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The juvenile myoclonic epilepsy mutant of the calcium channel β_4 subunit displays normal nuclear targeting in nerve and muscle cells

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Abbreviations: Ca_V, voltage-gated calcium channel; PP2A, protein phosphatases 2A; HP1γ, herterochromatin 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 β subunits with promoters and the epigenetic machinery in the nucleus. Mutations of the calcium channel β_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 β_4 subunit R482X mutation resulted in altered transcriptional regulation and consequently in neurological disease. Here we examined the nuclear targeting properties of the truncated $\beta_{4b(1–481)}$ subunit in tsA-201 cells, skeletal myotubes, and in hippocampal neurons. Contrary to expectation, nuclear targeting of $\beta_{4b(1–481)}$ was not reduced compared with full-length β_{4b} in any one of the three cell systems. These findings oppose an essential role of the β_4 distal C-terminus in nuclear targeting and challenge the idea that the nuclear function of calcium channel β_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 β subunits of voltage-gated calcium channels promote membrane expression and modulate the gating properties of Ca_V1 and Ca_V2 calcium channels. In humans four genes encode $Ca_V\beta$ subunits and abundant alternative splicing further increases the molecular heterogeneity of the β subunit family. Co-expression studies demonstrated that all β isoforms promote membrane expression of any Ca_V1 and Ca_V2 channel isoform and modulate their gating properties in a similar way.[1]

These highly promiscuous isoform interactions generate a considerable functional redundancy of β subunits. Consequently, loss-of-function mutations and knockouts of β subunit genes caused a disease phenotype primarily in those tissues that express exclusively the mutated β isoform.[2-5] In contrast, in brain, where all four β subunit genes are expressed, phenotypes were mild or non-existent, most likely because the channel function of the mutated β isoform was compensated by other β isoforms.[6-8] However, there is one notable exception: spontaneous mutations of the β_4 subunit lead to idiopathic generalized epilepsy and episodic ataxia in humans and in mice.[9,10] In cerebellum β_4 and $\alpha_2\delta$-2 are the major subunit partners of the P/Q-type ($Ca_V2.1$) calcium channel. Interestingly, mutations of all three subunit isoforms ($Ca_V2.1$, $\alpha_2\delta$-2 and β_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 β_4 mutants.

Recently, we and others discovered that specific β_4 subunit isoforms can also accumulate in the nucleus.[13-16] This unexpected finding suggested a role of β_4 in channel-independent cell functions. Furthermore, in excitable cells nuclear export of β_{4b} was shown to be activity-dependent. We demonstrated that in skeletal myotubes and in hippocampal neurons β_{4b} 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 β_{4c} isoform has previously been shown to interact with the nuclear protein HP1γ, 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 β_4 mutations. If mutated β_4 subunits differ from wildtype β_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 β_4 may cause the neurological deficits observed in human patients and in mouse models with mutations in the β_4 gene. Consistent with this idea, Tadmouri et al.[16] reported that the ataxia mutation R482X resulted in a C-terminally truncated β_4 protein, which failed to be targeted into the nucleus and

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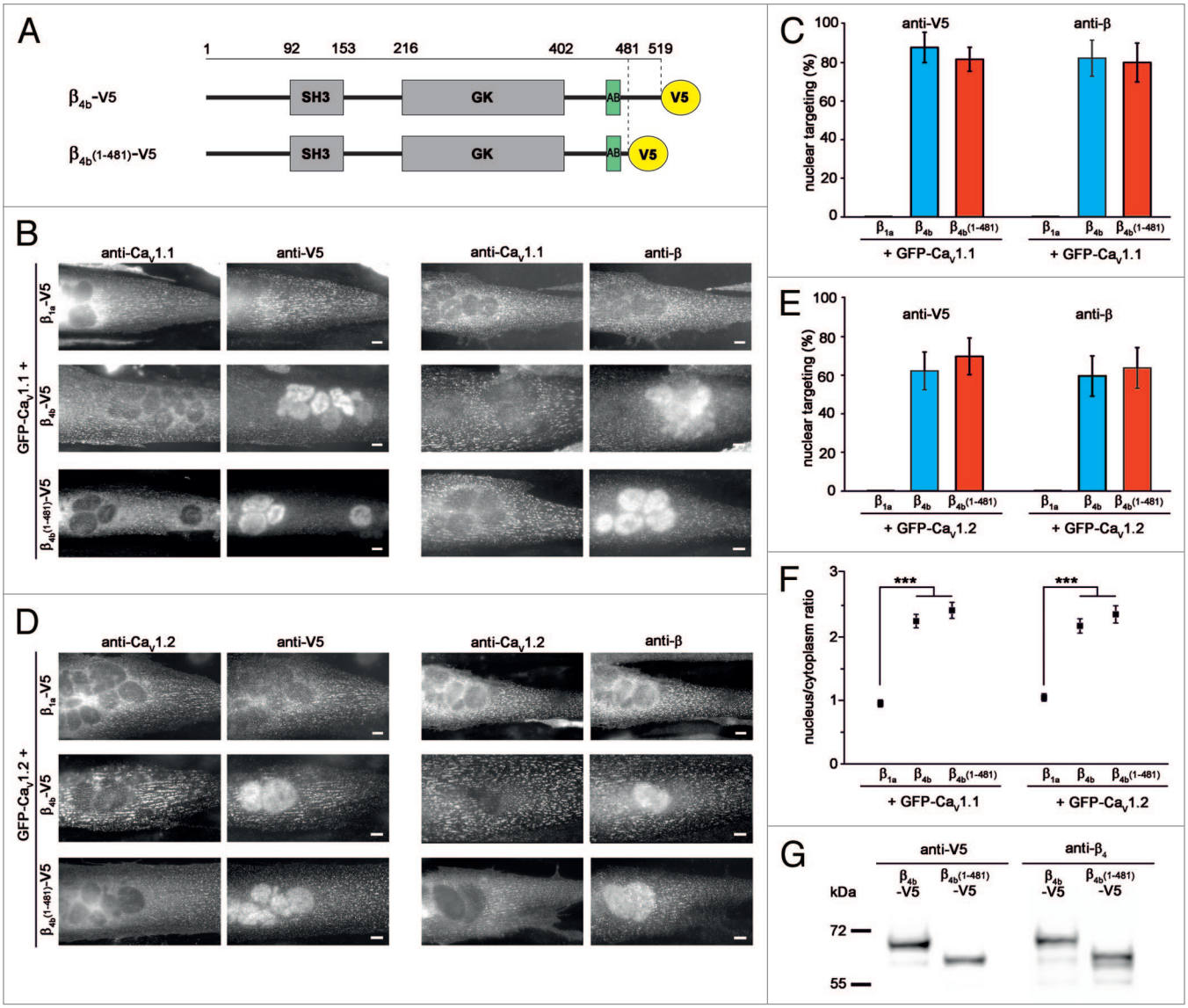

Figure 1. Nuclear targeting of V5-tagged wildtype and mutant β_{4b} subunits in dysgenic myotubes. (**A**) Domain structure of the full-length β_{4b}-V5 and truncated $\beta_{4b(1-481)}$-V5 subunits. Colored symbols indicate positions of antibody epitopes and numbers above indicate amino acid positions at domain borders and truncation site. (**B**) Representative double-immunofluorescence images of myotubes transfected with β_{1a}-V5, β_{4b}-V5, and $\beta_{4b(1-481)}$-V5 together with GFP-Ca_V1.1, 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**) ($\beta_{4b,}$ $\beta_{4b(1-481)}$: N = 3; anti-V5 n = 120, anti-β n = 150). (**D**) Double-immunofluorescence images of myotubes transfected with β_{1a}-V5, β_{4b}-V5, and $\beta_{4b(1-481)}$-V5 together with GFP-Ca_V1.2, labeled with anti-GFP and anti-V5 (left) or anti-β (right). (**E**) Fraction of myotubes showing nuclear targeting, transfected and labeled as in (**D**) ($\beta_{4b,}$ $\beta_{4b(1-481)}$: N = 4; anti-V5 n = 150, anti-β n = 210). Note that all β subunits co-cluster with the Ca_V1 channels throughout the myotubes, but only the two β_{4b} 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_V1.1 ($\beta_{4b,}$ $\beta_{4b(1-481)}$: N = 3, n = 60; β_{1a}: N = 1, n = 20): $F_{(2,137)} = 23.8$ $P = 1.3\ e^{-09}$; ANOVA for β subunits co-expressed with GFP-Ca_V1.2 ($\beta_{4b,}$ $\beta_{4b(1-481)}$: N = 4, n = 80; β_{1a}: N = 1, n = 20): $F_{(2,177)} = 1.7$; $P = 2.6\ e^{-05}$ (P values in the figure are for post-hoc analysis; ***$P < 0.001$). Scale bars, 10μm. (**G**) western blot analysis of β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 in dysgenic myotubes with anti-V5 and anti-β_4 antibodies reveals that both proteins are expressed at similar levels and at the expected size, 15 s exposure (n = 4).

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 β_{4b} isoform in HEK293 cells resulted in differential gene expression.[19]

Because we recently could demonstrate that in neurons only those β_4 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 β_{4b} 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 β_{4b} subunits may account for the neurological phenotypes in humans and mice with mutations in the *CACNB4* gene.

Table 1. Nuclear Targeting

Dysgenic myotubes										
	β_{1a}-V5		β_{4b}-V5		$\beta_{4b(1-481)}$-V5		*t* test	
	anti-V5	anti-β_1	anti-V5	anti-β_4	anti-V5	anti-β_4	anti-V5	anti-β_4
+ GFP-$Ca_V1.1$	0,0%	0,0%	87,2 ± 7,8%	81,7 ± 9,3%	81,1 ± 6,2%	79,4 ± 10,0%	$P = 0.57$	$P = 0.88$
+ GFP-$Ca_V1.2$	0,0%	0,0%	62,1 ± 9,8%	59,6 ± 10,4%	69,6 ± 9,5%	63,8 ± 10,5%	$P = 0.89$	$P = 0.79$
			β_{4b}		$\beta_{4b(1-481)}$		*t* test	
				anti-β_4		anti-β_4		anti-β_4
+ GFP-$Ca_V1.1$				90,6 ± 1,1%		90,8 ± 2,1%		$P = 0.91$
+ GFP-$Ca_V1.2$				74,6 ± 3,7%		78,8 ± 2,8%		$P = 0.41$
tsA-201								
	β_{1a}-V5		β_{4b}-V5		$\beta_{4b(1-481)}$-V5		*t* test	
	anti-V5	anti-β_1	anti-V5	anti-β_4	anti-V5	anti-β_4	anti-V5	anti-b4
w/o	0 ± 0,0%	0 ± 0,0%	98,9 ± 0,6%	98,9 ± 1,1%	99,8 ± 0,7%	94,4 ± 2,2%	$P = 0.79$	$P = 0.15$
+ GFP-$Ca_V1.2$/ $\alpha_2\delta$-1	0 ± 0,0%	0 ± 0,0%	60,6 ± 1,1%	64,4 ± 4,8%	65,6 ± 10,1%	63,3 ± 8,4%	$P = 0.65$	$P = 0.91$

*t test between β_{4b}-V5 and $\beta_{4b(1-481)}$-V5, β_{1a}-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 β_{4b} subunits in skeletal myotubes

In order to analyze the nuclear targeting properties of the wildtype and ataxia mutant of the β_4 subunit in muscle and nerve cells, we generated a truncated β_{4b} construct lacking the 39 C-terminal residues ($\beta_{4b(1-481)}$)[10,16] (**Fig. 1A**). Both the wildtype β_{4b} and truncated $\beta_{4b(1-481)}$ were V5-tagged at the C-terminus to enable specific localization of the heterologous β_4 subunits in neurons expressing also endogenous β_4. Extensive previous analysis[15,17] demonstrated that the V5-tagged β_4 subunits can functionally interact with Ca_V channels in the membrane and show normal nuclear targeting properties when expressed in muscle or nerve cells. Western blot analysis of the full-length β_{4b}-V5 and the truncated $\beta_{4b(1-481)}$-V5 constructs confirms that the two β_4 subunits express as intact proteins of the expected size (**Fig. 1G**).

First we expressed the full-length β_{4b}-V5 and truncated $\beta_{4b(1-481)}$-V5 subunits in dysgenic myotubes. These muscle cells lack the endogenous $Ca_V1.1$ channel but otherwise express the full complement of calcium signaling proteins, including the auxiliary $Ca_V\beta_{1a}$ and $\alpha_2\delta$-1 subunits and the ryanodine receptor. Therefore transfection with $Ca_V\alpha_1$ subunits reconstitutes the calcium channel in dysgenic myotubes and incorporates the heterologous β subunits into the functional excitation-contraction coupling apparatus.[20] Morphologically this is seen as co-clusters of the heterologously expressed $Ca_V\alpha_1$ and β subunits in peripheral couplings and developing triads. When the β subunit constructs (β_{1a}-V5, β_{4b}-V5, $\beta_{4b(1-481)}$-V5) were co-expressed with the pore-forming subunit GFP-$Ca_V1.1$ and immunolabeled with antibodies against GFP and the V5 tag or the β_1 or β_4 proteins, the calcium channel subunits co-clustered at the cell surface (**Fig. 1B**). Qualitatively, co-clustering of β_{4b}-V5 and $Ca_V1.1$ was not different from that of β_{1a}-V5 and $Ca_V1.1$, although quantitatively co-clustering of the native skeletal muscle subunit partners (β_{1a}-V5 and $Ca_V1.1$) was more robust than that of the heterologous pair (β_{4b}-V5 and $Ca_V1.1$).[21] Nevertheless, co-clustering with $Ca_V1.1$ confirms previous findings showing that β_{4b} can interact with $Ca_V1.1$[15,21] and it further demonstrates that the C-terminal truncation does not perturb the interaction of $\beta_{4b(1-481)}$-V5 with the skeletal muscle calcium channel complex. Consistent with previous findings,[22,23] normal incorporation of Ca_V subunits in triads and peripheral couplings was not limited to the skeletal muscle $Ca_V1.1$ channel. Also co-expression of the β_{4b}-V5 constructs and GFP-$Ca_V1.2$ resulted in the typical clustered distribution of both channel subunits in dysgenic myotubes (**Fig. 1D**) and the truncated $\beta_{4b(1-481)}$-V5 isoform was as efficiently incorporated into the calcium channel complex as the full-length β_{4b}-V5.

In addition to its incorporation into the channel complexes at the membrane β_{4b}-V5 accumulated in the nuclei of the dysgenic myotubes (**Fig. 1B**). As previously shown[15] this nuclear targeting was specific to the β_{4b}-V5 isoform and rarely observed with β_{1a}-V5. Unexpectedly however, the truncated $\beta_{4b(1-481)}$-V5 construct also accumulated in the nuclei of the myotubes. Co-clustering with the $Ca_V1.1$ channel and the accumulation of both β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 in the nuclei was observed with the V5 tag antibody (**Fig. 1B**, left panels) as well as with the β_4 antibody (**Fig. 1B**, right panels). The prevalence of nuclear targeting was quantified by assessing the fraction of transfected differentiated myotubes showing nuclear V5 or β_4 staining (**Fig. 1C**; **Table 1**). Whereas no myotubes with nuclear targeting of β_{1a}-V5 were observed, the β_{4b}-V5 constructs were targeted into the nuclei of approximately 80% of the myotubes when co-expressed with $Ca_V1.1$, both when stained with the V5 or with the β_4 antibody. Most importantly, the truncated $\beta_{4b(1-481)}$-V5 was as frequently found in the nuclei

Table 2. Nucleus/Cytoplasm ratio

Dysgenic myotubes			
	β_{4b}-V5	$\beta_{4b(1-481)}$-V5	ANOVA*
+ GFP-$Ca_V1.1$	2.25 ± 0.11	2.42 ± 0.13	$P < 0.001$
+ GFP-$Ca_V1.2$	2.03 ± 0.11	2.17 ± 0.12	$P < 0.001$
Hippocampal neurons			
	β_{4b}-V5	$\beta_{4b(1-481)}$-V5	*t* test
DIV1	1.55 ± 0.03	1.42 ± 0.03	$P = 0.66$
DIV2	1.47 ± 0.02	1.52 ± 0.02	$P = 0.21$
DIV3	1.36 ± 0.01	1.44 ± 0.01	$P = 0.79$
DIV5	0.8 ± 0.02	0.75 ± 0.01	$P = 0.12$
DIV14	0.72 ± 0.01	0.78 ± 0.02	$P = 0.84$
DIV21	0.81 ± 0.01	0.72 ± 0.01	$P = 0.33$
DIV21 + TTX	1.54 ± 0.03	1.57 ± 0.03	$P = 0.77$

*For ANOVA paramenters and n values refer to the legends of **Figure 1** (dysgenic myotubes) and **Figure 2** (hippocampal neurons).

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

To examine whether these targeting properties depended on the co-expressed α_1 subunit, the experiments were repeated with β_{1a}-V5, β_{4b}-V5, and $\beta_{4b(1-481)}$-V5 co-expressed with the cardiac/neuronal $Ca_V1.2$ channel isoform. **Figure 1D** shows that β_{4b}-V5 and $\beta_{4b(1-481)}$-V5, but not β_{1a}-V5, were targeted into the nuclei of the myotubes. This was equally seen when labeled with the V5 or with the β 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 β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 (**Fig. 1E and F**; **Tables 1 and 2**). Together these results demonstrate that—when co-expressed with L-type calcium channel α_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 β_{4b}-V5 subunit.

To exclude the possibility that the C-terminal V5 tag affected the nuclear targeting properties of the heterologous β_{4b} subunits, we generated two corresponding β_{4b} constructs (β_{4b} and $\beta_{4b(1-481)}$) without the V5 tag (**Fig. 2A**). When expressed in dysgenic myotubes together with either GFP-$Ca_V1.1$ or GFP-$Ca_V1.2$ and immunolabeled with the anti-β_4 antibody, both β_{4b} and $\beta_{4b(1-481)}$ were observed in co-clusters with the Ca_V1 subunits, confirming their expected association with the calcium channels in the membrane, and both β_{4b} and $\beta_{4b(1-481)}$ were concentrated in the nuclei (**Fig. 2B**). Semiquantitative analysis showed that the untagged β_{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 β_{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 β_{4b} nor to the truncated C-terminus of $\beta_{4b(1-481)}$ alters their nuclear targeting properties.

Nuclear targeting of the full-length β_{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 β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 in cultured hippocampal neurons. These neurons express all β isoforms in pre- and post-synaptic compartments throughout the neurons.[24,25] In addition the β_{4b} isoform is specifically targeted into the nuclei of young (DIV1–4) and electrically silenced differentiated neurons (DIV17).[15,17] Here we transfected hippocampal neurons with β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 and immunolabeled them with anti-V5 to specifically detect the recombinant β_{4b}-V5 constructs. Both β_{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 β_{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 β_{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

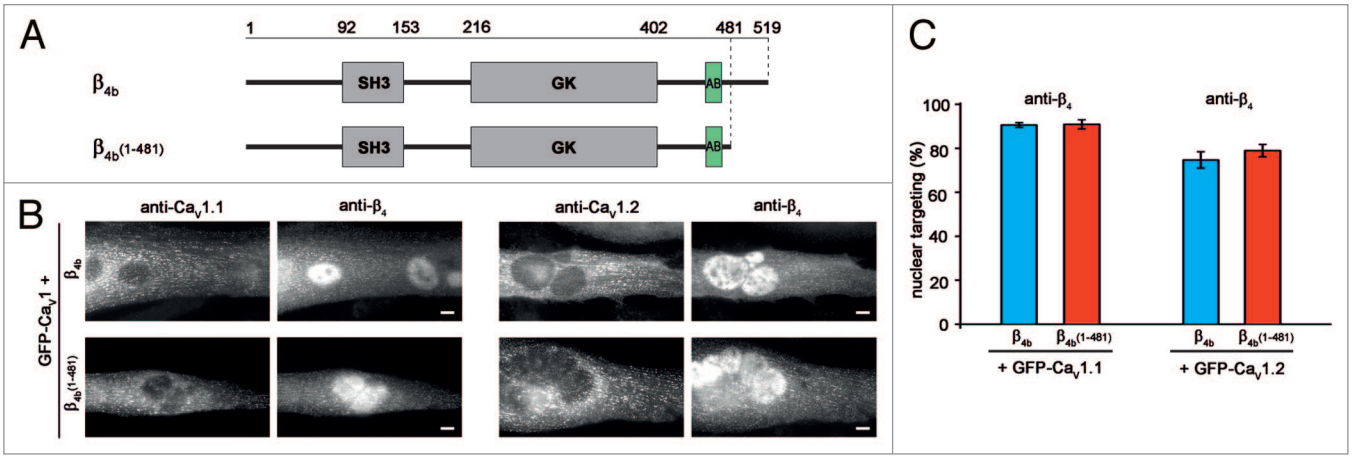

Figure 2. Nuclear targeting of untagged wildtype and mutant β_{4b} subunits in dysgenic myotubes. (**A**) Domain structure of the full-length β_{4b} and truncated $\beta_{4b(1-481)}$ subunits. Colored symbols indicate positions of antibody epitopes and numbers above indicate amino acid positions at domain borders and truncation site. (**B**) Representative double-immunofluorescence images of myotubes transfected with β_{4b}, and $\beta_{4b(1-481)}$ together with GFP-Ca_V1.1 (left) or GFP-Ca_V1.2 (right), labeled with anti-GFP and anti-β_4. C: Fraction of myotubes showing nuclear targeting, transfected and labeled as in (**B**) (β_4, $\beta_{4(1-481)}$ with GFP-Ca_V1.1: N = 4 n = 360; (β_4, $\beta_{4(1-481)}$ with GFP-Ca_V1.2: N = 3 n = 240).

stage as well as the lack thereof in differentiated neurons was identical for β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 (**Table 2**).

Because previously we detected that nuclear export of β_{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 µM TTX just prior to fixation and immunolabeling. As shown in **Figure 3A and B**, TTX treatment restored nuclear targeting of both β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 to the same levels as observed in the young neurons. Thus, the full-length β_{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 β_{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 β_{4b} does not interfere with its nuclear targeting properties in muscle and nerve cells. Because previous studies reported a failure of nuclear targeting of $\beta_{4b(1-481)}$ 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 β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 alone and in combination with GFP-Ca_V1.2 and $\alpha_2\delta$-1 subunits. As above, β_{1a}-V5 was used as control and all conditions were immunolabeled and analyzed with anti-V5 as well as with specific β_1 and β_4 antibodies. When the β subunits were expressed alone, β_{1a}-V5 was localized in the cytoplasm but not in the nucleus. In contrast, both β_{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-Ca_V1.2, the α_1 and β subunits formed co-aggregates in the cell periphery (**Fig. 4B**), indicative of expression of the channel complexes in the plasma membrane. In addition, the β_{4b}-V5 subunits, but not β_{1a}-V5 or GFP-Ca_V1.2, also accumulated in the nuclei. Again, no differences in the membrane and nuclear distribution patterns of β_{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 β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 (**Fig. 4C and D**). Whereas no cells were detected where nuclear staining of β_{1a}-V5 was above cytoplasmic staining levels, full-length and truncated β_{4b}-V5 subunits were concentrated in almost all nuclei of tsA-201 when expressed alone. When co-transfected with Ca_V1.2 and $\alpha_2\delta$-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 β_{4b}-V5 or $\beta_{4b(1-481)}$-V5. In both conditions the prevalence of nuclear staining appeared somewhat lower when labeled with the β_4 antibody (**Table 1**), most likely due to reduced sensitivity of the β antibodies compared with anti-V5. In total this analysis demonstrates that β_{4b} subunits are specifically targeted into the nuclei; that this nuclear targeting was independent of co-expression of Ca_V1.2; 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 β_4 protein lacking the 39 C-terminal amino acids. In humans this mutation has been linked with juvenile myoclonic epilepsy.[10] When coexpressed with Ca_V2.1 in Xenopus oocytes the truncated β_{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 β_4 subunit normally associated with the pore-forming calcium channel Ca_V2.1, facilitated its incorporation in the plasma membrane and modulated its

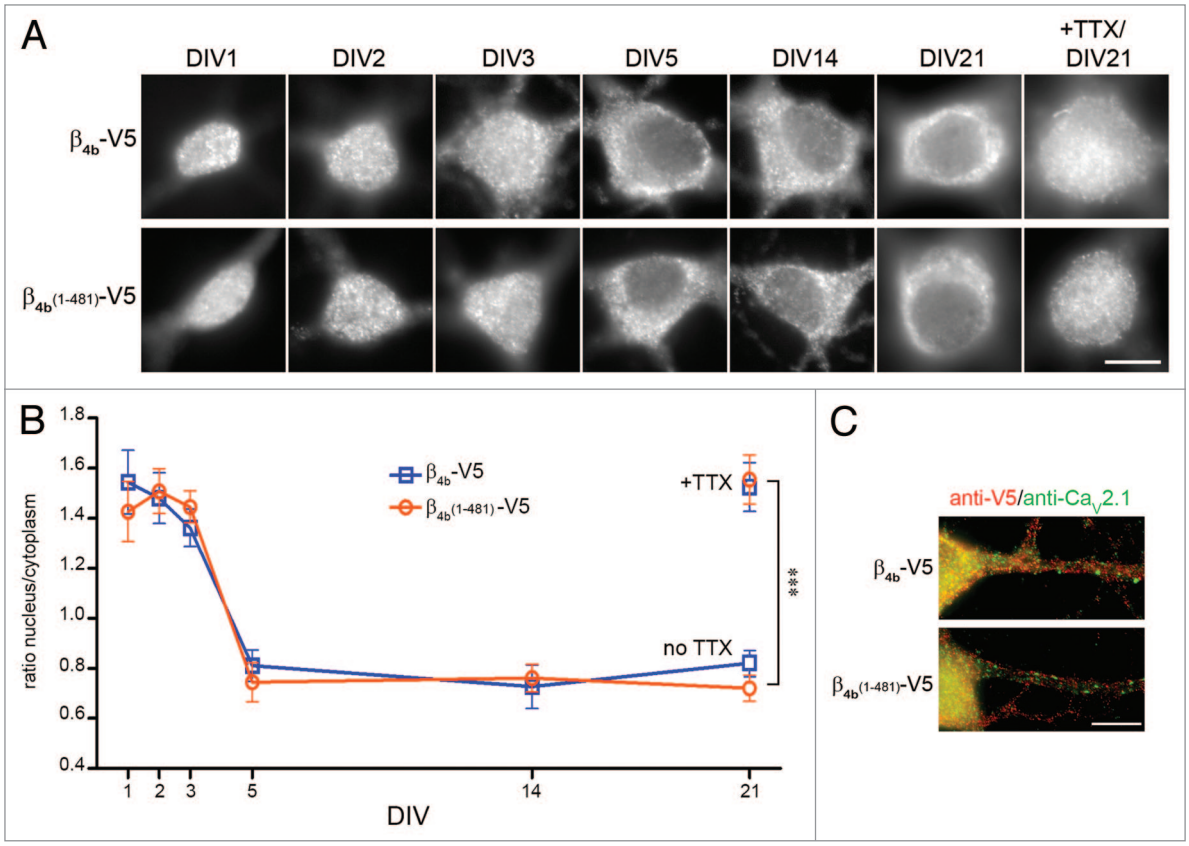

Figure 3. Nuclear targeting of full-length β_{4b} and truncated $\beta_{4b(1-481)}$ subunits in hippocampal neurons differentiating in culture. (**A**) Cultured hippocampal neurons were transfected at DIV 0 (4h after plating) with either β_{4b}-V5 or $\beta_{4b(1-481)}$-V5, fixed and immunolabeled with 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-$Ca_V2.1$ show similar distribution of β_{4b} and $\beta_{4b(1-481)}$ partially overlapping with synaptic $Ca_V2.1$ clusters. Scale bars, 10 μm.

gating properties. This interpretation is corroborated by our present findings, where we consistently observed co-clustering of the truncated $\beta_{4b(1-481)}$ subunits with $Ca_V1.1$ and $Ca_V1.2$ in skeletal muscle triads and peripheral junctions, co-aggregation of $\beta_{4b(1-481)}$-V5 with $Ca_V1.2$ in the plasma membrane of tsA-201 cells, and clustering of $\beta_{4b(1-481)}$-V5 throughout the axons and dendrites of hippocampal neurons. Thus, despite the truncation of the C-terminus tagged and untagged $\beta_{4b(1-481)}$ 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 β_{4b} 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 β_4 proteins—as well as mice with loss-of-function mutations of the primary calcium channel partner of β_4 in cerebellum, $Ca_V2.1$, develop similar ataxic and epileptic phenotypes.[9,26-29] These similarities of phenotypes are consistent with a synaptic defect being the primary cause of the ataxia and epilepsy also in β_{4b} mutants. However, a recent study demonstrated that ablation of $Ca_V2.1$ function specifically in cerebellar granule cell synapses did not generate ataxia and epilepsy.[30] In comparison to the severe impairment of synaptic function in $Ca_V2.1$ knockouts the expected effects from the β_4R482X mutation would be rather mild. In particular since there may be functional compensation by other β subunit isoforms expressed in the cerebellum.[17,25] Consequently, other, ideally unique properties of β_4 subunits may be the primary cause of the neurological phenotype.

One such unique property of the β_4 subunit is its ability to accumulate in the cell nucleus.[13-15] Because β_4 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 β_4 to regulate genes in neurons depends on the nuclear targeting properties of its splice variants[17] and heterologous expression of full-length β_{4b} and truncated $\beta_{4b(1-481)}$ resulted in differential gene regulation in HEK293 cells.[19] Thus, a model has been suggested, according to which β_{4b} 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

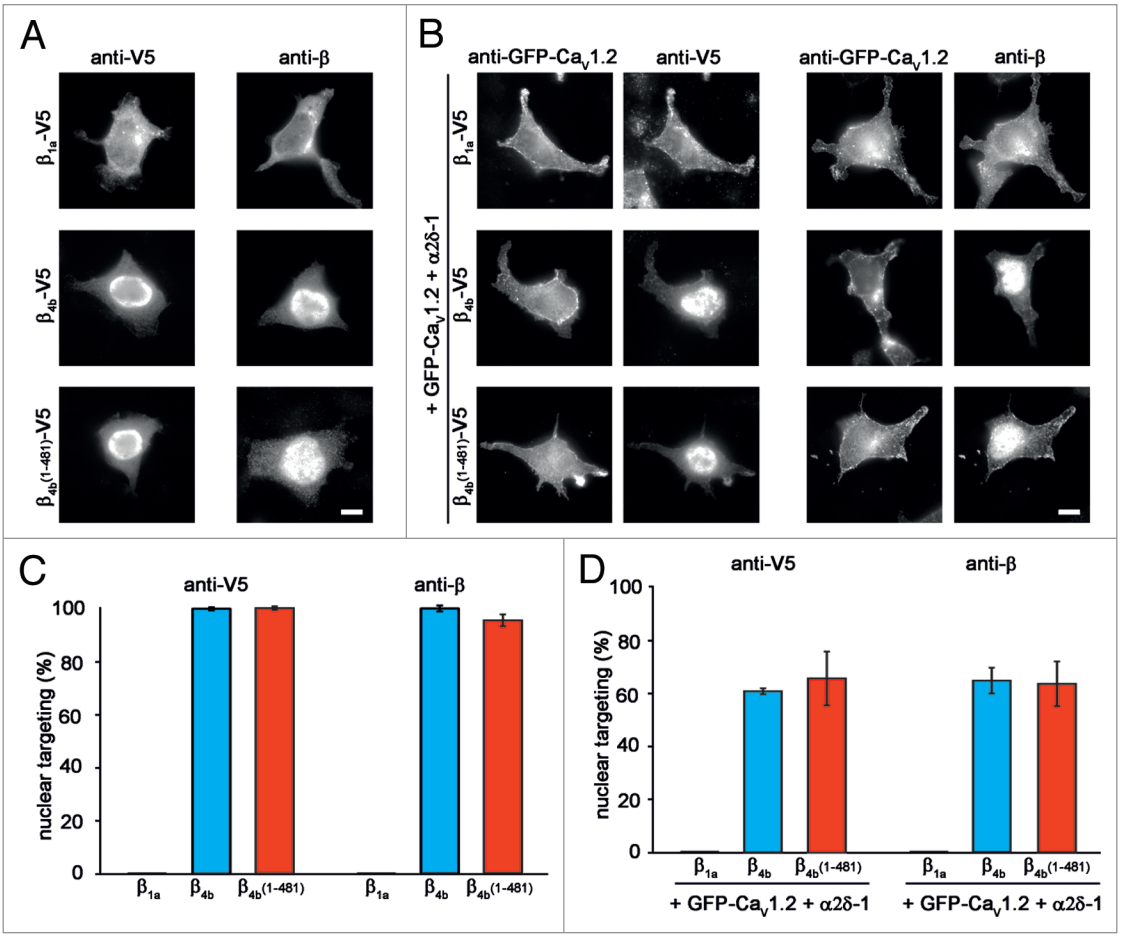

Figure 4. Nuclear targeting of full-length β_{4b} and truncated $\beta_{4b(1-481)}$ subunits in tsA-201 cells. tsA-201 cells were transfected with β_{1a}-V5, β_{4b}-V5 and $\beta_{4b(1-481)}$-V5 alone, or in combination with GFP-$Ca_V1.2/\alpha_2\delta$-1 and immunolabeled with anti-GFP and anti-V5 or anti-β. (**A**) Representative immunofluorescence images of β subunits expressed alone; and (**B**), of β subunits expressed together with GFP-$Ca_V1.2/\alpha_2\delta$-1. (**C**) Fraction of cells showing nuclear targeting when β subunits were expressed alone (N = 3; anti-V5 n = 180, anti-β n = 90); and (**D**), when β subunits were expressed together with $Ca_V1.2/\alpha_2\delta$-1 (N = 3; anti-V5 n = 180, anti-β n = 90). Note that the β subunits are cytoplasmic in the absence of an α_1 subunit, but co-aggregate in the plasma membrane in the presence of $Ca_V1.2$. Nuclear staining is reduced in the presence of $Ca_V1.2/\alpha_2\delta$-1, but still equally abundant with both β_{4b} constructs. Scale bars, 10 µm.

well as the nuclear translocation, of the β_4/B56δ/PP2A complex is totally impaired by the premature R482X mutation of β_4."[19]

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 β_{4b}. Normal nuclear targeting of $\beta_{4b(1-481)}$ was observed in differentiated excitable cells (myotubes and hippocampal neurons), in which both β_{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 $Ca_V1.2$ α_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.[16] 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.[16] In contrast, the equal nuclear targeting of full-length β_{4b} and truncated $\beta_{4b(1-481)}$ subunits observed here indicates that other variable regions of β_{4b} determine its nuclear targeting properties. Previously we demonstrated the importance of N-terminal residues of β_{4b} for isoform-specific nuclear targeting.[15] This was further corroborated by our recent discovery of a new splice variant (β_{4e}), which essentially lacks the variable N-terminus and displays no nuclear targeting.[17] The localization of β_{4b} subunits in the nucleus is further regulated by a CRM1-dependent nuclear export mechanism.[15] Consistent with the existence of an activity-dependent β_{4b} nuclear export mechanism, we observed β_{4b} nuclear targeting in young and electrically silent, but not in differentiated hippocampal neurons.[15,17] Moreover we showed that nuclear localization of β_{4b} was lost upon KCl depolarization in myotubes and increased after blocking electrical activity with TTX in myotubes and in hippocampal neurons.[15,17] In the

present study we extended these observations to the truncated $\beta_{4b(1–481)}$, 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 $\beta_{4b(1–481)}$ 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_4 and they raise serious concerns about the effects of the mutation on gene regulation.[19] Nevertheless, it is important to note that even when nuclear targeting of $\beta_{4b(1–481)}$ remained intact, this does not exclude the possibility that within the nucleus the interactions of the C-terminally truncated $\beta_{4b(1–481)}$ with B56γ/PP2A and HP1γ might be perturbed. Whereas this possibility would preclude a model according to which complex formation of β_4 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 $\beta_{4b(1–481)}$ differentially regulate genes in HEK293 cells.[19]

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_4 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-$Ca_V1.1$ (NM_001101720) and GFP-$Ca_V1.2$ (X15539),[31] pβA-β1a-V5 (M25514),[24] and pβA-β4b-V5 (L02315),[15] $\alpha_2\delta$-1 (NM_001082276),[32] pβA-β4b (L02315).[17] To construct pβA-$\beta_{4b(1–481)}$-V5 the pβA-β_{4b}-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 pbA-β_{4b}-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/SalI digested and cloned into the respective sites of pβA-β_{4b}-V5 (L02315) yielding pβA-$\beta_{4b(1–481)}$-V5. To construct pβA-$\beta_{4b(1–481)}$, pβA-β_{4b}-V5 (L02315) was used as a template and the 3′ cDNA sequence coding for the C-terminus of β_{4b} 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-β_{4b}-V5, yielding pβA-$\beta4b_{(1–481)}$. Note that to be consistent with published literature[10,16,19] we named the truncated β_{4b} construct $\beta_{4b(1–481)}$. However in the *Cacnb4* gene (L02315) the R-to-X mutation occurs at amino acid position 481, and not at position 482, as previously described, so 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.[33] 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.[34-36] Neurons were plated on poly-L-lysine-coated glass coverslips in 60-mm culture dishes at a density of ~3500 cells/cm^2. 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-β_{4b}-V5 and pβA-$\beta_{4b(1–481)}$-V5 constructs immediately after plating for 4 h using Lipofectamine 2000-mediated transfection reagent (Invitrogen) as previously described[36] a total amount of 0.05 μg DNA was used per each condition. Transfected neurons were used for experiments from DIV 1 onwards.

tsA-201 cell culture and transfection

tsA-201 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.44 M $NaHCO_3$, 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% CO_2. 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% CO_2 for 24 h prior to fixation.

Immunocytochemistry and microscopy

Cells were immunostained as described in[37] for myotubes and in[36] for neurons. Briefly, cells were fixed in 4% paraformaldehye/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-β_1 (1:2000) and anti-β_4 (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-Ca_V1 staining (green channel) and nuclear staining of the β subunits was analyzed after switching to the red filter channel.[15] 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 dysgenic 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_4 fluorescence intensity, the analysis was performed by a semi-automated procedure using a custom programmed Metamorph Macro journal as described in.[17]

Statistical analysis

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 pβA-β_{4b}-V5 or pβA-$\beta_{4b(1-481)}$-V5 were trypsinized, centrifuged, resuspended and lysed in RIPA buffer (50 mM TRIS-HCl, pH 8; 150 mM $NaCl_2$; 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 semidry-blotting system (Roth). The blot was incubated with mouse anti-V5 (1:5000; Invitrogen) or mouse anti-β_4 (1:10,000; Neuromab) 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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