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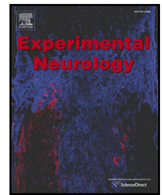

Research Paper

Phenylketonuria: Direct and indirect effects of phenylalanine

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ABSTRACT

High phenylalanine concentrations in the brain due to dysfunctional phenylalanine hydroxylase (Pah) are considered to account for mental retardation in phenylketonuria (PKU). In this study, we treated hippocampal cultures with the amino acid in order to determine the role of elevated levels of phenylalanine in PKU-related mental retardation. Synapse density and dendritic length were dramatically reduced in hippocampal cultures treated with phenylalanine. Changes in cofilin expression and phosphorylation status, which were restored by NMDA, as well as reduced activation of the small GTPase Rac1, likely underlie these structural alterations. In the Pah^{enu2} mouse, which carries a mutated *Pah* gene, we previously found higher synaptic density due to delayed synaptic pruning in response to insufficient microglia function. Microglia activity and C3 complement expression, both of which were reduced in the Pah^{enu2} mouse, however, were unaffected in hippocampal cultures treated with phenylalanine. The lack of a direct effect of phenylalanine on microglia is the key to the opposite effects regarding synapse stability *in vitro* and in the Pah^{enu2} mouse. Judging from our data, it appears that another player is required for the inactivation of microglia in the Pah^{enu2} mouse, rather than high concentrations of phenylalanine alone. Altogether, the data underscore the necessity of a lifelong phenylalanine-restricted diet.

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1. Introduction

Phenylketonuria (PKU; OMIM # 261600) is the most common inherited disorder of amino acid metabolism. Mutations in the gene coding for phenylalanine hydroxylase, which converts phenylalanine (Phe) into tyrosine, result in elevated Phe concentrations in plasma and cerebrospinal fluid (CSF) of patients. If untreated, PKU results in severe mental retardation. For many disorders associated with mental retardation, such as Down syndrome or fragile X syndrome, alterations in neuronal morphology and anomalies in neuronal growth have been found (Irwin et al., 2000; Kaufmann and Moser, 2000; Marin-Padilla, 1976). There are observations suggesting this is also true of PKU. Treatment of newborn rats with Phe during the first 21 days of their lives results in malformation of dendritic trees, with reduced lengths of neurites (Cordero et al., 1983) and reduced synaptic density in the neocortex (Horster et al., 2006). Furthermore, it has been shown that the expression of cytoskeletal proteins, such as α- and β-tubulin, in the cortex of young rats is affected by Phe (de Freitas et al., 1997). One of the major cytoskeleton proteins is actin, which exists in two forms: F-actin (filamentous) and G-actin (globular). The proper shape and size of neurons and synapses relies on the continuous assembly and disassembly of F-actin, which depends on the activity of cofilin, a protein that controls actin polymerization. Cofilin itself is strongly regulated: when phosphorylated on Ser3 by LIM domain kinase1 (LIMK1), cofilin activity is inhibited, and this, in turn, prevents the disassembly of F-actin and stabilizes, for instance, dendritic spines on neurons (Bramham, 2008). LIMK1, in turn, is regulated by the small GTPase Rac1 (Yang et al., 1998), which exhibits reduced activity in cortical neurons after treatment with elevated Phe (Zhang et al., 2007).

In the Pah^{enu2} mouse, a model of PKU with Phe concentrations similarly elevated to those in PKU patients, we have recently shown a delay of synaptic pruning after birth, resulting in an increased synapse density in the hippocampus of adult mutants. The delay in synaptic pruning was paralleled by insufficient activation of microglia (Horling et al., 2015). Zhan et al. demonstrated that synaptic pruning, which is directed at the elimination of weak synapses shortly after birth, depends on microglia activity (Zhan et al., 2014). Proper microglia function, in turn, requires neuronal activity, and can be mediated by C3 complement expression in neurons. Consistently, we found impaired long-term potentiation (LTP) and impaired paired-pulse facilitation (PPF) in acute hippocampal slices of the Pah^{enu2} mutants, pointing to disturbed synaptic transmission (Horling et al., 2015). As a result, reduced phagocytosis by microglia accounts for the failure of synaptic pruning and synaptic rearrangement of the network after birth (Bahrini et al., 2015).

In this study, we used hippocampal slice cultures to test whether the effects in the Pah^{enu2} mouse are in fact due to elevated Phe concentrations. Unlike our results in the Pah^{enu2} mouse, we found synapse loss

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in response to Phe, but unchanged activity of microglial cells in these cultures. Alterations of the spine cytoskeleton very likely underlie the neurotoxic effects of Phe on neuronal structures. Obviously, reduced activity of microglia in the *Pah*^{enu2} mouse is not a direct effect of elevated levels of Phe.

2. Materials and methods

2.1. Animals

Homozygous *Pah*^{enu2} mice and wild type (WT) C57BL/6 mice were housed on a 12 h light-dark cycle with food and water *ad libitum*. Genetic characterisation was performed as previously described (Horling et al., 2015). DNA was extracted from tissue using the Extract-N-Amp™ Tissue PCR Kit (Sigma-Aldrich). Subsequent PCR amplification, followed by Alw26I digestion differentiates mutant from wild type animals. All experiments were carried out in accordance with the institutional guidelines for animal welfare.

2.2. Dispersion culture

Primary hippocampal neurons were prepared from rat pups at embryonic day 18 (E18), as described by Brewer (1997), with slight modifications. Briefly, after decapitation, the hippocampi were carefully removed and digested with 0.5% trypsine (Biochrom). The scattered cells were resuspended in plating medium and plated on poly-L-lysine-coated (20 μg/cm^2; Sigma-Aldrich) coverslips in 24-well culture dishes (Thermo Scientific), or in poly-L-lysine-coated 6-well culture dishes at a density of 7.5×10^4 cells/well or 3.5×10^5 cells/well, respectively. Following a 3 h incubation (37 °C; 5% CO_2), the cells were washed with pre-chilled PBS to eliminate unattached cells and cell debris. The medium was changed to culture medium. The plating medium consisted of MEM medium (Gibco), 0.15% glucose, 10% FCS and 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco). The culture medium consisted of Neurobasal without Phenol Red (Gibco), 2% B27 (Gibco), 2 mM L-glutamine (Sigma), and 0.1 mg/ml streptomycin and 100 U/ml penicillin (Gibco). The medium was replaced twice a week for fourteen days.

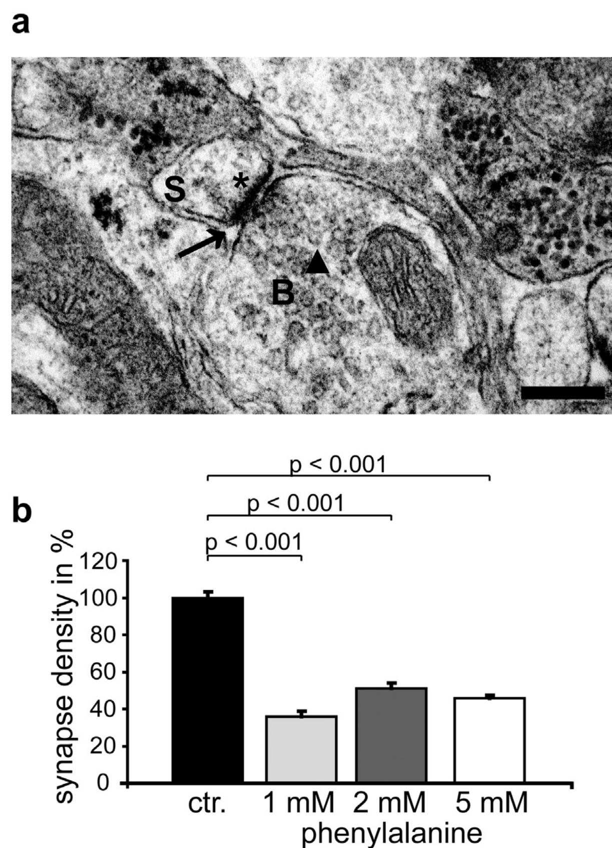

Fig. 1. Spine synapse density in hippocampal slice cultures. (a) Ultrastructure of a CA1 hippocampal spine synapse (B: bouton, S: spine, ↑: synaptic cleft, ▲: vesicle, *: postsynaptic density; scale bar: 200 nm). (b) Stereological determination of synapse density in response to various doses of Phe. (n = 3 independent experiments and 20 images per treatment; mean ± SEM; $p \leq 0.05$).

2.3. Organotypic hippocampal slice culture

375 μm slices of hippocampus and entorhinal cortex from 5 day-old C57BL/6 mice were prepared and cultivated as described by Stoppini et al. (1991). Only undamaged slices were used for further experiments. The sections were placed on translucent membranes with 0.4 μm pore size (Millicell-CM, Millipore) and cultivated in 6-well plates filled with 1 ml culture medium (50% MEM, 25% HBSS, 25% heat-inactivated horse serum, 2 mM glutamine, 0.044% $NaHCO_3$). The cultures were kept *in vitro* for 21 days at 37 °C in a humidified, CO_2-enriched atmosphere. The medium was changed every second day.

2.4. Culture treatment

After a recovery time of three days, slice cultures and dispersion cultures were treated with diverse concentrations of Phe (Sigma-Aldrich). The final Phe concentrations were 1 mM, 2 mM and 5 mM. For some experiments dispersion cultures were stimulated with 50 μM NMDA for 10 min, prior to lysis or fixation of the neurons.

2.5. TUNEL assay

The *In Situ* Cell Death Detection Kit, Fluorescein (Roche) was used to exclude the possibility of apoptotic effects of the applied Phe concentrations. The assay was performed according to the manufacturer's instructions. Briefly, cells were fixed in 4% PFA and unspecific binding sites were blocked with 3% H_2O_2 in methanol. For permeability, cells were incubated in 0.1% TX-100 in 0.1% sodium acetate for 2 min. Afterwards, TUNEL reaction mixture, containing enzyme solution and labeling solution, was added to the cells for 1 h at 37 °C. In a final step, the nuclei were stained with DAPI (4′,6-diamidin-2-phenylindol, Sigma-Aldrich), and the cells were mounted with Fluorescence Mounting Medium (Dako).

2.6. Transfection of hippocampal dispersion culture

For Sholl analysis, the neurons were transfected with pEGFP after 10 days *in vitro* (DIV10) using the Effectene Transfection Reagent Kit (Qiagen) and the recommended protocol, with some alterations. Briefly, pEGFP DNA was diluted with buffer to a concentration of 0.1 μg/μl and enhancer (1:8). Effectene Transfection Reagent was added to the solution and incubated for 10 min. Finally, the transfection complex was added drop-wise to the cells. After 1.5 h, the neurons were washed with pre-chilled PBS and cultivated for another 24 h in culture medium.

2.7. Immunohistochemistry and image acquisition

Dispersion cultures were fixed with 4% PFA after 3 or 10 days in culture, depending on the experimental approach, for immunohistochemistry. The fixed cells were stored in PBS at 4 °C until further use. Organotypic slices cultured for 21 days were fixed with 4% PFA overnight, followed by dehydration with 25% glucose for several hours. After blocking unspecific binding sites with 5% NGS (Sigma-Aldrich), the neurons were incubated with the primary antibodies over night at 4 °C, followed by incubation with the secondary antibody solution for

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