Acute reduction of neuronal RNA binding Elavl2 protein and Gap43 mRNA in mouse hippocampus after kainic acid treatment

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ABSTRACT

Activity-dependent gene regulation in neurons has been hypothesized to be under transcriptional control and to include dramatic increases in immediate early genes (IEGs) after neuronal activity. In addition, several reports have focused on post-transcriptional regulation, which could be mediated by neuronal post-transcriptional regulators, including RNA binding proteins (RNABPs). One such protein family is the neuronal Elavls (nElavls; Elavl2, Elavl3, and Elavl4), whose members are widely expressed in peripheral and central nervous system. Previous reports showed that Elavl3 and 4 are up-regulated following repeated stimulation such as during cocaine administration, a seizure, or a spatial discrimination task. In this study, we focused on *Elavl2*, a candidate gene for schizophrenia and studied its role in neuronal activity. First we found that Elavl2 has a cell-type specific expression pattern that is highly expressed in hippocampal CA3 pyramidal neurons and hilar interneurons using Elavl2 specific antibody. Second, unexpectedly, we discovered that the Elavl2 protein level in the hippocampus was acutely down-regulated for 3 h after a kainic acid (KA)-induced seizure in the hippocampal CA3 region. In addition, level of *Gap43* mRNA, a target mRNA of Elavl2 is decreased 12 h after KA treatment, thus suggesting the involvement of Elavl2 in activity-dependent RNA regulation.

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1. Introduction

RNA binding proteins (RNABPs) are a major contributor of post-transcriptional gene regulation through their direct binding to specific RNA targets *in vivo*. This is particularly true for neurons, in which neuronal, activity-dependent gene regulation, including alternative splicing and translational control of target RNA by RNABPs, has been reported to be associated with facilitating synaptogenesis [1,2]. Therefore, activity-dependent, post-transcriptional regulation is thought to be responsible both for spatiotemporal gene expression and for the formation of complex neural circuits that lead to higher ordered brain functions, such as learning or memory. Furthermore, impairments of this type of regulation are associated with various neurological disorders, such

as autism spectrum disorder (ASD), mental retardation, Alzheimer's disease (AD), and amyotrophic lateral sclerosis (ALS) [3—5].

Neuronal ELAV-like proteins (nElavls) are neuron specific RNABPs, which include Elavl2, Elavl3, and Elavl4 (also known as HuB, HuC, and HuD). They have been identified as autoimmune antigens in the sera of patients with paraneoplastic neurologic disorder (PND) [6]. Many *in vitro* and *in vivo* studies have revealed that nElavls play important roles in multiple steps of neuronal development [7—9]. One such role during neurodevelopment is the function of nElavl in post-transcriptional control. *In vitro* modeling has revealed that Elavl4 stimulates Cap-dependent translation through its direct binding to eIF4A and the poly-A sequence of target mRNA [10]. Moreover, this function is mediated by the direct binding of Elavl4 to active Akt1, which leads to the activation of translational initiation via mammalian Target of Rapamycin (mTOR) [11]. In contrast, the control of nElavl expression itself is also associated with several brain functions. For example, nElavl proteins are up-regulated in the hippocampus after learning in a radial maze and after several neuronal activities [12,13]. Additionally, previous experiments in which Elavl3 is knocked down in these trained mice resulted in decreased mRNA expression of *Gap43*,

which is both a crucial component of axogenesis and an nElavl target [13]. This same experimental group also showed that phosphokinase C (PKC α) directly binds to and phosphorylates nElavl proteins and recruits them to the cytoplasm, which results in *Gap43* translation [14]. Furthermore, after spatial learning using the Morris water maze, Elavl4 expression increases, which induces the up-regulation of Gap43, even one month after spatial training [15]. These reports suggest that nElavls could regulate the activity-dependent, mRNA stability and translational activation of target mRNAs by controlling their expression level and localization, thereby manipulating the complex processes of synaptogenesis and axogenesis *in vivo*.

Here, we show that Elavl2, a schizophrenia susceptibility gene in Asian populations [16], has a region- and cell type-specific expression pattern in the hippocampus. There, it is strictly restricted to the CA3 pyramidal neurons and hilar interneurons, which indicates that Elavl2 is differentially expressed and regulated from other nElavl family members. Moreover, we find that Elavl2 is acutely decreased at the protein-expression level in the CA3 after kainic acid-induced neuronal activity, followed by the reduction of Elavl2 target transcript, *Gap43* mRNA.

2. Materials and methods

2.1. Animals and seizure induction using kainic acid

All animal care and treatment procedures were performed in accordance with the institutional guidelines approved by the Experimental Animal Care Committee of the Keio University School of Medicine (approved No.09091). In all experiments, adult, male, CD-1 mice (8 weeks of age) were used. Kainic acid (KA; Sigma, K0250) was dissolved in sterile phosphate-buffered saline (PBS) and administered intraperitoneally at 20 mg/kg body weight. Same amounts of sterile PBS were administered to control (Sham) mice, as above. After the KA administration, seizure activities, such as lying motionless or adopting a rigid posture with tail extension, myoclonic contracture of the neck, forelimb clonus, and rearing were observed at each time point.

2.2. Quantitative RT-PCR

Total RNA was extracted from dissected hippocampi of three sham and KA-treated mice, using Trizol reagent (Invitrogen) and a RNeasy kit (QIAGEN). Then, 1 μg of total RNA was reverse transcribed with a iScript cDNA synthesis kit (BioRad). The amount of cDNA was quantified with SYBR Premix Ex Taq II (Takara) and StepOnePlus real-time PCR system (Life technology).

2.3. Western blotting

Hippocampi sections were dissected and homogenized in MAPK-lysis buffer that contained 10 mM Tris—HCl (pH7.6), 50 mM NaCl, 30 mM Na_3PO_4, 50 mM NaF, 20 mM β-glycerophosphoric acid, 1% Triton X-100, and Complete EDTA-free protease inhibitor (Roche). Protein concentrations were measured with BCA assay kit (Thermo Fisher Scientific), and 10 μg of protein was separated on each lane by SDS-PAGE. Then, protein was electroblotted onto a polyvinylidene difluoride membrane. The membranes were preblocked with Tris-buffered saline containing 2% skim milk (wt/v) for 1 h at room temperature. They were then incubated with primary antibodies at 4 °C overnight, followed by an additional incubation with an HRP-conjugated secondary antibody at room temperature for 90 min. The protein amount was quantified as a measure of intensity of chemiluminescence with ECL prime western blotting detection reagent (GE Healthcare) on the LAS4000 mini detector (GE Healthcare).

2.4. Immunofluorescence microscopy

Animals were perfused with 4% (w/v) paraformaldehyde in PBS and brains were removed. The brains were post fixed in the same fixative at 4 °C overnight and were then sliced into 50-μm sections using a Vibratome (Leica). The sections were incubated in HistoVT One antigen retrieval solution (Nacalai tesque) for 30 min at 70 °C and preblocked with a Tris-NaCl blocking buffer (PerkinElmer) for 1 h at room temperature. Then, sections were incubated with primary antibodies at 4 °C overnight, followed by incubation with an Alexa-conjugated secondary antibody for 90 min at room temperature. Slices were analyzed with a LSM700 Laser Scanning Confocal Microscope (Carl Zeiss Microscopy).

2.5. Antibodies

The following primary antibodies were used in this study: rabbit anti-Elavl2 (Sigma, H1538, 1:1000), mouse anti-actin (Sigma, A1978, 1:1000) and human anti-Pan-nElavls (gift from Dr. Darnell R. B., 1:2000) for immunoblotting; and rabbit anti-Elavl2 (Sigma, H1538, 1:400), rabbit anti-c-Fos (Calbiochem, PC38, 1:400), mouse anti-parvalbumin (Sigma, P3088, 1:400), mouse anti-Calbindin-D-28K (Sigma, C9848, 1:400) and human anti-Pan-nElavls (gift from Dr. Darnell R. B., 1:800) for immunohistochemistry.

3. Results

3.1. Elavl2 is specifically expressed in the CA3 pyramidal neurons and hilar interneurons in the hippocampus

All neurons express sets of nElavl family mRNA and proteins; however, each nElavl shows a different expression pattern in its mRNA [17]. These different sets of expression pattern for the same family member are thought to predicate the diversity and complexity of neuronal development and the functions of the brain. In a recent report, the *ELAVL2* gene has been shown to be a schizophrenia-related, top candidate gene in Asian populations [16]. To discriminate Elavl2 and other Elav members, on the basis of expression, we first analyzed detailed Elavl2 protein expression by using an anti-Elavl2 specific antibody and pan-neuronal Elavl antiserum. Immunohistochemistry revealed that Elavl2 expression is restricted to specific neurons, including the CA3 pyramidal neurons of the hippocampus, but on the other hand the other nElavl proteins are expressed in all neurons (Fig. 1A—C). This agrees with a previous *in situ* hybridization analysis for Elavl2 mRNA [17]. In addition, we observed that Elavl2 protein is expressed in parvalbumin-positive interneurons and parvalbumin-negative basket neurons by double immunostaining (Fig. 1D). This unique expression indicates that Elavl2 is a useful marker for specific neurons in hippocampus and implies an involvement of Elavl2 in diversity and specificity of post-transcriptional regulation in various neuron cell types. Moreover, Elavl2 protein localization is predominantly cytoplasmic in any neuron (Fig. 1), which suggests that the major function of Elavl2 is translational control or mRNA stabilization rather than splicing control, which is generally a nuclear event.

3.2. Acute reduction of Elavl2 protein level depending on kainic acid-derived neuronal activity

The unique expression of Elavl2 in the hippocampus is likely to be involved in neuronal activity because both of the high-affinity kainate receptors (GluK4, and GluK5) and their auxiliary subunits