Functional Study of NIPA2 Mutations Identified from the Patients with Childhood Absence Epilepsy

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Abstract

Recently many genetic mutations that are associated with epilepsy have been identified. The protein NIPA2 (non-imprinted in Prader-Willi/Angelman syndrome region protein 2) is a highly selective magnesium transporter encoded by the gene NIPA2 in which we have found three mutations (p.I178F, p.N244S and p.N334_E335insD) within a population of patients with childhood absence epilepsy (CAE). In this study, immunofluorescence labeling, inductively coupled plasma-optical emission spectroscopy (ICP-OES), MTT metabolic rate detection and computational modeling were utilized to elucidate how these mutations result in CAE. We found in cultured neurons that NIPA2 (wild-type) proteins were localized to the cell periphery, whereas mutant proteins were not effectively trafficked to the cell membrane. Furthermore, we found a decrease in intracellular magnesium concentration in the neurons transfected with mutant NIPA2, but no effect on the survival of neurons. To understand how low intracellular magnesium resulted in hyperexcitability, we built and analyzed a computational model to simulate the effects of mutations. The model suggested that lower intracellular magnesium concentration enhanced synaptic N-methyl-D-aspartate receptor (NMDAR) currents. This study primarily reveals that a selective magnesium transporter NIPA2 may play a role in the pathogenesis of CAE.

Introduction

Epilepsy is a collection of neurologic diseases characterized by unprovoked and recurrent seizures. Childhood absence epilepsy (CAE) is considered a crucial type of genetic generalized epilepsy (GGE). Previous studies have only found mutations in ion channel genes associated with CAE, such as CACAN1H [1–3], which encodes a T-type Ca2+ channel. Recently, some non-ion channel genes leading to the CAE have also been identified, such as NIPA2 [4]. NIPA2 is located at 15q11.2, a region associated with GGE. In our previous study, we found three NIPA2 mutations in a population of patients with CAE. They included two missense mutations (c.332A>T, p.I178F; c.731A>G, p.N244S) and one novel small insertion (c.1002_1003insGAT, p.N334_E335insD) [4].

NIPA2 encodes the non-imprinted in Prader-Willi/Angelman syndrome region protein 2 (NIPA2) [5]. NIPA2 consists of 360 amino acids and has 9 transmembrane protein domains. It belongs to the NIPA family of proteins. The NIPA family members are integral membrane proteins which function as magnesium transporters and include NIPA1, NIPA2, NIPA3 and NIPA4 [6,7]. NIPA1, NIPA3 and NIPA4 transport Mg2+ as well as other cations. NIPA2 is a highly selective magnesium transporter located in the cytomembrane and the early endosome [7]. Its function is to transfer extracellular Mg2+ into the cytoplasm [8]. To date, no functional study about NIPA2 mutations has been reported. Functional studies from NIPA1 mutations have shown that mutations disrupt transport of the protein to the extracellular membrane, resulting in an accumulation of the proteins in the cytoplasm [9]. Based on this finding we hypothesize that NIPA2 mutations also affect transport, resulting in accumulation of the proteins in the cytoplasm and a decreased intracellular Mg2+ concentration.

Mg2+ participates in the gating and activation of channels and receptors, such as N-methyl-D-aspartate receptors (NMDARs) [10,11], which play a role in synaptic plasticity [12,13]. Absence epilepsy is thought to be generated by pathological behavior in the thalamocortical loop [14]. NMDARs are widely distributed among excitatory neurons in the thalamus and cortex. Therefore, it is easy to imagine how decreased intracellular Mg2+ concentration caused by the NIPA2 mutation may result in epilepsy.

Results

The accumulation of NIPA2 mutant proteins in cytoplasm

In a previous genetic study, we discovered three mutations in the NIPA2 Mg2+ transporter gene associated with a form of
childhood absence epilepsy [4]. Studies of NIPA1 mutations found that the mutations prevented transport of the NIPA1 protein to the extracellular membrane, resulting in an accumulation of the proteins in the endoplasmic reticulum (ER) [6]. NIPA2 was also responsible for transport of Mg\(^{2+}\) [8]. Production of NIPA2 was Mg\(^{2+}\) dependent; when extracellular Mg\(^{2+}\) was low the cell compensated with a significant increase in membrane expression of NIPA2 [7], presumably to restore the intracellular concentration to normal. Based on these findings, we hypothesized expression of NIPA2 [7], presumably to restore the intracellular

The first goal was to determine the location of the mutant NIPA2 proteins expressed in the cells. To do this, they were marked with red fluorescence protein (RFP) and the cell’s plasma membrane was labeled with a green fluorescence marker (DIO). We found that wild-type proteins were localized to the cell border, but all three mutant proteins were retained in the cytoplasm (Figure 1).

Cell viability of transfected neurons

A number of proteins accumulating in cytoplasm, especially in the endoplasmic reticulum (ER), may cause apoptosis through the unfolded protein response (UPR) and ER stress [15]. Therefore, we hypothesized that NIPA2 proteins trapped in the cytoplasm would influence cell viability. In this study, MTT metabolic rate detection was performed to observe whether cell viability of cultured neurons with NIPA2 mutations decreased. MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) is a yellow substrate that is enzymatically cleaved in living cells to yield a dark blue formazan product. This process requires active mitochondria, and cells that have recently died do not cleave significant amounts of MTT. Primary cultured neurons were transfected with the wild-

![Image](58x151 to 296x326)

**Figure 1. The localization of NIPA2-WT and the mutants in cultured neurons.** CM: cytomembrane marked by DIO (a cytomembrane marker; green); NIPA2 was labeled by red fluorescence. MERGE: overlapped image. WT: wild-type. NIPA2-WT proteins (red) were distributed at cytomembrane, overlapped with DIO (green), but the three mutant proteins (I178F, N244S, N334_E335insD; red) were trapped in cytoplasm and showed no overlap with the plasma membrane. n = 4 experiments, scale bar: 5 μm. doi:10.1371/journal.pone.0109749.g001

Intracellular Mg\(^{2+}\) concentrations decreased in neurons with mutations

The accumulation of NIPA2 proteins in cytoplasm did not affect cell viability, but due to the location of the proteins in the cytoplasm, we hypothesized that it might decrease intracellular Mg\(^{2+}\) concentration. The four members of the NIPA family all are Mg\(^{2+}\) transporters; however, only NIPA2 is highly selective for Mg\(^{2+}\) [7]. NIPA2 plays a vital role in Mg\(^{2+}\) influx [8,16], and incorrect localization may decrease the intracellular Mg\(^{2+}\) concentrations significantly. To test this, we applied inductively coupled plasma-optical emission spectroscopy (ICP-OES) to

![Image](315x213 to 535x402)

**Figure 2. Cell viability of neurons with wild-type or mutant NIPA2.** Cell viability was measured by MTT detection. The absorbance of formazan was proportional to metabolic activity of the cells. Relative cell viability was calculated as: (mean absorbance of transfected group - mean absorbance of basal temperature control)/(mean absorbance of naıve group - mean absorbance of basal temperature control). Naıve: the cultured neurons without transfection; Overexpression: neurons transfected with NIPA2\(^{WT}\); Ins: the small insertion (N334_E335insD); sIRNA: neurons transfected with NIPA2-sIRNA. Neurons were co-transfected with the mutant and NIPA2-sIRNA in the groups (I178F-sIRNA, N244S-sIRNA and ins-sIRNA). The group (WT-sIRNA) represented the neurons transfected with NIPA2\(^{WT}\) and NIPA2-sIRNA. The mutant groups (I178F, N244S and Ins) represented the neurons transfected only with the mutant. n = 9 experiments. No significant differences between naıve group and any other condition were found. doi:10.1371/journal.pone.0109749.g002
measure extracellular/intracellular Mg$^{2+}$ concentration. Results were shown in Figure 3. For the missense mutant I178F cells we found a small but insignificant decrease on intracellular Mg$^{2+}$ concentration. However, for the missense mutant N244S and the small insertion (N334_E335insD) mutation there was a significant decrease in the concentration of intracellular Mg$^{2+}$ by 62% and 35% respectively (analyzed by Prism 5.0, One-way ANOVA, $p = 0.0037$). Compared to the naive group the N244S+siRNA (t-test, $p = 0.0066$, **), Ins+siRNA ($p = 0.0034$, **), and siRNA ($p = 0.0046$, **) had significantly lower intracellular Mg$^{2+}$ concentration, but the group I178F+siRNA did not ($p = 0.5435$). In no case did the mutations significantly alter the extracellular Mg$^{2+}$ concentration (Prism 5.0, One-way ANOVA, $p = 0.3414$).

**Modeling: Effects of low intracellular Mg$^{2+}$ on NMDA receptor currents**

We hypothesized that low intracellular Mg$^{2+}$ may affect the NMDAR currents. To test this hypothesis, we used an NMDAR synaptic model [17,18] and modified it to simulate the effects of intracellular Mg$^{2+}$. The NMDAR was blocked by intracellular Mg$^{2+}$ [19]; decreasing intracellular Mg$^{2+}$ increased the NMDAR currents. A simulation of the post-synaptic conductance during three bursts of synaptic inputs in normal and low intracellular Mg$^{2+}$ was shown in Figure 4. When intracellular Mg$^{2+}$ was set at one-tenth the normal value (0.1 mM), the amplitude of postsynaptic potential during the tonic phase of the burst was increased (Figure 4).

**Discussion**

NIPA2, a highly selective Mg$^{2+}$ transporter, may play an important role in the blocking of NMDARs via the regulation of Mg$^{2+}$ concentration, and thus has a vital effect on the excitability of neural network excitability. Our previous genetic study was the first to identify three mutations in Chinese patients with CAE [4]. From these findings, we infer that NIPA2 mutations may affect neural network behavior. However, no functional studies have been done to understand how NIPA2 mutations result in CAE. To reveal the mechanism by which NIPA2 mutations cause CAE, we measured cell viability, protein localization, and extracellular/intracellular Mg$^{2+}$ concentration in neuronal cultures. We then measured cell excitability in computational models. In a protein localization assay, we found that NIPA2 mutations led to the incorrect localization of the NIPA2 protein in neurons, resulting in a decrease of intracellular Mg$^{2+}$. In computational models, we inferred that a decrease in intracellular Mg$^{2+}$ concentration enhanced NMDAR currents, putatively resulting in the pathogenesis of CAE.

Incorrect protein localization affects Mg$^{2+}$ transport in transfected neurons with NIPA2 mutations

Due to the function of NIPA2, i.e. the transportation of Mg$^{2+}$ into the cytoplasm [8,16], we have predicted that incorrect protein localization may decrease the concentration of intracellular Mg$^{2+}$. In this study, we have observed the significant decrease in intracellular Mg$^{2+}$ in a mutant model (N244S, N334_E335insD) and a knock-down model, while the concentration of extracellular Mg$^{2+}$ was not changed significantly (within the detection precision of ICP-OES).

However, decreased intracellular Mg$^{2+}$ was not seen in all mutants. No significant change in intracellular Mg$^{2+}$ concentration was detected in the neurons with the I178F mutation, yet these mutant proteins were retained in cytoplasm as the neurons with other mutants. In a previous study it was found that NIPA1 mutant proteins were distributed in the cytoplasm but its function in Mg$^{2+}$ transport was diminished [7]. We infer that in

Figure 3. The concentration of extra/intracellular Mg$^{2+}$ measured by ICP-OES. There was no significant change of extracellular Mg$^{2+}$ concentration among the groups compared to naive cells; the mutant N244S and Ins dramatically decreased the concentration of intracellular Mg$^{2+}$, though the mutant I178F and the overexpression group were not different from the naive group. Naive: the cultured neurons without transfection; Overexpression: neurons transfected with NIPA2 (WT); Ins: N334_E335insD. siRNA: neurons transfected with NIPA2-siRNA; the mutant NIPA2-wt and NIPA2-siRNA; neurons transfected with NIPA2-wt and NIPA2-siRNA; the mutant NIPA2-wt and NIPA2-siRNA; neurons transfected with NIPA2-siRNA: the group (WT+siRNA); the mutant+siRNA group (I178F+siRNA, N244S+siRNA, Ins+siRNA); neurons transfected with the mutant and NIPA2-siRNA. Relative concentration was calculated as: Mg$^{2+}$ concentration of the transfected group/Mg$^{2+}$ concentration of naive group. n = 4 experiments.

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some of the mutations both the distribution of the protein and Mg²⁺ transport are affected, while in the mutant I178F only the transport is affected. In the mutant I178F, the small amount of protein in the cytomembrane is able to effectively transport Mg²⁺ into the neuron. Some protein accumulation in the neurons with the mutant I178F may not significantly affect intracellular Mg²⁺ concentration. Therefore, we believe that while the I178F mutation may inhibit NIPA2 protein trafficking to cell membranes, it does not also affect the function at the cell membrane. In contrast N244S and N334_E335insD decrease both the cytomembrane concentration and Mg²⁺ transport.

**NIPA2 mutations did not affect cell viability of transfected neurons**

NIPA1, another member of NIPA family, is similar to NIPA2 in terms of biological features. NIPA1 is a transmembrane protein, and also functioned as an Mg²⁺ transporter [20]. Earlier studies of NIPA1 mutant proteins found that they accumulated in the ER and caused cellular toxicity [21]. Thus we tested whether neurons with NIPA2 mutations encountered the same problems. In this study, mutant NIPA2 proteins trapped in cytoplasm were observed; however, the accumulation of mutant proteins did not affect the viability of the neurons. We infer that the degree of accumulation caused by NIPA1 mutations may be more serious than that caused by NIPA2 mutations. The apoptosis resulting from excessive UPR and ER stress in the cells with NIPA1 mutations may not occur in the transfected neurons with NIPA2 mutations. Thus we have found no significant change in viability in the transfected neurons with NIPA2 mutations.

**NIPA2 mutations may enhance NMDAR currents**

Mg²⁺ participates in the gating and activation of channels and receptors, such as NMDARs [10,11], which play a role in modulations of neural excitability [12,13]. Intracellular Mg²⁺ blocks the activation of NMDAR channels to modulate synaptic strength. Based on our findings that NIPA2 mutations decrease intracellular Mg²⁺, we hypothesize that the mutations may cause enhanced NMDAR currents. In our computational model, we have observed that low intracellular Mg²⁺ increases NMDAR-related synaptic currents significantly. It supports our hypothesis intracellular Mg²⁺ increases NMDAR currents. But further electrophysiological experiments are needed to test this theory. Since there is not a specific blocker of the Mg²⁺ transporter to build a model of low intracellular Mg²⁺ for extracellular recording, computational modeling has been used in this study to test the effect of low intracellular Mg²⁺. We believe a mutant animal model may be ideal and needed to further study the effect of these mutations.

**NIPA2 mutations may contribute to childhood absence epilepsy**

We have found the third mutations lead to functional changes, suggesting they may be pathogenic. The NIPA2 mutations may play a role in the pathogenesis of CAE. CAE is known as a multigenic disease. CACAN1H and other genes associated with CAE have been reported [1–3]. Each of them may increase risk for the development of CAE. NIPA2 is no exception. The three NIPA2 mutations identified in our previous study support this issue [4]. However, Hildebrandt and his colleagues have not found any NIPA2 mutations in a large Caucasian cohort [22]. We suppose different frequencies between the Caucasian and Chinese population and low incidence may account for the negative result of Hildebrandt’s study [23]. In addition, only typical CAE patients were involved in our previous study, but several forms of GGE were included in Hildebrandt’s study. The inconsistency of the cases is probably another reason why they have obtained a negative result [23]. We believe further studies with Caucasian and other populations are needed to profoundly discuss the association between NIPA2 mutation and CAE.

**Summary**

We demonstrate that NIPA2 mutant proteins accumulate in the cytoplasm, lowering intracellular Mg²⁺. Low intracellular Mg²⁺ concentration may enhance NMDAR currents. This study describes functional changes of a highly selective Mg²⁺ transporter NIPA2 mutations. This study may provide insights to the elaboration of pathogenic mechanism and the development of further treatments for childhood absence epilepsy.

**Materials and Methods**

**Ethics statement**

Primary cultured neurons were prepared from pregnant Sprague-Dawley (SD) rats at the gestational age of 16–18 days. The rats were used in accordance with protocols approved by the experimental animal sciences of Peking University Health Science Center Institutional Animal Care and Use Committee (IACUC).

**Cell culture**

Primary cultured neurons were prepared from pregnant SD rats at the gestational age of 16–18 days. The procedures were all in accordance with ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357). Pregnant SD rats were anaesthetized by intraperitoneal injection of 10% chloral hydrate, and fetal rats were obtained by cesarean. The fetal cerebral cortices were dissected with 0.5 mM EDTA and 0.5 mM cysteine-HCl (Sigma) in Earle’s balanced salt solution, and then the cortices were gently triturated. The triturated cortices were digested at 37°C for...
15–25 min with 1 mg/ml papain (0.5–2 units/mg, Sigma) before dissociated cells were suspended in the plating medium (Minimum Essential Medium containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin). The number of dissociated cells was counted. 2–5 ml of cell suspension was planted on 15–25 min with 1 mg/ml papain (0.5–2 units/mg, Sigma) before dissociated cells were suspended in the plating medium (Minimum Essential Medium containing 10% fetal bovine serum, 100 IU/ml penicillin and 100 IU/ml streptomycin). The number of dissociated cells was counted. 2–5 ml of cell suspension was planted on the coverslips (Sigma) in 24-well plates at a density of 1.5–3.0 × 10^5 cells/ml. Cells were incubated at 37°C in a 95% O_2, 5% CO_2 humidified incubator for 4-6 h. Then the plating medium was replaced with the culture medium (Neurobasal A (Gibco BRL)) medium containing 2% B27 and 0.5 mM L-glutamine (Gibco BRL)). The culture medium was replaced every 3–4 days.

### Constructs and transfections of NIPA2 HSV vectors

Different groups of cultured neurons were transfected with NIPA2 (wild-type), NIPA2-siRNA, missense mutants (I178F, N244S) and a small insertion (N334_335EinsD) (MOI value = 1.5). Herpes simplex virus (HSV) vectors with NIPA2 (wild-type, mutations or siRNA) were conducted by OrienGene Biotechnology Ltd. (Beijing, China). To identify transfected proteins all vectors were labeled with red fluorescent protein (RFP). The concentrated liquid containing the vectors was diluted by culture medium Neurobasal A (Gibco BRL) medium containing 2% B27 and 0.5 mM L-glutamine (Gibco BRL). The diluted liquid was concentrated liquid containing the vectors was diluted by culture medium Neurobasal A (Gibco BRL) medium containing 2% B27 and 0.5 mM L-glutamine (Gibco BRL). The diluted liquid was added to coverslips with cultured neurons, and then the coverslips were incubated at 37°C in a 95% O_2, 5% CO_2-humidified incubator. The medium was replaced after the first 24 h, and every 3 days thereafter. Transfected cells were either processed for MTT testing, immunofluorescence labeling, or Mg^{2+} concentration measurement.

### Immunofluorescence

To observe the localization of NIPA2 proteins, cultured cells were washed with 0.1 M phosphate-buffered saline (PBS) three times before being fixed in 4% paraformaldehyde for 10 min. After that, the cells were incubated with DIO (1:500; Abcam), a fluorescent microscope (DMIRB, Leica, Germany). The steps were all performed at room temperature.

### Cell viability test

Viability of the transfected cells was measured by the MTT test. The cell mass was used as an estimate of the cell mass of each group.

\[
\text{Mg}^{2+}\text{concentration measured by ICP-OES, } \text{Weight}_{\text{cell}}
\]

where \(\text{Mg}^{2+}\text{concentration measured by ICP-OES, } \text{Weight}_{\text{cell}}\) is the weight of the cell mass of each group.

### Computational modeling: NMDAR model with intracellular Mg^{2+} dependence

The NMDAR glutamate receptor played a vital role in synaptic plasticity and development as well as epilepsy. NMDAR was activated by glutamate and either D-serine or glycine and was blocked by extracellular and intracellular Mg^{2+} [10,11,19]. To simulate the effects of intracellular Mg^{2+} block of NMDAR receptors [19], we modified an NMDAR model by Destexhe et al. [17,18] from MODEL DB. In Destexhe’s model, the NMDAR channel was blocked by extracellular Mg^{2+} but not intracellular Mg^{2+}. To incorporate the influence of intracellular Mg^{2+} concentrations on NMDAR synaptic transmission, we modified a term in the model to account for the intracellular and extracellular Mg-block. The equation describing Mg-block in the NMDAR model was as follows:

\[
Mg_{\text{block}} = \frac{1}{1 + [Mg]_ZK_0^{-1}e^{-\frac{g_{\text{Mg}}}{RT}}} \times \frac{1}{1 + [Mg]_ZK_i^{-1}e^{-\frac{g_{\text{Mg}}}{RT}}}
\]

where \(K_0\) of intracellular Mg^{2+} = 3.4 [26], \(K_i\) of intracellular Mg^{2+} = 0.95 [19], \(g_{\text{Mg}}\) = 0.0668, and the definition of the components were defined in Destexhe’s model [17,18].

The effect of Mg^{2+} block on NMDAR conductance \(G_{\text{NMDA}}\) was modeled as follows [17,18]:

\[
G_{\text{NMDA}} = G_{\text{NMDA}}^{\text{max}} \times O \times Mg_{\text{block}}
\]
where $G_{\text{NMDA}}^{\max}$ is the maximal conductance, and $O$ is the proportion of ligand-gated channels that are open [17,18].

Author Contributions
Conceived and designed the experiments: HX YJ TN. Performed the experiments: HX TN. Analyzed the data: HX YJ TN. Contributed reagents/materials/analysis tools: YZ PZ JW YW XW TN YJ. Wrote the paper: HX YJ TN.

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