J Appl Biomed
DOI: 10.32725/jab.2024.019
journal homepage: http://jab.zsf.jcu.cz

Journal of Applied Biomedicine

Original research article

Anti-NMDAR1 antibody impairs dendritic branching in immature cultured neurons

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Abstract
Anti-*N*-methyl D-aspartate receptor (anti-NMDAR) encephalitis is an autoimmune disorder characterized by IgG antibodies targeting NMDAR. The prevalence is remarkably higher in women and some develop the condition during pregnancy. While immunotherapies have shown good outcomes for pregnant mothers and their infants, the impact on early neurodevelopment remains elusive. This study investigates the effects of anti-NMDAR antibody on the development of primary cortical cultures. Anti-NMDAR antibody was administered to the cultures at day *in vitro* 5 for the following 5 days to assess dendritic branching and arbor complexity, and at day *in vitro* 14 for measuring the expression of brain-derived neurotrophic factor (BDNF) and synaptic proteins. Immature cultured neurons treated with anti-NMDAR antibody exhibited impaired dendritic branching and arbor complexity. Interestingly, BDNF expression was unaffected in mature neurons. Additionally, GluN1 expression, a mandatory NMDAR subunit, was significantly reduced, while no significant alterations were observed in PSD-95, gephyrin and synaptophysin expression. These findings shed light on the structural and synaptic impacts of anti-NMDAR antibody on immature neurons, providing evidence for their consequences in early neuronal development.

Keywords: Anti-NMDAR encephalitis; BDNF; Dendritic branching; Neuronal development; Synaptic proteins

Highlights:
- Anti-NMDAR antibody decreased dendritic branching in cultured neurons.
- Anti-NMDAR antibody decreased the expression of the mandatory NMDAR subunit GluN1.
- BDNF expression was unaffected after exposure to anti-NMDAR antibody.
- No difference in PSD-95, gephyrin, and synaptophysin expression.

Introduction

Anti-*N*-methyl D-aspartate receptor (anti-NMDAR) encephalitis is an autoimmune disorder characterized by the presence of immunoglobulin G (IgG) antibodies targeting NMDAR, which has an essential role in synaptic plasticity, underlying the neurobiology of learning and memory. Although the disease is rare (Dalmau et al., 2019), it is associated with a range of clinical manifestations, including psychiatric symptoms, seizures, memory deficits, and decreased levels of consciousnes (Dalmau et al., 2007).

The prevalence of anti-NMDAR encephalitis is considerably higher in women, who account for approximately 80% of patients. Around 40% of all affected patients have an associated underlying neoplasm, mostly ovarian teratomas (Titulauer et al., 2013). Some women develop the condition during pregnancy, which brings unique challenges and concerns (Dalmau et al., 2011; Kumar et al., 2010). While immunotherapies have shown good outcomes in treating pregnant patients – with most of their newborns healthy and appearing to have normal development – there is a small fraction of infants that present transient neurologic symptoms or die of brain edema (Joubert et al., 2020). These cases underscore the need for a comprehensive understanding of the condition, especially its impact on early neuronal development.

The anti-NMDAR antibodies responsible for the encephalitis specifically recognize epitopes within the amino terminal domain of the NMDAR mandatory GluN1 subunit (Gleichman et al., 2012). Cellular, molecular and behavioral effects of these antibodies have been previously studied using primary neuronal cultures and animal models. Notably, patients' antibodies induce a reversible internalization of surface NMDARs (Hughes et al., 2010) and disrupt their interaction with the synaptic anchoring protein Ephrin-B2 receptor (Mikasova et al., 2012). Moreover, they impair NMDAR-mediated currents and synaptic plasticity (Hughes et al., 2010; Kreye et al., 2016; Mikasova et al., 2012; Würdemann et al., 2016), leading to

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http://doi.org/10.32725/jab.2024.019
Submitted: 2024-04-04 • Accepted: 2024-08-20 • Prepublished online: 2024-09-19
J Appl Biomed 22/3: 136–140 • EISSN 1214-0287 • ISSN 1214-021X

memory deficits, anhedonia, and depressive-like behavior in animals (Li et al., 2015; Planagumà et al., 2015).

In animal models, placental transfer of patients' NMDAR antibodies has been shown to induce severe, yet reversible synaptic and neurodevelopmental alterations (García-Serra et al., 2020). Specifically, it impaired dendritic branching and delayed the centrosome elimination stage during the early development of cultured neurons (Okamoto et al., 2022). To shed further light on these structural and synaptic effects, this study investigates the effects of anti-NMDAR antibodies on dendritic branching and synaptic protein expression in developing cortical neurons in culture.

Materials and methods

Primary cortical cultures

All experimental procedures have been approved by the NIMH Committee for Animal Research Ethics in line with the Animal Protection Code of the Czech Republic and the European Union Directive 2010/63/EU. Primary cortical cultures from embryonic day 18 Wistar rats were prepared as described previously (Jorratt et al., 2023). Briefly, once brains were isolated, the meninges were removed and cortices were incubated in Hank's balanced salt solution (HBSS) (Thermo Fisher Scientific 14175095). After washing 3 times with HBSS, tissue was dissociated mechanically using a 0.9 (B. Braun 4657519) and 0.45 mm needle (B. Braun 4657683). Dissociated cells were filtered through 100 μm pore cell strainer (VWR 732-2759) and re-suspended in seeding medium, which contained DMEM (Biowest L0104-500) supplemented with 10% FBS (Biowest S1810-500), and 1% Penicillin-Streptomycin (Thermo Fisher Scientific 15070063). Cells were plated at 20 000 cells/cm^2 on 13 mm coverslips coated with poly-*L*-lysine in 24 well-plates for immunostaining, and at 125 000 cells/cm^2 on poly-*L*-lysine coated 6 well-plates for Western blot and ELISA. Primary cortical cultures were incubated at 37 °C and 5% CO_2. During the next day, the seeding medium was replaced with a growth medium that contained Neurobasal medium (Thermo Fisher Scientific, 21103049) with 2% B27 serum-free supplement (Thermo Fisher Scientific, A3582801), 2 mM *L*-Glutamine (Thermo Fisher Scientific, 25030149), and 1% Penicillin-Streptomycin. Every 4–5 days, half of the growth medium was replaced with a fresh medium. Primary cortical cultures were treated at day *in vitro* (DIV) 5 for immunocytochemistry and at DIV 14 for ELISA and western blot analyses. Sterile double-distilled water (control) and a commercial anti-NMDAR1 antibody (8 μg/ml, Alomone) were used for the 5-day treatment.

Immunocytochemistry

Primary cortical cultures were washed for 5 min with phosphate buffer solution (PBS), fixed with paraformaldehyde (4% in PBS and 2% sucrose) for 15 min, and washed three times with PBS for 5 min each. Afterwards, cells were incubated in blocking solution (10% FBS in 0.1% Triton X-100) for 1 h followed by overnight incubation with chicken anti-Microtubule-associated Protein 2 (MAP2) antibody (1:10 000; Abcam ab5392). Then, cells were washed with PBS three times for 5 min and incubated with anti-chicken IgG secondary antibody conjugated to Alexa Fluor 488 (1:500; Jackson ImmunoResearch) for 1 h. After washing with PBS as previously, cells were mounted onto microscope slides using Fluoroshield mounting media (Sigma-Aldrich). Leica DMi8 (Leica Microsystems) laser scanning microscope controlled by LAS X software (Leica Microsystems) was used for image acquisition, using a 40 × oil immersion objective (NA = 1.3). The analysis of images was performed using ImageJ with SNT v3.2.11 plugin (Arshadi et al., 2021). All images had been analysed by an experimenter blinded to treatment conditions.

Western blot and ELISA

After treatment, primary cortical cultures were washed with ice-cold PBS and collected with cell scraper in RIPA buffer with Protease and Phosphatase Inhibitor Cocktail (Sigma-Aldrich). Samples were incubated on ice for 15 min, sonicated three times, and centrifuged at 13 000 g for 5 min at 4 °C. Protein concentration was determined by BCA protein assay. 20 μg of proteins were loaded in 4–20% TGX Stain-Free gel (Bio-Rad) and run at 200 V constant. Gels were activated to visualize the total content of loaded protein according to the manufacturer's instructions. PVDF membranes with transferred proteins were blocked in 5% nonfat dry milk (in PBS) for 1 h and incubated overnight at 4 °C with the primary antibodies in 1% nonfat dry milk. The following antibodies were used: gephyrin (1:1000, Synaptic systems 147111), GluN1 (1:1000, Thermo Fisher Scientific 32-0500), PSD-95 (1:500, Abcam ab192757), and synaptophysin (1:1000, Abcam ab32594). Afterwards, membranes were washed 5 times with PBST (0.1% Tween-20 in PBS) and incubated with horseradish peroxidase-conjugated antibody for 1 h. Finally, membranes were washed as before and incubated with Clarity ECL substrate (Bio-Rad) for 5 min. Image Lab 6 software (Bio-Rad) was used for blot imaging and analysis of the optical density of the protein bands. Levels of γ-aminobutyric acid (GABA) and brain-derived neurotrophic factor (BDNF) were measured using an ELISA kit (LDN Labor Diagnostika Nord #BAE-2500R and Biosensis #BEK-2211, respectively), according to the manufacturer's guidelines. Absorbance was measured in a plate reader (Tecan Infinite M200 Pro) and concentration was calculated based on the standard curve.

Data analysis and statistics

Data are represented as mean ± SD. Data distribution was evaluated by Shapiro–Wilk test. Unpaired *t*-test and ANOVA were used to compare the differences between groups. Significant differences were considered for statistical tests with p-value < 0.05. All statistical tests were performed using R software (version 4.0.5) and RStudio (version 1.4.1717).

Results

Anti-NMDAR antibody decreases dendritic branching and arbor complexity

In order to measure morphological changes, cortical cultures were treated from DIV 5 with anti-NMDAR antibody or sterile double distilled water (control) for 5 days. Fig. 1A shows representative pictures and traces of neurons after both treatments. Anti-NMDAR antibody impaired dendritic branching of immature neurons by decreasing total dendritic length (control: 0.79 ± 0.32 (mean ± SD) mm, antibody: 0.60 ± 0.28 mm, p = 0.019), number of branch points (control: 7.48 ± 2.38, antibody: 5.47 ± 3.04, p = 0.008) and number of total branches (control: 20.2 ± 5.06, antibody: 15.7 ± 6.74, p = 0.008), without changing the number of primary branches (control: 6.80 ± 1.41, antibody: 6.30 ± 1.24) (Fig. 1B–E). When measuring dendritic arbor complexity through Sholl analysis (Fig. 1F), anti-NMDAR antibody also decreased the total number of intersections (control: 66.5 ± 25.9, antibody: 50.3 ± 21.9, p = 0.017) and maximum number of intersections (control: 10.80 ± 2.42, antibody: 8.97 ± 2.70, p = 0.010) (Fig. 1G–H).

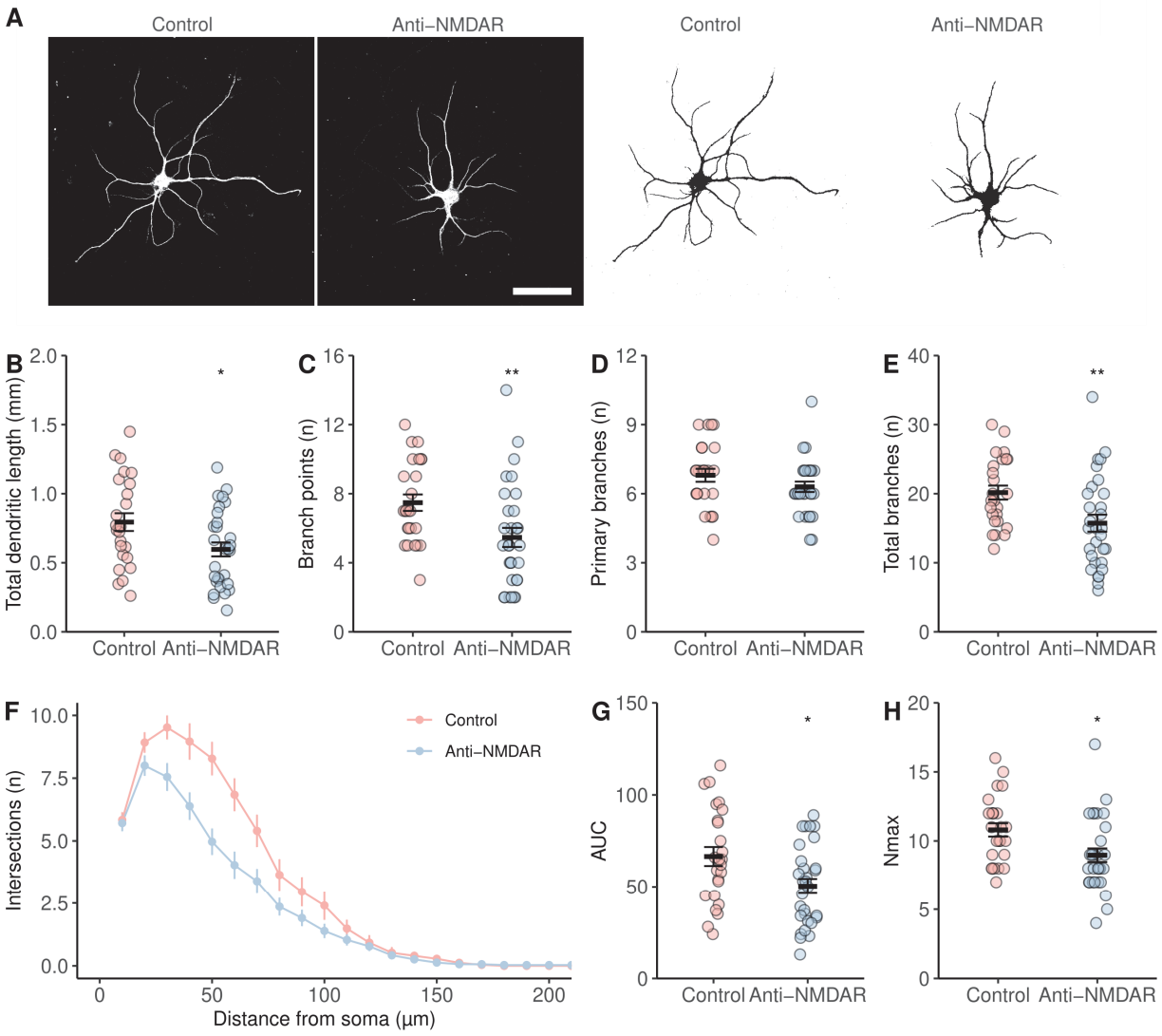

Fig. 1. Dendritic branching and arbor complexity is impaired by anti-NMDAR antibody. (**A**) Representative pictures (left) and traces (right) of cortical neurons treated at DIV 5 for 5-day treatment. Scale bar = 50 µm. (**B–E**) Dendritic branching measured by (**B**) total dendritic length, (**C**) number of branch points, (**D**) number of primary branches and (**E**) number of total branches. (**F**) Sholl analysis showing the number of intersections along with the distance from the soma, (**G**) area under curve (AUC) and (**H**) maximum number of intersections (Nmax). 25 and 30 neurons, obtained from three independent experiments, were analyzed for the control and anti-NMDAR groups, respectively. Data are represented as means ± SEM. Unpaired *t*-test.

Anti-NMDAR antibody decreases GluN1 expression

We measured the protein expression of gephyrin and PSD-95, postsynaptic scaffolding proteins that cluster synaptic proteins at inhibitory and excitatory synapses, respectively, together with the presynaptic vesicle protein synaptophysin. Compared to the control, anti-NMDAR antibody did not alter the postsynaptic markers gephyrin (ratio: 1.05 ± 0.56), PSD-95 (0.79 ± 0.58), and the presynaptic marker synaptophysin (0.87 ± 0.72) (Fig. 2A–B). However, the mandatory NMDAR subunit GluN1 was significantly decreased (0.51 ± 0.29, p = 0.041) (Fig. 2B). Since the role of BDNF in neuritogenesis is well known, we assessed BDNF levels in cell lysate and culture medium using an ELISA kit. Data were obtained from 3-4 independent experiments of cultured neurons treated at DIV 14 for 5 days. After normalizing for the total amount of protein, BDNF expression of control cultures (180 ± 72.6 pg/mg) was not significantly different from those treated with anti-NMDAR antibody (164 ± 49.8 pg/mg) (Fig. 2C). Released BDNF levels were undetectable in the culture medium. Finally, as an additional proxy of inhibitory function, we measured intracellular (from cell lysate) and extracellular (from culture medium) GABA levels using an ELISA kit. Amounts of protein (normalized by total protein levels) were not different between the control (intracellular: 3.52 ± 1.91 ng/µg, extracellular: 75.8 ± 55.9 ng/ml) and anti-NMDAR antibody (intracellular: 7.27 ± 5.46 ng/µg, extracellular: 216 ± 268 ng/ml) (Fig. 2D–E).

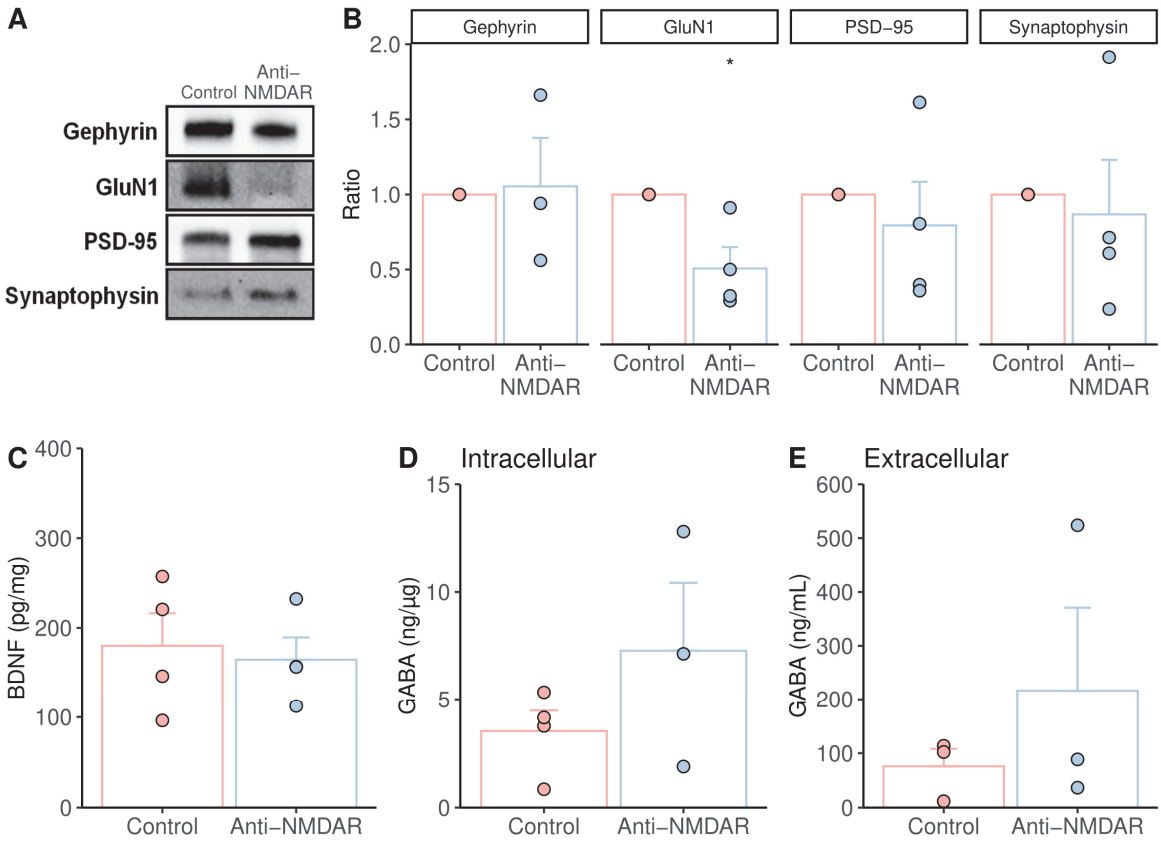

Fig. 2. Anti-NMDAR antibody decreases GluN1 expression without altering other synaptic protetins. Western blot analysis of synaptic markers and ELISA were performed in cortical cultured neurons treated at DIV 14 for 5 days. (**A**) Western blot images of PSD-95, gephyrin, synaptophysin and GluN1. (**B**) Mean densitometric analysis of proteins levels normalized to control. BDNF and GABA levels were determined by ELISA (n = 3–4 independent experiments). (**C**) Intracellular BDNF and (**D**) intracellular GABA levels were normalized to protein content. (**E**) Extracellular refers to GABA in the culture medium. Data are represented as means ± SEM. Each dot represents data from one independent experiment. Unpaired t-test.

Discussion

We have demonstrated that anti-NMDAR antibody significantly decreases dendritic branching and arbor complexity in immature cultured neurons, but it does not affect BDNF expression. Additionally, presence of the antibody decresed GluN1 expression, but not other proteins associated with excitatory or inhibitory synapses. Although we treated neurons with commercial anti-NMDAR1 antibodies, these were shown to have similar effects on impairing NMDAR function as those observed with patients' CSF antibodies (Würdemann et al., 2016). Moreover, the epitope of the antibody we used corresponds to amino acid residues in the extracellular domain (385–399), which partially overlap with the residues used for inducing immunization against NMDAR in mice model of anti-NMDAR encephalitis (Domise et al., 2019).

Placental transfer of anti-NMDAR antibodies decreases NMDAR clusters density and cortical plate thickness, induces neurodevelopmental alterations and depressive-like behavior, and impairs social-spatial memory in adolescent mice (García-Serra et al., 2020). In order to study structural changes, we sought to evaluate changes in neuronal morphology upon treatment with anti-NMDAR antibodies in cultured neurons. While it has previously been reported that no changes in morphology were observed following a 24-hour treatment of patients' NMDAR antibodies in cultured neurons (Hughes et al., 2010), that study was conducted on mature neuronal cultures. Instead, we focused on the early-stage of neuronal development, which is particularly relevant in cases of pregnant patients. Our results of 5 days treatment with NMDAR antibodies in the early stage of cultured neurons show decreased dendritic branching and arbor complexity, which is in line with a recent study using patients' NMDAR antibodies (Okamoto et al., 2022). These findings underscore the importance of protecting the developing neurons of the fetus by early immunotherapy in cases of anti-NMDAR encephalitis during pregnancy. Importantly, we observed that anti-NMDAR antibodies do not alter the expression of BDNF in mature neurons. The reason for not measuring it in immature neurons is due to the lower expression at that stage (Arévalo and Deogracias, 2023; Ding et al., 2021; Taft and Turrigiano, 2013).

We report that commercial anti-NMDAR antibodies reduced GluN1 expression as has been shown using patients' NMDAR antibodies through protein expression measurements and cluster density (Hughes et al., 2010; Mikasova et al., 2012; Planagumà et al., 2015). We also showed that anti-NMDAR antibodies do not alter the protein expression of the excitatory postsynaptic marker PSD-95 and presynaptic marker synaptophysin in matured neurons. Those results

are in line with studies showing that patients' NMDAR antibodies do no alter the expression and clusters of PSD-95 and α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPAR) (Hughes et al., 2010; Planagumà et al., 2015). Regarding inhibitory synapse, patients' antibodies decreased its density on excitatory neurons (Moscato et al., 2014) and drove cortical networks to a hyperexcitable state by a reduced synaptic output of inhibitory neurons (Andrzejak et al., 2022). We found that the expression of the inhibitory postsynaptic protein gephyrin is not altered, nor the expression and release of GABA.

Conclusion

We have shown that anti-NMDAR antibody impairs dendritic branching and arbor complexity, and that while the expression of GluN1 is decreased, other synaptic proteins remain unaltered. Given the pivotal role of morphological development in synaptic organization, our findings provide evidence of the importance of early immunotherapy for anti-NMDAR encephalitis during pregnancy.

Funding
This study was supported by GAUK 365121 (Charles University Grant Agency), NIMH (National Institute of Mental Health) Internal Grant 333 and Charles University research program Cooperatio-Neurosciences.

Ethical aspects and conflict of interest
The authors have no conflict of interest to declare.

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