Molecular and Cellular Neuroscience 65 (2015) 52–57

Contents lists available at ScienceDirect

Molecular and Cellular Neuroscience

journal homepage: www.elsevier.com/locate/ymcne

Synaptic structure and function are altered by the neddylation inhibitor MLN4924

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ARTICLE INFO

Article history:
Received 28 October 2014
Revised 26 January 2015
Accepted 17 February 2015
Available online 19 February 2015

Keywords:
Ubiquitin
NEDD8
MLN4924
Synapse
Hippocampus

ABSTRACT

The posttranslational modification of proteins by the ubiquitin-like small molecule NEDD8 has previously been shown to be vital in a number of cell signaling pathways. In particular, conjugation of NEDD8 (neddylation) serves to regulate protein ubiquitination through modifications to E3 ubiquitin ligases. Despite the prevalence of NEDD8 in neurons, very little work has been done to characterize the role of this modifier in these cells. Here, we use the recently developed NEDD8 Activating Enzyme (NAE) inhibitor MLN4924 and report evidence of a role for NEDD8 in regulating mammalian excitatory synapses. Application of this drug to dissociated rat hippocampal neurons caused reductions in synaptic strength, surface glutamate receptor levels, dendritic spine width, and spine density, suggesting that neddylation is involved in the maintenance of synapses.

1. Introduction

Control of synaptic strength is achieved in part through posttranslational modifications to synaptic proteins (Lu and Roche, 2012). In addition to the well-studied role of phosphorylation at the synapse, ubiquitination has recently emerged as an important regulatory mechanism for a number of proteins located both pre- and postsynaptically (Mabb and Ehlers, 2010; Yamada et al., 2013). Ubiquitination controls the targeting of specific substrates to 26S proteasomes and lysosomes for degradation and can also mediate subcellular distribution and function. Substrate specificity is accomplished by E3 ubiquitin ligases, which are responsible for the final step in the ubiquitination process. These enzymes are divided into a number of different families based on their structure and function, and their activity can be regulated through posttranslational modifications.

The conjugation of the ubiquitin-like protein NEDD8, termed neddylation, has been identified as a regulatory mechanism for a number of E3 ligases and components of E3 complexes (Rabut and Peter, 2008). Notably, all members of the cullin family of proteins, which comprise multi-subunit E3 ligase complexes, are known to be neddylated (Jones et al., 2008). While the exact biological effects of neddylation are still unclear, ample evidence suggests that the conjugation of NEDD8 to cullin proteins stimulates the ligase activity of cullin-based E3 complexes (Bennett et al., 2010; Podust et al., 2000; Read et al., 2000). Members of other E3 ligase families have also been shown to be substrates for neddylation, including the RING finger ubiquitin ligase Mdm2 and the HECT ligase Smurf1 (Xie et al., 2014; Xirodimas et al., 2004). This suggests that neddylation plays a critical role in regulating ubiquitination. Interestingly, many ubiquitin ligases known to be neddylated have also been demonstrated to regulate neuronal function and synaptic strength through the ubiquitination of synaptic proteins (Chen and Matesic, 2007; Cheng et al., 2011; Choo et al., 2012; Colledge et al., 2003; Zhu et al., 2005). However, little work has been done to directly explore the role of neddylation in neurons.

The recent generation of the NEDD8 Activating Enzyme (NAE) inhibitor MLN4924 has enabled a number of studies exploring the roles of neddylation in various cell types (Fig. 1A) (Bennett et al., 2010; Gu et al., 2014; Soucy et al., 2009). Though initially created as a potential tumor inhibitor, this drug allows for the pharmacological exploration of NEDD8 function. Here, we demonstrate that inhibition of neddylation in cultured hippocampal rat neurons affects synaptic strength and dendritic spine morphology. Our results provide evidence that neddylation plays a role in the maintenance of excitatory synapses.

Abbreviations: NEDD8, neural precursor cell expressed, developmentally downregulated 8; AMPAR, α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor; Ubc12, ubiquitin carrier protein 12; RING, really interesting new gene; HECT, homologous to the E6-AP carboxyl terminus; Mdm2, mouse double minute 2 homolog; GFP, green fluorescent protein; DMSO, dimethyl sulfoxide; BSA, bovine serum albumin; mEPSC, miniature excitatory postsynaptic current

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http://dx.doi.org/10.1016/j.mcn.2015.02.010
1044-7431/

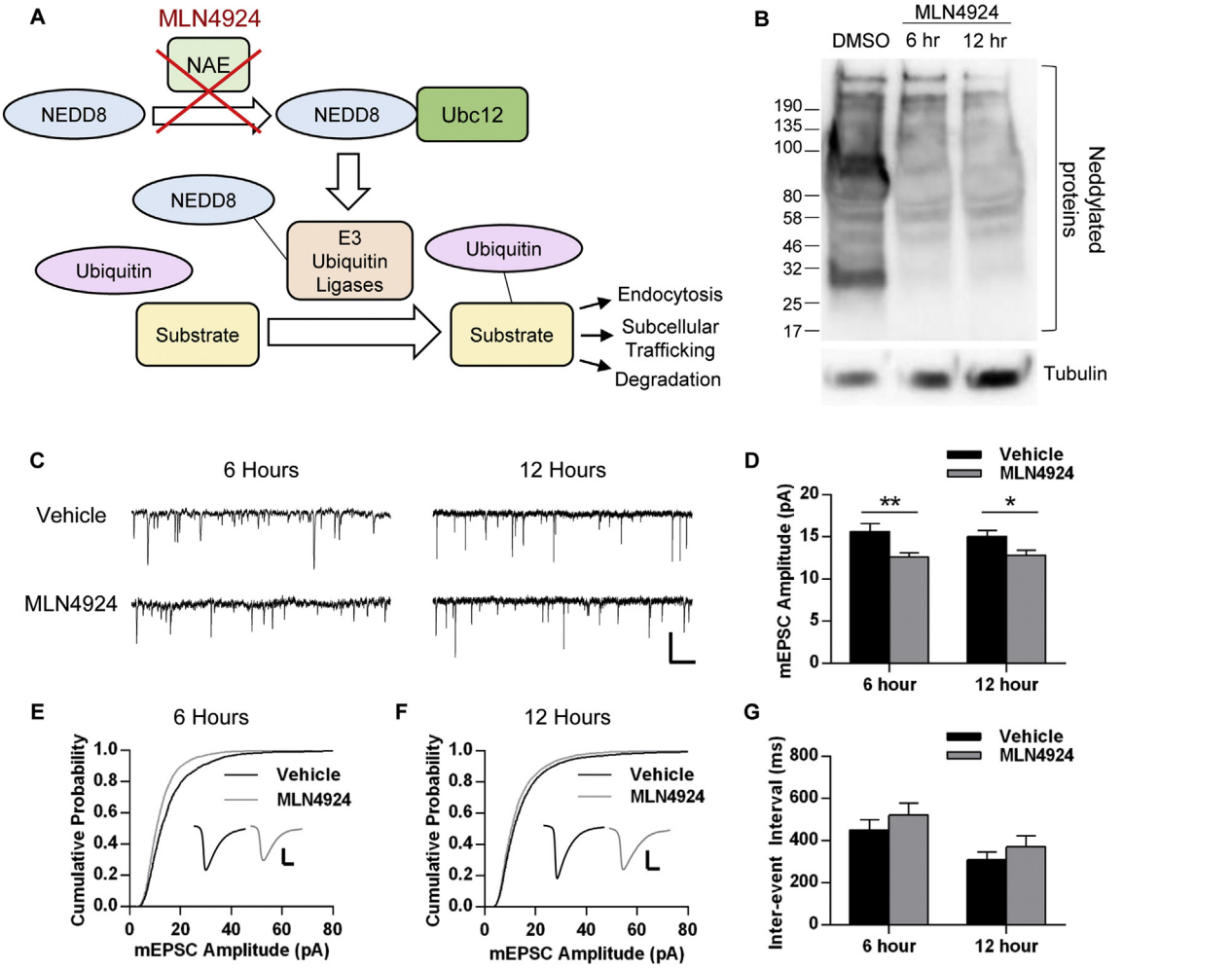

Fig. 1. Inhibition of neddylation with MLN4924 reduces mEPSC amplitude. A, Diagram depicting process of neddylation and action of the drug MLN4924. Under normal conditions, NEDD8 Activating Enzyme (NAE) permits NEDD8 to be conjugated to its intermediate E2 enzyme (Ubc12) and then conjugated to its target substrates, which include a number of E3 ubiquitin ligases. The stabilizing and activating effects of NEDD8 conjugation allow the E3 ligases to conjugate ubiquitin to their specific protein substrates, which serves as a signal for surface protein endocytosis, trafficking between subcellular compartments, or degradation via proteasomes or lysosomes. MLN4924 inhibits this regulatory pathway through direct inhibition of NAE. B, Western blot depicting inhibition of NEDD8 conjugation in neurons after treatment with MLN4924. Dissociated cortical rat neurons (DIV 19–25) were treated with DMSO (vehicle) for 12 h or with 1 μM MLN4924 for 6 or 12 h. Cells were lysed in the presence of 1,10-orthophenanthroline, then lysates were resolved on 4–20% SDS-PAGE and probed for NEDD8 and tubulin (n = 2 independent experiments). C–G, Dissociated hippocampal rat neurons (DIV 19–25) were infected with 2-gene GFP for 16–20 h. Neurons were treated with 1 μM MLN4924 or DMSO (vehicle) for the last 6 or 12 h of infection and mEPSCs were recorded from GFP-positive pyramidal-like neurons. C, Representative mEPSC traces recorded from neurons in each condition. Scale bar represents 500 ms, 20 pA. D, Average mEPSC amplitudes of neurons recorded from the four conditions; n > 19 cells per condition across 3 independent experiments. E, Cumulative probability plot of all mEPSC events recorded from cells treated with MLN4924 for 6 h; $p < 0.001$, Kolmogorov–Smirnov test; n = 2003, 2224 events. F, Cumulative probability plot from mEPSCs in 12-hour conditions; $p < 0.001$, K–S test; n = 5781, 5350 events. Insets depict averaged waveform from all events in each condition, scale bar represents 5 ms, 5 pA. G, Average inter-event intervals of neurons in each condition. Graphs depict mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, Student's t test.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies purchased are as follows: pAb surface (N-terminal) GluA1, pAb (C-terminal) GluA1 (Millipore); mAb GFP (Neuromab, UC Davis); pAb GFP (Invitrogen); mAb tubulin (Sigma-Aldrich); and pAb NEDD8 (generous gift from Dr. Eric Bennett). Reagents purchased are as follows: tetrodotoxin (TTX), bicuculline (Tocris Bioscience); and MLN4924 (Active Biochem, Maplewood, NJ).

2.2. Infections and drug treatments

Hippocampal cultures were infected with double subgenomic Sindbis virion (2-gene GFP) at 19–25 DIV and allowed to express for 14–22 h to fill cells for visualization. All drug treatments were conducted within cell media at indicated concentrations for indicated amounts of time, with DMSO of equal volume used as control.

2.3. Neuronal cultures

Dissociated hippocampal or cortical neurons from postnatal day 1 rat pups of either sex were plated onto poly-D-lysine-coated glass coverslips at a density of 45,000 cells/cm^2 (hippocampal) or onto coated 6-well plastic dishes at ~500,000 cells per well (cortical) and were maintained in B27-supplemented Neurobasal media (Invitrogen) until ≥14 days in vitro (DIV), as previously described (Schwarz et al., 2010; Scudder et al., 2014).

2.4. Western blots

To test the efficacy of MLN4924, cultured cortical neurons were lysed in a lysis buffer containing 8 M urea, 75 mM NaCl, 50 mM Tris (pH 8.2), 2 mM 1,10-orthophenanthroline, 1 mM NaF, 1 mM β-glycerophosphate, 1 mM sodium orthovanadate, and 1× protease inhibitor cocktail (Roche). For GluA1 experiments, neurons were lysed