vivo* MRS. For example, absolute values of GABA, glutamate, glutamine, and multiple choline-containing compounds are readily quantified in intact tissue with the present technique. Although MR-visible neurochemical concentrations are a unique measure distinct from tissue extracts or microdialysis, the fact that the present values were determined in integral tissue samples closely resembles the cellular environment of neurochemicals measured with *in vivo* ^{1}H-MRS. For example, glucose and lactate notwithstanding, HR-MAS ^{1}H-MRS values presented herein closely match those determined in the rat brain *in vivo* at 9.4 T (Pfeuffer *et al* 1999; Tkáč *et al*, 2003) Nonetheless, species differences exist in absolute values of MR-visible neurochemicals, most notably the paucity of taurine in human brain relative to levels in rodent brain. Concerns over postmortem dependent alterations in metabolite levels that occur despite rapid tissue extraction (eg increased lactate) are mitigated by comparison to appropriate vehicle-treated controls. The mean results for NAA, Cre, Glu, and Gln in the present report (6.0, 4.5, 7.6, and 1.6, nmol/mg wet weight, respectively) are similar to the relative proportions of these metabolites in human studies: NAA is 10–50% greater than Cre (Bustillo *et al*, 2001), much greater than Gln (Theberge *et al*, 2003), and similar to Glu (Theberge *et al*, 2003). Levels of MR-visible choline in human studies actually represent several choline-contaning moieties in the brain, including choline, phosphorylcholine, and glycerophosphorylcholine, whereas the high spectral resolution of the present method allows measurement of choline separate from other choline-containing metabolites. Regarding regional variations in NAA and how these compare between rats and humans, a consensus remains to be established. In our previous human study (Bustillo *et al*, 2001), NAA was 12.7 in frontal lobe and 11.6 in striatum in healthy volunteers similar to the current rat study where cortical NAA was highest and striatal concentrations were lowest.

Finally, our design was cross-sectional and a longitudinal study may be more powerful to detect within-subject changes. Future animal studies with repeated *in vivo* ^{1}H-MRS measures over several months would address this limitation.

In summary, we found no evidence in rodents that a prolonged exposure to haloperidol, more consistent with clinical practice, results in NAA reductions in several brain regions. These data suggest that the preliminary findings describing NAA reductions early in the course of schizophrenia (Bustillo *et al*, 2002) may not be entirely accounted for by treatment with antipsychotic medications.

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