主論文の要旨

# Neuronal PAS domain protein 4 (Npas4) controls neuronal homeostasis in pentylenetetrazole-induced epilepsy through the induction of Homer1a

（Neuronal PAS domain 4（Npas4）は Homer1a の誘導を介して
ペンチレンテトラゾール誘発性てんかんにおける
神経の恒常性を制御する）

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## Introduction

Epilepsy is a common and refractory neurological disorder. Current anti-epileptic drugs primarily enhance the threshold for seizures without a significant anti-epileptogenic effect. Furthermore, approximately 20% of epileptic patients continue to have seizures even though they are receiving pharmacological treatment. The key to preventing and curing epilepsy is to elucidate the mechanisms underlying epileptogenesis. Neuronal intrinsic homeostatic scaling-down of excitatory synapse has been implicated in pathogenesis of epilepsy to prevent the neuronal circuits from hyper-excitability. Recent findings suggest a role for neuronal PAS domain protein 4 (Npas4), an activity-dependent neuron-specific transcription factor, in epileptogenesis; however, the underlying mechanism by which Npas4 regulates epilepsy remains unclear. In the present study, we showed that activation of the Npas4 signaling pathway after convulsive seizures plays a crucial role in intrinsic homeostatic scaling during epileptogenesis using an animal model of epilepsy, pentylenetetrazol (PTZ)-induced kindling. Furthermore, we found that Npas4 controlled the homeostatic scaling capacity of hippocampal neurons through the introduction of Homer1a, which regulates the surface expression of the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) GluA1 subunit.

## Materials and Methods

*Npas4* KO mice and C57BL/6 mice were administered 45mg/kg PTZ (i.p.) for the acute seizure model, and 25 mg/kg PTZ every 48 h for the kindling model. Mice showing more than three consecutive stage 4 seizure levels were defined as kindled mice. Levels of *Npas4* and *cFos* mRNA were determined by real-time PCR using an ABI PRISM 7300 real-time PCR system. *In situ* hybridization of *Npas4* was carried out using the DIG RNA Labeling kit and DIG Nucleic Acid Detection Kit. For fluorescence *in situ* hybridization (FISH) of *Homer1a*, signals were visualized by incubation with an Alexa 546 anti-sheep IgG antibody. Protein expressions of Npas4 and Homer1a were analyzed by immunohistochemistry or immunoblotting. Homer1a promoter activity in COS7 cells was analyzed using the Dual-Luciferase Reporter Assay System. In electrophysiological recordings, we measured field excitatory postsynaptic potentials (fEPSPs) or AMPAR-medicated miniature excitatory postsynaptic currents (mEPSCs) from mossy fiber（MF)-CA3 synapses or associational/commissural fibers (AC)-CA3 synapses. To express Homer1a in the hippocampus of *Npas4* KO mice, adeno-associated virus (AAV) was injected into the hippocampus through a glass microinjection capillary tube at a rate of 0.1 μl/min (0.5 μl/site, six sites). All data are expressed as means ± SEM. A one-way, two-way, or three-way analysis of variance (ANOVA) was used, followed by Tukey's test. Significant differences between two groups were assessed using the Student's T-test.

**Results**

A total of 16 injections of PTZ (25 mg/kg), a $GABA_A$ receptor antagonist, were required for the development of kindling, whereas no marked change in the seizure score was observed with repeated saline injections and a repeated saline plus single treatment with PTZ at the last injection (Fig. 1a). The expression level of Npas4 mRNA was significantly higher after the repeated PTZ treatment than in saline-treated or single PTZ-treated mice (Fig. 1b). Npas4 KO mice developed kindling more rapidly than their wild-type littermates (Fig. 1c). The expression of Npas4 mRNA was significantly increased in the hippocampus 1 h after the PTZ treatment, and the expression was the most prominent in the entire hippocampal CA subregions and dentate gyrus (Fig. 2a,b). Immunoblotting and immunohistochemistry analyses revealed that Npas4 protein levels were increased in the hippocampus 2 h after the PTZ treatment (Fig. 2c,d). Based on previous chromatin immunoprecipitation (ChIP) sequencing screens and microarray studies on Npas4 target genes, we focused on Homer1a. The expression of Homer1a mRNA and protein in the hippocampus increased 2 h and 4 h, respectively, after seizure activity (Fig. 3a,b). The PTZ-stimulated induction of Homer1a mRNA and protein was attenuated in the hippocampus of Npas4 KO mice (Fig. 4 a,b). The combination of FISH and immunohistochemical analyses revealed that Homer1a mRNA co-localized with the Npas4 protein 2 h after the convulsive seizure response (Fig. 4c). Surface AMPAR GluA1 subunit levels significantly decreased 24 h after the PTZ treatment, and the reductions were attenuated in Npas4 KO mice (Fig. 5a). PTZ significantly reduced the fEPSP/PSFV ratio at AC-CA3 synapses in wild-type mice, whereas it had no effect on that in Npas4 KO mice (Fig. 5b). PTZ also significantly reduced the average amplitude of mEPSCs in wild-type mice, but not in Npas4 KO mice (Fig. 5c). We bilaterally microinjected AAV, which promotes the expression of Homer1a, into the hippocampus of Npas4 KO mice (Fig. 6a,b). The AAV-mediated expression of Homer1a resulted in lower AMPAR GluA1 subunit levels in the hippocampal plasma membrane fraction than in that from AAV-EGFP-transfected Npas4 KO mice (Fig. 6c). The development of kindling was more strongly suppressed in AAV-Homer1a-microinjeted Npas4 KO mice than in AAV-EGFP-microinjected Npas4 KO mice, whereas the seizure threshold was not affected after the first injection (Fig. 6d).

**Discussion**

We showed that Npas4 was up-regulated within 2 h of seizure activity in a single PTZ-induced convulsive seizure model and repeated PTZ-induced kindling model. Treatment with PTZ has been shown to increase the release of glutamate by blocking $GABA_A$ receptors. Therefore, Npas4 may be immediately induced in response to excitatory glutamatergic inputs after seizure responses. Homer1 belongs to a family of scaffolding

proteins that localize at the PSD. Homer1 proteins are primarily classified into two isoforms. The long form of Homer1 (Homer1L) is constitutively expressed and consists of an N-terminal EVH1-binding domain followed by a coiled-coil domain that mediates dimerization with other Homer proteins. Homer1a is the short form of Homer1 and is induced in an activity-dependent manner. Homer1a has the ability to interact with PSD target proteins, but cannot self-assemble because it lacks the C-terminal coiled-coil domain. Therefore, Homer1a is regarded as a dominant negative regulator that interferes with PSD complexes. It has been demonstrated that Npas4 interacts with Homer1a gene in the mouse brain using ChIP sequencing analysis. The promoter region of Homer1 contains several response elements for transcription factors including CRE-binding protein (CREB) and Npas4. The PTZ treatment increased Homer1a mRNA and protein levels, and the induction of Homer1a was markedly attenuated in the hippocampus of Npas4 KO mice. Furthermore, co-localization of the Npas4 protein and Homer1a mRNA was observed in the hippocampus of PTZ-treated mice. These results indicate that Homer1a is one of the Npas4 target genes. PTZ treatment decreased the surface expression of the AMPAR GluA1 subunit and AMPAR-mediated mEPSCs at AC-CA3 synapses in wild-type mice. The application of AAV-Homer1a to the hippocampus of Npas4 KO mice promoted the down-regulation of surface AMPAR GluA1 subunit expression and normalized the facilitated development of kindling induced by PTZ. Our results suggest that PTZ treatment leads to a down-regulation of postsynaptic AMPARs through Npas4-Homer1a to induce compensatory changes in excitatory synaptic transmission.

**Conclusion**

The main finding of the present study is that Npas4 controls neuronal homeostatic scaling during epileptogenesis through the induction of Homer1a as negative feedback machinery for the management of epilepsy.