The juvenile myoclonic epilepsy mutant of the calcium channel $\beta_4$ subunit displays normal nuclear targeting in nerve and muscle cells

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‡These authors contributed equally to this work.; Keywords: CACNB4, $\beta_4$ subunit, voltage-gated Ca$^{2+}$ channel, hippocampal neurons, dysgenic myotubes

Abbreviations: Ca$\alpha$, voltage-gated calcium channel; PP2A, protein phosphatases 2A; HP1$\gamma$, heterochromatin protein 1 gamma; DIV, days in vitro

Voltage-gated calcium channels regulate gene expression by controlling calcium entry through the plasma membrane and by direct interactions of channel fragments and auxiliary $\beta$ subunits with promoters and the epigenetic machinery in the nucleus. Mutations of the calcium channel $\beta_4$ subunit gene (CACNB4) cause juvenile myoclonic epilepsy in humans and ataxia and epileptic seizures in mice. Recently a model has been proposed according to which failed nuclear translocation of the truncated $\beta_4$ subunit R482X mutation resulted in altered transcriptional regulation and consequently in neurological disease. Here we examined the nuclear targeting properties of the truncated $\beta_4$ subunit in tsA-201 cells, skeletal myotubes, and in hippocampal neurons. Contrary to expectation, nuclear targeting of $\beta_4$ was not reduced compared with full-length $\beta_4$ in any one of the three cell systems. These findings oppose an essential role of the $\beta_4$ distal C-terminus in nuclear targeting and challenge the idea that the nuclear function of calcium channel $\beta_4$ subunits is critically involved in the etiology of epilepsy and ataxia in patients and mouse models with mutations in the CACNB4 gene.

Introduction

The auxiliary $\beta$ subunits of voltage-gated calcium channels promote membrane expression and modulate the gating properties of Ca$\alpha_1$ and Ca$\alpha_2$ calcium channels. In humans four genes encode Ca$\alpha_\beta$ subunits and abundant alternative splicing further increases the molecular heterogeneity of the $\beta$ subunit family. Co-expression studies demonstrated that all $\beta$ isoforms promote membrane expression of any Ca$\alpha_1$ and Ca$\alpha_2$ channel isoform and modulate their gating properties in a similar way.

These highly promiscuous isoform interactions generate a considerable functional redundancy of $\beta$ subunits. Consequently, loss-of-function mutations and knockouts of $\beta$ subunit genes caused a disease phenotype primarily in those tissues that express exclusively the mutated $\beta$ isoform.\(^3\)\(^-\)\(^5\) In contrast, in brain, where all four $\beta$ subunit genes are expressed, phenotypes were mild or non-existent, most likely because the channel function of the mutated $\beta$ isoform was compensated by other $\beta$ isoforms.\(^6\)\(^-\)\(^8\) However, there is one notable exception: spontaneous mutations of the $\beta_4$ subunit lead to idiopathic generalized epilepsy and episodic ataxia in humans and in mice.\(^9\)\(^-\)\(^10\) In cerebellum $\beta_3$ and $\alpha_\delta$-2 are the major subunit partners of the P/Q-type (Ca$\alpha_2.1$) calcium channel. Interestingly, mutations of all three subunit isoforms (Ca$\alpha_2.1$, $\alpha_\delta$-2 and $\beta_4$) result in an epileptic and ataxic phenotype.\(^9\)\(^,\)\(^11\)\(^,\)\(^12\) This is consistent with the notion that a deficiency of the P/Q-type channel function causes the neurological disease in $\beta_4$ mutants.

Recently, we and others discovered that specific $\beta_4$ subunit isoforms can also accumulate in the nucleus.\(^13\)\(^-\)\(^16\) This unexpected finding suggested a role of $\beta_4$ in channel-independent cell functions. Furthermore, in excitable cells nuclear export of $\beta_4$ was shown to be activity-dependent. We demonstrated that in skeletal myotubes and in hippocampal neurons $\beta_4$ accumulated in the nuclei during early development and in electrically quiescent cells and that it was rapidly exported in response to depolarization.\(^15\)\(^,\)\(^17\) Because a truncated $\beta_4$-4b isoform has previously been shown to interact with the nuclear protein HP1$\gamma$, a possible function in gene regulation has been suggested.\(^14\)\(^,\)\(^18\) This calcium channel-independent nuclear function provided an alternative explanation for the etiology of the severe neurological phenotype of $\beta_4$ mutations. If mutated $\beta_4$ subunits differ from wildtype $\beta_4$ isoforms with regard to their nuclear targeting properties or their ability to interact with nuclear proteins, then the loss of the nuclear function of $\beta_4$ may cause the neurological deficits observed in human patients and in mouse models with mutations in the $\beta_4$ gene. Consistent with this idea, Tadmouri et al.\(^16\) reported that the ataxia mutation R482X resulted in a C-terminally truncated $\beta_4$, protein, which failed to be targeted into the nucleus and...
consequently did not interact with the regulatory protein complex shown to repress tyrosine hydroxylase expression. Moreover, in a follow-up study the same group showed that heterologous expression of the full-length and truncated β4b isoform in HEK293 cells resulted in differential gene expression.19 Because we recently could demonstrate that in neurons only those β4 splice variants capable of targeting to the nucleus also regulated genes,17 we set out to examine the nuclear targeting function of the truncated ataxia mutant in nerve and muscle cells. Unexpectedly, however, wildtype and truncated β4b variants displayed identical nuclear targeting properties. Thus, the data presented here contradict the previous report and challenge the idea that differences in transcriptional regulation due to differential nuclear targeting of the wildtype and mutated β4b subunits may account for the neurological phenotypes in humans and mice with mutations in the CACNB4 gene.

**Figure 1.** Nuclear targeting of V5-tagged wildtype and mutant β4b subunits in dysgenic myotubes. (A) Domain structure of the full-length β4b-V5 and truncated β4b(1–481)-V5 subunits. Colored symbols indicate positions of antibody epitopes and numbers above indicate amino acid positions at domain borders and truncation site. (B) Representative double-immunofluorescence images of myotubes transfected with β4b-V5, β4b(1–481)-V5, and β1a-V5 together with GFP-Ca, labeled with anti-GFP and anti-V5 (left) or anti-β (right). (C) Fraction of myotubes showing nuclear targeting, transfected and labeled as in (B) (β4b, β4b(1–481): N = 3; anti-V5 n = 120, anti-β n = 150). (D) Double-immunofluorescence images of myotubes transfected with β4b-V5, β4b(1–481)-V5, and β1a-V5 together with GFP-Ca, labeled with anti-GFP and anti-V5 (left) or anti-β (right). (E) Double-immunofluorescence images of myotubes showing nuclear targeting, transfected and labeled as in (D) (β4b, β4b(1–481): N = 4; anti-V5 n = 150, anti-β n = 210). Note that all β subunits co-cluster with the Ca, channels throughout the myotubes, but only the two β4b subunit constructs accumulate in the nuclei. (F) Nucleus/cytoplasm ratios of myotubes labeled with anti-V5. ANOVA for β subunits co-expressed with GFP-Ca 1.1 (β4b, β4b(1–481): N = 3, n = 60; β1a: N = 1, n = 20): F(2,137) = 23.8, P = 1.3 e-09; ANOVA for β subunits co-expressed with GFP-Ca 1.2 (β4b, β4b(1–481): N = 4, n = 80; β1a: N = 1, n = 20): F(2,137) = 1.7, P = 2.6 e-01 (P values in the figure are for post-hoc analysis; ***P < 0.001). Scale bars, 10µm. (G) Western blot analysis of β4b-V5 and β4b(1–481)-V5 in dysgenic myotubes with anti-V5 and anti-β4b antibodies reveals that both proteins are expressed at similar levels and at the expected size, 15 s exposure (n = 4).
Table 1. Nuclear Targeting

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|---------------------------|
| **Dysgenic myotubes**     |
|                           |
| α4-V5 | β4-V5 | β4(0-461)-V5 | t test |
| **anti-V5** | **anti-β4** | **anti-β4** | **anti-β4** | **anti-β4** | **anti-β4** |
| + GFP-Ca 1,1 | 0 ± 0,0% | 0 ± 0,0% | 64,4 ± 4,8% | 63,3 ± 8,4% |
| + GFP-Ca 1,2 | 0 ± 0,0% | 0 ± 0,0% | 60,6 ± 1,1% | 65,6 ± 10,1% |

*P test between β4-V5 and β4(0-461)-V5, β4-V5 is shown as comparison. For n values refer to the legends of Figure 1 (dysgenic myotubes) and Figure 3 (tsA-201)

Results

Similar incorporation into calcium channel complexes and nuclear targeting of the full-length and truncated β4 subunits in skeletal myotubes

In order to analyze the nuclear targeting properties of the wildtype and ataxia mutant of the β4 subunit in muscle and nerve cells, we generated a truncated β4 construct lacking the 39 C-terminal residues (β4(1–481))10,16 (Fig. 1A). Both the wildtype β4 and truncated β4(1–481) were V5-tagged at the C-terminus to enable specific localization of the heterologous β4 subunits in neurons expressing also endogenous β4. Extensive previous analysis15,17 demonstrated that the V5-tagged β4 subunits can functionally interact with CaV1.1 channel and the accumulation of both CaV subunits in triads and peripheral couplings was not limited to the skeletal muscle CaV1.1 channel. Also co-expression of the β4-V5 constructs and GFP-Ca1.2 resulted in the typical clustered distribution of both channel subunits in dysgenic myotubes (Fig. 1D) and the truncated β4(1–481)-V5 isoform was as efficiently incorporated into the calcium channel complex as the full-length β4-V5.

In addition to its incorporation into the channel complexes at the cell surface (Fig. 1B). Qualitatively, co-clustering of β4-V5 and Ca1.1 was not different from that of β4-V5 and Ca1.1, although quantitatively co-clustering of the native skeletal muscle subunit partners (β4-V5 and Ca1.1) was more robust than that of the heterologous pair (β4-V5 and Ca1.1).21 Nevertheless, co-clustering with Ca1.1 confirms previous findings showing that β4 can interact with Ca1.115,21 and it further demonstrates that the C-terminal truncation does not perturb the interaction of β4(1–481)-V5 with the skeletal muscle calcium channel complex. Consistent with previous findings,12,23 normal incorporation of Ca4 subunits in triads and peripheral couplings was not limited to the skeletal muscle Ca4 channel. Also co-expression of the β4-V5 constructs and GFP-Ca1.2 resulted in the typical clustered distribution of both channel subunits in dysgenic myotubes (Fig. 1D) and the truncated β4(1–481)-V5 isoform was as efficiently incorporated into the calcium channel complex as the full-length β4-V5.

Table 3:

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| α4-V5 | β4-V5 | β4(0-461)-V5 |
| + GFP-Ca 1,1 | 0 ± 0,0% | 0 ± 0,0% |
| + GFP-Ca 1,2 | 0 ± 0,0% | 0 ± 0,0% |

*P test between β4-V5 and β4(0-461)-V5, β4-V5 is shown as comparison. For n values refer to the legends of Figure 1 (dysgenic myotubes) and Figure 3 (tsA-201)

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as the full-length $\beta_{4b}$ construct. To determine whether the extent of nuclear targeting differed between full-length $\beta_{4b}$ and the truncated $\beta_{4b(1–481)}$-V5, we analyzed the nucleus to cytoplasm ratio of the anti-V5 labeled constructs (Fig. 1F). The nucleus/cytoplasm ratio of the control $\beta_{1a}$-V5 staining was near 1, owing to the uniform distribution of $\beta_{1a}$-V5 clusters throughout the myotubes. In contrast, the nucleus/cytoplasm ratio of the truncated $\beta_{4b}$-V5 constructs were above 2, reflecting their strong nuclear staining. The nucleus/cytoplasm ratio of the truncated $\beta_{4b-V5}$ was not statistically different from that of the full-length $\beta_{4b}$-V5 (Table 1).

To examine whether these targeting properties depended on the co-expressed $\alpha_1$ subunit, the experiments were repeated with $\beta_{1a}$-V5, $\beta_{1d}$-V5, and $\beta_{4b(1–441)}$-V5 co-expressed with the cardiac/neuronal Ca2.1.2 channel isoform. Figure 1D shows that $\beta_{1a}$-V5 and $\beta_{1d(1–441)}$-V5, but not $\beta_{4a}$-V5, were targeted into the nuclei of the myotubes. This was equally seen when labeled with the V5 or with the $\beta$ antibody. Also counting the frequency of myotubes with nuclear targeting and analyzing the nucleus/cytoplasm ratios failed to detect any significant difference in the nuclear targeting of $\beta_{4b}$-V5 and $\beta_{4b(1–441)}$-V5 (Fig. 1E and F; Tables 1 and 2). Together these results demonstrate that—when co-expressed with L-type calcium channel $\alpha_1$ subunits in dysgenic myotubes—the truncated ataxia mutant $\beta_{4b(1–481)}$-V5 was efficiently targeted into the nuclei of as many cells as the full-length $\beta_{4b}$-V5 subunit.

To exclude the possibility that the C-terminal V5 tag affected the nuclear targeting properties of the heterologous $\beta_{4b}$ subunits, we generated two corresponding $\beta_{4b}$ constructs ($\beta_{4b}$ and $\beta_{4b(1–441)}$) without the V5 tag (Fig. 2A). When expressed in dysgenic myotubes together with either GFP-Ca2.1.1 or GFP-Ca2.1.2 and immunolabeled with the anti-$\beta$ antibody, both $\beta_{4b}$ and $\beta_{4b(1–441)}$ were observed in co-clusters with the Ca2.1 subunits, confirming their expected association with the calcium channels in the membrane, and both $\beta_{4b}$ and $\beta_{4b(1–441)}$ were concentrated in the nuclei (Fig. 2B). Semiquantitative analysis showed that the untagged $\beta_{4b}$ subunits were targeted into the nuclei as efficiently as the V5-tagged versions (compare Figure 2C with Figure 1C and E). Again, there were no statistically significant differences in number of myotubes showing nuclear targeting between the full-length $\beta_{4b}$ and the truncated $\beta_{4b(1–481)}$ isoform (Table 1). These experiments clearly demonstrate that fusing a V5 antibody-tag to the C-terminus of neither the full-length $\beta_{4b}$ nor to the truncated C-terminus of $\beta_{4b(1–481)}$ alters their nuclear targeting properties.

### Nuclear targeting of the full-length $\beta_{4b}$-V5 and the truncated $\beta_{4b(1–481)}$-V5 subunits in cultured hippocampal neurons

Because we did not detect reduced nuclear targeting properties of the truncated $\beta_{4b(1–481)}$-V5 subunit in the skeletal myotubes, we decided to directly compare nuclear targeting of $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 in cultured hippocampal neurons. These neurons express all $\beta$ isoforms in pre- and post-synaptic compartments throughout the neurons.24,25 In addition the $\beta_{4b}$ isoform is specifically targeted into the nuclei of young (DIV1–4) and electrically silenced differentiated neurons (DIV17).26,27 Here we transfected hippocampal neurons with $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 and immunolabeled them with anti-V5 to specifically detect the recombinant $\beta_{4b}$-V5 constructs. Both $\beta_{4b}$-V5 subunits were localized in a punctate distribution pattern in the soma and throughout the neuronal processes (Fig. 3C, indicative of their incorporation in pre- and postsynaptic calcium channel complexes. In neurons at DIV1, 2, and 3 $\beta_{4b}$-V5 as well as $\beta_{4b(1–481)}$-V5 also labeled the neuronal nuclei, whereas at later developmental stages (DIV5, 14, and 21) the nuclei were devoid of $\beta_{4b}$ staining (Fig. 3A). Measuring the fluorescent staining intensity of the nucleus and cytoplasm of neurons and calculating the nucleus/cytoplasm ratio showed a high ratio during the first three days in culture followed by a rapid decline and continued low nucleus/cytoplasm ratio from DIV5 onward (Fig. 3B). Importantly, the nuclear targeting at the early developmental
stage as well as the lack thereof in differentiated neurons was identical for $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 (Table 2).

Because previously we detected that nuclear export of $\beta_{4b}$ in differentiated neurons was activity dependent, we examined whether this was also the case for the truncated $\beta_{4b(1–481)}$-V5 subunit. Therefore we blocked spontaneous electric activity in three weeks old (DIV21) hippocampal neurons by overnight incubation with 1 $\mu$M TTX just prior to fixation and immunolabeling. As shown in Figure 3A and B, TTX treatment restored nuclear targeting of both $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 to the same levels as observed in the young neurons. Thus, the full-length $\beta_{4b}$-V5 and the truncated $\beta_{4b(1–481)}$-V5 subunits do not differ with respect to their nuclear targeting properties in neurons. Both accumulate in the nuclei of young, presumably electrically silent hippocampal neurons, and in differentiated neurons when electrical activity is blocked.

Similar nuclear targeting properties of the full-length $\beta_{4b}$-V5 and the truncated $\beta_{4b(1–481)}$-V5 subunits in tsA-201 cells

So far our results demonstrate that truncation of the C-terminus of $\beta_{4b}$ does not interfere with its nuclear targeting properties in muscle and nerve cells. Because previous studies reported a failure of nuclear targeting of $\beta_{4b}$ in CHO and HEK293 cells, we next examined the possibility that this failed nuclear targeting of $\beta_{4b(1–481)}$ might be particular to non-excitable cells. Therefore we also analyzed the nuclear targeting properties in tsA-201 cells transfected with $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 alone and in combination with GFP-CaV1.2 and $\alpha_\delta$-1 subunits. As above, $\beta_{4b}$-V5 was used as control and all conditions were immunolabeled and analyzed with anti-V5 as well as with specific $\beta$ and $\beta_4$ antibodies. When the subunits were expressed alone, $\beta_{4b}$-V5 was localized in the cytoplasm but not in the nucleus. In contrast, both $\beta_{4b}$-V5 and the truncated $\beta_{4b(1–481)}$-V5 accumulated in the nuclei of tsA-201 cells (Fig. 4A). When co-expressed with GFP-CaV1.2, the $\alpha_1$ and $\beta$ subunits formed co-aggregates in the cell periphery (Fig. 4B), indicative of expression of the channel complexes in the plasma membrane. In addition, the $\beta_{4b}$-V5 subunits, but not $\beta_{4b}$-V5 or GFP-CaV1.2, also accumulated in the nuclei. Again, no differences in the membrane and nuclear distribution patterns of $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 were observed (Fig. 4B).

Semiquantitative analysis confirmed that a similar fraction of transfected tsA-201 cells showed nuclear targeting of $\beta_{4b}$-V5 and $\beta_{4b(1–481)}$-V5 (Fig. 4C and D). Whereas no cells were detected where nuclear staining of $\beta_{4b}$-V5 was above cytoplasmic staining levels, full-length and truncated $\beta_{4b}$-V5 subunits were concentrated in almost all nuclei of tsA-201 when expressed alone. When co-transfected with $\alpha_1$-1.2 and $\alpha_4\delta$-1 the fraction of tsA-201 cells with nuclear targeting was reduced to approximately 60% of tsA-201 cells, but again there was no difference between cells transfected with $\beta_{4b}$-V5 or $\beta_{4b(1–481)}$-V5. In both conditions the prevalence of nuclear staining appeared somewhat lower when labeled with the $\beta_4$ antibody (Table 1), most likely due to reduced sensitivity of the $\beta$ antibodies compared with anti-V5. In total this analysis demonstrates that $\beta_{4b}$ subunits are specifically targeted into the nucleus; that this nuclear targeting was independent of co-expression of $\alpha_1$-1.2; and that truncation of the C-terminus did not reduce the targeting efficiency of $\beta_{4b(1–481)}$-V5.

**Discussion**

The premature-termination mutation R482X of the CACNB4 gene gives rise to a calcium channel $\beta_4$ protein lacking the 39 C-terminal amino acids. In humans this mutation has been linked with juvenile myoclonic epilepsy. When coexpressed with $\alpha_2$-1.2 in Xenopus oocytes the truncated $\beta_{4b}$ subunit resulted in calcium currents with slightly increased current amplitudes and an accelerated fast time constant of inactivation. This demonstrated that the R482X mutant $\beta_4$ subunit normally associated with the pore-forming calcium channel $\alpha_2$-1.2, facilitated its incorporation in the plasma membrane and modulated its...
gating properties. This interpretation is corroborated by our present findings, where we consistently observed co-clustering of the truncated β4b(1–481) subunits with CaV1.1 and CaV1.2 in skeletal muscle triads and peripheral junctions, co-aggregation of β4b(1–481)-V5 with CaV1.2 in the plasma membrane of tsA-201 cells, and clustering of β4b(1–481)-V5 throughout the axons and dendrites of hippocampal neurons. Thus, despite the truncation of the C-terminus tagged and untagged β4b(1–481) subunits can associate with L-type and non-L-type calcium channels and appears to be normally incorporated into native calcium channel complexes in skeletal muscle cells and neurons.

This raises the question as to whether the modest functional differences between calcium channels containing the wildtype or truncated β4b subunits\(^{10}\) can be responsible for the neuronal disease phenotype. In fact, lethargic mice—which carry a mutation in the Cacnb4 gene resulting in the total lack of the β4 proteins—as well as mice with loss-of-function mutations of the primary calcium channel partner of β4 in cerebellum, CaV2.1, develop similar ataxic and epileptic phenotypes.\(^{9,26-29}\) Consequently, other, ideally unique properties of β4 subunits may be the primary cause of the neurological phenotype.

One such unique property of the β4 subunit is its ability to accumulate in the cell nucleus.\(^{13-15}\) Because β4 interacts with nuclear proteins involved in epigenetic regulation of genes,\(^{14,16,18}\) altered gene regulation might play a role in the etiology of epilepsy in patients with the R482X mutation. Indeed, the ability of β4 to regulate genes in neurons depends on the nuclear targeting properties of its splice variants\(^{47}\) and heterologous expression of full-length β4b and truncated β4b(1–481) resulted in differential gene regulation in HEK293 cells.\(^{28}\) Thus, a model has been suggested, according to which β4b forms a complex with B56δ/PP2A that is translocated into the nucleus where, in combination with HP1γ, it modifies histone H3 and consequently transcriptional regulation.\(^{16,19}\) Most importantly for the issue addressed in the present study, Tadmouri et al.\(^{16}\) asserted that “the formation, as

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**Figure 3.** Nuclear targeting of full-length β4b and truncated β4b(1–481) subunits in hippocampal neurons differentiating in culture. (A) Cultured hippocampal neurons were transfected at DIV 0 (4h after plating) with either β4b-V5 or β4b(1–481)-V5, fixed and immunolabeled with an antibody against the C-terminal V5 epitope at 1, 2, 3, 5, 14 and 21 d in culture (DIV). At DIV21 one set of cultures was treated with 1µM TTX over night to block spontaneous electrical activity. Scale bar, 10µm. (B) Nucleus/cytoplasm ratio of cultures shown in (A); including the TTX-treated DIV21 neurons; N = 5, n = 15–21 (** = P < 0.001, unpaired t test). (C) DIV 21 hippocampal neurons double-labeled with anti-V5 and anti-CaV2.1 show similar distribution of β4b and β4b(1–481) partially overlapping with synaptic CaV2.1 clusters. Scale bars, 10 µm.
well as the nuclear translocation, of the $\beta_4$/B56δ/PP2A complex is totally impaired by the premature R482X mutation of $\beta_4$.

Our results presented here do not support this notion. In contrast, we examined the nuclear targeting of the truncated $\beta_4b(1–481)$ subunit in three different cell models and found no difference of its nuclear targeting properties compared with full-length $\beta_4b$. Normal nuclear targeting of $\beta_4b(1–481)$ was observed in differentiated excitable cells (myotubes and hippocampal neurons), in which both $\beta_4b$ constructs were also incorporated in native calcium signaling complexes (triads and synapses). But also when expressed in tsA-201 cells, with and without the CaV1.2 $\alpha_1$ subunit, the truncated $\beta_4b(1–481)$ construct was targeted to the nucleus. Furthermore, in myotubes nuclear targeting of $\beta_4b(1–481)$ was similarly observed with V5-tagged and untagged constructs. This excludes the possibility that the differences between our findings and those of Tadmouri et al. resulted from differences in the used $\beta_4b(1–481)$ constructs or from distinct nuclear targeting mechanisms in different cell types.

Their data indicate the importance for nuclear targeting of C-terminal residues and of intramolecular SH3/GK interactions. In contrast, the equal nuclear targeting of full-length $\beta_4b$ and truncated $\beta_4b(1–481)$ subunits observed here indicates that other variable regions of $\beta_4b$ determine its nuclear targeting properties. Previously we demonstrated the importance of N-terminal residues of $\beta_4b$ for isoform-specific nuclear targeting. This was further corroborated by our recent discovery of a new splice variant ($\beta_4e$), which essentially lacks the variable N-terminus and displays no nuclear targeting. The localization of $\beta_4b$ subunits in the nucleus is further regulated by a CRM1-dependent nuclear export mechanism. Consistent with the existence of an activity-dependent $\beta_4b$ nuclear export mechanism, we observed $\beta_4b$ nuclear targeting in young and electrically silent, but not in differentiated hippocampal neurons. Moreover we showed that nuclear localization of $\beta_4b$ was lost upon KCl depolarization in myotubes and increased after blocking electrical activity with TTX in myotubes and in hippocampal neurons. In the

Figure 4. Nuclear targeting of full-length $\beta_4b$ and truncated $\beta_4b(1–481)$ subunits in tsA-201 cells. tsA-201 cells were transfected with $\beta_4b$-V5, $\beta_4b$-V5 and $\beta_4b$-V5 alone, or in combination with GFP-CaV1.2/$\alpha_2\delta-1$ and immunolabeled with anti-GFP and anti-V5 or anti-$\beta_4b$. (A) Representative immunofluorescence images of $\beta_4b$ subunits expressed alone; and (B), of $\beta_4b$ subunits expressed together with GFP-CaV1.2/$\alpha_2\delta-1$. (C) Fraction of cells showing nuclear targeting when $\beta_4b$ subunits were expressed alone (N = 3; anti-V5 n = 180, anti-$\beta_4b$ n = 90); and (D), when $\beta_4b$ subunits were expressed together with CaV1.2/$\alpha_2\delta-1$ (N = 3; anti-V5 n = 180, anti-$\beta_4b$ n = 90). Note that the $\beta_4b$ subunits are cytoplasmic in the absence of an $\alpha_1$ subunit, but co-aggregate in the plasma membrane in the presence of CaV1.2. Nuclear staining is reduced in the presence of CaV1.2/$\alpha_2\delta-1$, but still equally abundant with both $\beta_4b$ constructs. Scale bars, 10 µm.
present study we extended these observations to the truncated β_{4b(1–481)} which in response to TTX treatment accumulated in the nuclei of differentiated hippocampal neurons as potently as full-length β_{4b}.

Taken together, our present results clearly demonstrate that nuclear targeting and nuclear export properties of β_{4b} and β_{4b(1–481)} are indistinguishable, both in excitable cells and in heterologous expression systems. These findings contest a role of the R482X epilepsy mutation in perturbing nuclear targeting of β_{4b} and they raise serious concerns about the effects of the mutation on gene regulation. Nevertheless, it is important to note that even when nuclear targeting of β_{4b(1–481)} remained intact, this does not exclude the possibility that within the nucleus the interactions of the C-terminally truncated β_{4b(1–481)} with B56γ/PP2A and HP1γ might be perturbed. Whereas this possibility would preclude a model according to which complex formation of β_{4b} and B56γ is a prerequisite for nuclear translocation, it would still be consistent with many of the biochemical data of Tadmouri et al. (2012) as well as with the observation that β_{4b} and β_{4b(1–481)} differentially regulate genes in HEK293 cells.

Clearly the function of the β_{4b} subunit in the nucleus is still far from being understood. Additional experiments will be necessary to resolve the conflicting findings as well as to settle the important problem as to whether the nuclear function of calcium channel β_{4b} subunits is critically involved in the etiology of epilepsy and ataxia in patients and mouse models with mutations in the CACNB4 gene.

Materials and Methods

Expression plasmids

Cloning procedures were previously described for: GFP-CA1.1 (NM_001101720) and GFP-CA1.2 (X15539), pβA-βα1-V5 (M25514), pβA-ββ4b-V5 (L02315), αδ-1 (NM_01082276), pβA-ββ4b (L02315). To construct pβA-ββ4b(1–481)-V5 the pβA-ββ4b-V5 (L02315) was used as a template, the deletions of amino acids 482–519 was introduced by SOE-PCR. Briefly, the 3′ cDNA sequence coding for the C-terminus of β_{4b} was PCR amplified with overlapping mutagenesis primers in separate PCR reactions using pβA-ββ4b-V5 (L02315) as template. Further the two separate PCR products were then used as templates for a final PCR reaction with flanking primers to connect the nucleotide sequences. This fragment was then BglII/Sall digested and cloned into the respective sites of pβA-ββ4b-V5 (L02315) yielding pβA-ββ4b(1–481)-V5. To construct pβA-ββ4b(1–481)-β2-V5 (L02315) was used as a template and the 3′ cDNA sequence coding for the C-terminus of β_{2} was PCR amplified with a modified reverse primer introducing a stop codon after residue 481. The PCR fragment was then EcoRV/XbaI digested and cloned into the respective sites of pβA-ββ4b(1–481)-V5, yielding pβA-ββ4b(1–481). Note that to be consistent with published literature we named the truncated β_{4b} construct β_{4b(1–481)} and the truncated constructs actually end with amino acid 480.

Myotube cell culture and transfection

Myotubes of the homozygous dysgenic (mdg/mdg) cell line GLT were cultured as previously described. At the onset of myoblast fusion, GLT cell cultures were transfected with plasmids coding for the calcium channel subunits using FuGeneHD transfection reagent (Promega) according to the manufacturer’s instructions. A total of 1 μg of plasmid DNA was used per 30 mm culture dish.

Hippocampal cultures

Low-density cultures of hippocampal neurons were prepared from 17 d-old embryonic BALB/c mice of either sex as described previously. Neurons were plated on poly-L-lysine-coated glass coverslips in 60-mm culture dishes at a density of ~3500 cells/cm². After plating, cells were allowed to attach for 3–4 h before transferring the coverslips neuron-side-down into a 60-mm culture dish with a glial feeder layer. For maintenance, the neurons and glial feeder layer were cultured in serum-free neurobasal medium (Invitrogen) supplemented with Glutamax and B27 supplements (Invitrogen). Ara-C (5 μM) was added 3 d after plating and once a week 1/3 of medium was removed and replaced with fresh maintenance medium.

Transfection of hippocampal neurons

Cultured hippocampal neurons were transfected with pβA-ββ4b-V5 and pβA-ββ4b(1–481)-V5 constructs immediately after plating for 4 h using Lipofectamine 2000-mediated transfection reagent (Invitrogen) as previously described a total amount of 0.05 μg DNA was used per each condition. Transfected neurons were used for experiments from DIV 1 onwards.

txA-201 cell culture and transfection

txA-201 cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 0.44 M NaHCO₃, 10% fetal calf serum (Gibco, 500–064), 2 mM glutamine (Sigma, G753) penicillin (10 units/ml) and streptomycin (10 μg/ml) and maintained at 37 °C in a humidified environment with 5% CO₂.

Cells were grown and transiently transfected when they reached about 80% of confluency with FuGeneHD transfection reagent (Roche Diagnostics) according to the manufacturer’s instructions. A total of 0.25 μg of plasmid DNA was used per 30 mm culture dish. Cells were replated 24 h after transfection onto 13 mm poly-L-lysine coated coverslips and kept at 30 °C, 5% CO₂ for 24 h prior to fixation.

Immunochemistry and microscopy

Cells were immunostained as described in for myotubes and in for neurons. Briefly, cells were fixed in 4% paraformaldehyde/4% sucrose in PBS (pF) at room temperature for 20 min and incubated in 5% normal goat serum in PBS containing 0.2% bovine serum albumin (BSA) and 0.2% Triton X-100 (PBS/BSA/Triton) for 30 min. Primary antibodies; mouse monoclonal anti-β₂ (1:2000) and anti-β₄ (1:500) (both NeuroMab, UC Davis/NIH NeuroMab Facility), mouse monoclonal anti-V5 (1:400; Invitrogen), polyclonal anti-GFP (1:10000; Molecular Probes, Eugene, OR, USA) were applied in PBS/BSA/Triton for 4 h at RT, washed in PBS and then stained with goat anti-rabbit Alexa 488 and/or goat anti-mouse Alexa 594 (1:4000, Molecular Probes) for 1 h at RT. After staining coverslips were washed and mounted in Vectashield to avoid photo bleaching. Preparations were analyzed on an AxioImager microscope (Carl Zeiss, Inc.,) using a 63x 1.4 NA
objective. 14-bit images were recorded with a cooled CCD camera (SPOT or INSIGHT; Diagnostic Instruments, Stirling Heights, MI, USA) and Metaview image processing software (Universal Imaging, Corp., West Chester, PA, USA). Figures were arranged in Adobe Photoshop CS6 (Adobe Systems Inc.) and where necessary contrast, black level and gamma were adjusted to optimally display the labeling patterns.

Nuclear targeting analysis in myotubes and tsA-201 cells

Cultures labeled with anti-GFP and anti-V5 or anti-β were systematically screened for transfected, well differentiated myotubes or tsA-201 cells based on the GFP-CaV1 staining (green channel) and nuclear staining of the β subunits was analyzed after switching to the red filter channel. Nuclear targeting was rated positive, when the fluorescence intensity of any nuclei in the myotube was above that of the cytoplasm. The degree of nuclear targeting in dygenic myotubes was determined by calculating the nucleus/cytoplasm ratio of the anti-V5 fluorescence intensity, after background subtraction using Metamorph software. Results are expressed as mean ± SEM. All data were organized in MS Excel and analyzed using ANOVA with Tukey post-hoc analysis in Excel with Daniel's XL toolbox.

Nuclear targeting analysis in neurons

The degree of nuclear targeting in cultured hippocampal neurons was determined by calculating the nucleus/cytoplasm ratio of the anti-β, fluorescence intensity, the analysis was performed by a semi-automated procedure using a custom programmed Metamorph Macro journal as described in. Results are expressed as means ± SEM except where otherwise indicated. “N” indicates the number of independent experiments and “n” the total number of analyzed cells. Data were organized and analyzed in Excel and GraphPad.

Western blot

DIV 7 GLTs expressing pβ-β4a-V5 or pβ-β4b-V5 were trypsinized, centrifuged, resuspended and lysed in RIPA buffer (50 mM TRIS-HCL, pH 8; 150 mM NaCl; 10 mM NaF; 0.5 mM EDTA; 0.10% SDS; 10% glycerol; 1% igepal; 1x Protease Inhibitor Complete cocktail (Roche)) with a pestle and left on ice for 30 min. The lysates were then purified by centrifugation (4000 g, 10 min, 4 °C). Protein concentrations were determined using a BCA assay (Thermo Scientific) according to manufacturer’s instructions. Thirty micrograms of protein were separated by SDS-PAGE (10%) at 196 V and 40 mA for 60 min and transferred to a PVDF membrane at 25 V and 100 mA for 3 h at 4 °C with a semidy-blotting system (Roth). The blot was incubated with mouse anti-V5 (1:5000; Invitrogen) or mouse anti-β (1:10,000; Neuronab) antibodies overnight at 4 °C and successively with HRP-conjugated secondary antibody (1:5000; Pierce) for 1 h at room temperature. The chemiluminescent signal was detected with ECL Supersignal West Pico kit (Thermo Scientific) and visualized with ImageQuant LAS 4000.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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