Activity and calcium regulate nuclear targeting of the calcium channel β_{4b} subunit in nerve and muscle cells

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Abbreviations: CaM, calmodulin; CRM-1, chromosomal region maintenance 1; DIV, days in-vitro; GFP, green fluorescent protein; GuK, guanylate kinase; LMB, leptomycin B; NES, nuclear export signal; NLS, nuclear localization signal; SH3, Src homology 3 domain; TTX, tetrodotoxin

Introduction

Voltage-gated calcium channels transduce membrane depolarization into cellular responses. In the nervous system they play important and diverse roles in synaptic transmission, in synaptic plasticity, and in the activity-dependent regulation of gene expression that underlies long-term adaptive processes during development and learning. Voltage-gated calcium channels are composed of a major pore-forming α_{1} subunit and three auxiliary subunits; the two integral membrane proteins α_{2}δ and γ, and a peripheral membrane protein the β subunit. The α_{1} subunit contains the conductance pore, activation and inactivation gates, and major modulatory and drug binding sites.

Calcium entering through voltage-gated calcium channels can regulate transcription by either activating cytoplasmic signaling cascades or by entering the nucleus and activating CaM-kinases and transcription factors locally. Two very recent studies indicate a new route of calcium channel-dependent gene regulation. Gomez-Ospina et al. report that the C-terminus of Ca_{1.2} is cleaved and translocated into the nucleus, where it associates with endogenous promoters and regulates transcription. Kordasiewicz et al. report that the C-terminus of Ca_{2.1} can be cleaved and actively transported into the nucleus. Nuclear targeting of C-terminal fragments with polyglutamine extensions appears to be involved in the cytotoxicity associated with spinocerebellar ataxia type 6. Together these studies provide evidence for hitherto unnoticed functions of calcium channel components that require their dissociation from the channel in the surface membrane and their translocation into the nucleus.

The β subunits of voltage-gated calcium channels are cytoplasmic peripheral membrane proteins comprising a conserved src homology domain 3 (SH3) and a guanylate kinase-like domain (GuK). Coexpression of α_{1} subunits with a β subunit in heterologous expression systems causes an increased membrane expression and altered gating properties of the channel complex. Knock-out of β_{1} and β_{2} resulted in lethal muscle and cardio-vascular phenotypes, respectively, whereas mice with neuron-specific knock-out of β_{1} and β_{2}, and β_{1} knock-out mice were all viable and showed little or no neuronal phenotype. In contrast, the lethargic mouse, a spontaneous β_{4} null-mutant, as well as humans with β_{4} mutations show absence epilepsy and ataxia. This indicates that β_{4} plays a unique and important role in neuronal functions that cannot be fully compensated by other β subunits.

Two recent studies suggest that β_{4} subunits may play a role in the nucleus, apart from its known functions in the calcium channel complex. Colecraft et al. noticed the nuclear localization of β_{4}-GFP heterologously expressed in cardiac myocytes. Hibino et al. isolated a short β_{4} splice variant (β_{4c}) from chicken cochlear

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Here we show for the first time that the release of \( \beta_{4b} \) from the nuclei into the cytoplasm via a CRM-1-dependent nuclear export mechanism. This unique function of \( \beta_{4b} \) puts this calcium channel subunit in a position to signal the activity status of calcium channels to the nucleus.

**Results**

The endogenous \( \beta_4 \) subunit is located in nuclei of granular cells and Purkinje cells in the cerebellar cortex. In an effort to study the distribution of the calcium channel \( \beta_4 \) subunits in the rodent brain, we observed strong immunoreactivity of \( \beta_4 \) in the cerebellar cortex and in neurons of the deep cerebellar nucleus (Fig. 1A). Brain sections from mouse and rat (Suppl. Fig. 1) were stained with monoclonal antibodies against the \( \beta_1 \) and \( \beta_4 \) subunits of voltage-gated calcium channels. Consistent with the published distribution pattern of \( \beta_4 \) transcripts, the strongest \( \beta_4 \) immunoreactivity was found in the molecular layer and in the granular cell layer of the cerebellar cortex (Fig. 1A and C). This labeling pattern was specific in that immunostaining with the \( \beta_1 \) antibody resulted in a clearly distinct distribution pattern (Fig. 1B and D). The strong staining of the molecular layer is consistent with the localization of calcium channels in the parallel fibers and in the dendritic tree of the Purkinje neurons. The strong \( \beta_4 \) labeling in the granular cell layer (Fig. 1C) and in Purkinje cells (Fig. 1E) demonstrates that also the cell bodies of these neurons contain \( \beta_4 \). Surprisingly, in the cell bodies of the granular cells and to some degree also in Purkinje cells, the strongest staining was found in the nuclei and not as expected for a membrane-associated protein in the cell periphery. No nuclear staining was observed with \( \beta_1 \) (Fig. 1D) or in controls, in which the primary antibody was omitted (not shown). Thus, in addition to the expected localization of \( \beta_4 \) in neuronal processes, the \( \beta_4 \) subunit is specifically located in nuclei of neurons in the cerebellar cortex. Western blot analysis of subcellular fractions from mouse cerebellum corroborated this finding. \( \beta_4 \) was specifically enriched in the membrane and nuclear fractions, but virtually absent from the cytosol fraction (Suppl. Methods). This subcellular distribution is consistent with the expected localization of \( \beta_4 \) subunits in the calcium channel complexes in the membrane and with the newly discovered localization of \( \beta_4 \) in the nuclei of cerebellar granule and Purkinje cells. Neurons in the deep cerebellar nuclei were also stained with the \( \beta_4 \) antibody (Fig. 1A). However, in contrast to Purkinje and granule cells, the majority of \( \beta_4 \)-expressing neurons in the cerebellar nuclei showed no nuclear staining (Suppl. Fig. 1). This indicates that \( \beta_{4b} \) nuclear targeting in the cerebellum is neuron type specific.

**The heterologously expressed \( \beta_{4b} \) subunit is specifically targeted into nuclei of dysgenic myotubes.** To examine whether \( \beta_4 \) nuclear targeting can be observed in other excitable cells and whether it is specific for this particular \( \beta \) isoform, we generated expression plasmids coding for C-terminally V5-tagged \( \beta \) subunits and expressed them in dysgenic myotubes. This \( \alpha_{4.1} \) null mutant muscle cell line expresses the endogenous \( \alpha_{4.1} \) and \( \beta_{1-4} \) subunits and can be readily reconstituted with homologous and heterologous \( \alpha \) subunits. 20-24 In contrast to undifferentiated cells, dysgenic myotubes form triads and are excitable, thus...
allowing the analysis of channel targeting and its function in activity-induced calcium signaling.

Previously we have shown that, when expressed without an α, subunit in dysgenic myotubes, the calcium channel GFP-β, subunit is diffusely distributed throughout the cytoplasm. Similarly the V5-tagged β subunit isoforms β,α-V5, β,β-V5, β,2a-V5, β,2b-V5, β,3-V5, β,α-V5, β,4b-V5 were all located in the cytoplasm and excluded from the myo-nuclei when expressed without an α, subunit (Fig. 2A).

In contrast, the β,α-V5 subunit was localized in the nuclei. Nuclear labeling intensity above that of the surrounding cytoplasm, was observed on average in 87% of transfected myotubes, whereas with β, and β, constructs nuclear staining was negligible, and for β, and β, it was about 18% (Fig. 2B).

On coexpression with the skeletal muscle α, subunit (GFP-α,) all examined β isoforms colocalized with GFP-α, in dense clusters throughout the myotubes (Fig. 2C). These clusters represent the normal localization of L-type calcium channels in myotubes, in junctions between the sarcoplasmic reticulum and either the plasma membrane or t-tubules, called peripheral couplings and triads, respectively. The redistribution of the β subunits from the cytoplasm to these junctions on coexpression with GFP-α, indicates that all β isoforms are capable of associating with the α, subunit in skeletal muscle triads. Moreover, normal association of the β subunit constructs with the α, subunit is an indication that expression levels are balanced and that normal targeting mechanisms were not overwhelmed by overexpression of heterologous channel subunits.

Most importantly for the present study, in 83% of the transfected myotubes the β,α-V5 subunit was localized in the nuclei, in addition to its colocalization with GFP-α, in the clusters (Fig. 2D). In myotubes transfected with any of the other β subunits this fraction was below 17%. These morphological results were corroborated by western blot analysis of cell fractions of dysgenic cultures transfected with β,α-V5 and GFP-α,; β,α-V5 was most abundant in the fraction enriched in nuclear proteins (Fig. 2E).

Together these results demonstrate that β,α, is able to accumulate in nuclei of excitable cells and that this property is highly specific to the β,α, isofrom. Specific β,α, nuclear targeting was also observed in tsA201 cells, LLC-PK1 pig kidney epithelial cell, and in Neuro-2A neuroblastoma cells (Suppl. Fig. 2).

The N-terminus of β,α, is necessary and sufficient for specific targeting of β,α, into nuclei. Because the various β isoforms greatly differ in the sequence of the N-terminus, we hypothesized that this domain may contain the signal necessary for the specific targeting of β,α,.

We tested this hypothesis by sequentially truncating 27, 38, and the entire 48 amino acids of the N-terminus of β,α-V5 (Fig. 3A). All truncated β,α-V5 constructs showed normal expression and association with GFP-α, in clusters (Fig. 3B). While nuclear targeting of β,α-V5(Δ1-27) was not different from that of full-length β,α-V5, nuclear targeting was greatly reduced in β,α-V5(Δ1-38) (Fig. 3C). This indicated that amino acids 28 to 38 of β,α, are necessary for its nuclear targeting. Interestingly, when the entire N-terminus was truncated (β,α-V5(Δ1-48)), the degree of β,α, nuclear targeting increased again. This could indicate
that other sites of the $\beta_{4b}$ are responsible for nuclear targeting and that partial truncation of the N-terminus interferes with the structural integrity of these sites. Alternatively, the increased nuclear targeting of $\beta_{4b}$-V5($\Delta$1-48) could simply result from passive entry of the truncated protein into the nucleus, because its calculated molecular mass of 50 kD is near the threshold for active nuclear transport.26

The $\beta_{4b}$ subunit does not contain a canonical nuclear localization signal (NLS) (WoLF PSORT, PredictNLS).27,28 However, the amino acid sequence 28 to 38 contains a pair of arginines flanked by a serine (Fig. 3D). We mutated all three residues to alanines, to examine whether this motif is part of the nuclear targeting signal of $\beta_{4b}$. Indeed the AAA mutant of $\beta_{4b}$-V5 displayed strongly reduced nuclear targeting compared to $\beta_{4b}$-V5 (Fig. 3E and F), demonstrating the importance of these residues in regulating the nuclear targeting properties of $\beta_{4b}$. Interestingly, site-directed mutagenesis of only the serine into alanine (S30A) showed only a weak reduction of $\beta_{4b}$ nuclear targeting (not shown), indicating that phosphorylation of serine 30 is not essential for regulating nuclear import.

To test whether sequences in the N-terminus of $\beta_{4b}$ are sufficient for targeting of a $\beta$ subunit into nuclei, we fused the entire N-terminus of $\beta_{4b}$ to the full-length $\beta_{2a}$-V5 subunit (Fig. 3D). Whereas $\beta_{2a}$-V5 was found in nuclei in less than 5% of transfected myotubes, the $\beta_{4b}$-NT-$\beta_{2a}$-V5 fusion protein was as efficiently targeted into the nuclei as $\beta_{4b}$-V5 itself (Fig. 3E and F). Together these results demonstrate that the N-terminus of $\beta_{4b}$ is both necessary and sufficient for the specific targeting of $\beta_{4b}$ into nuclei.

To further examine whether the N-terminal sequences which determine the specific targeting properties of $\beta_{4b}$ comprise a genuine nuclear import signal, we fused the entire N-terminus of $\beta_{4b}$ onto GFP and compared the nuclear targeting properties of this $\beta_{4b}$-NT-GFP construct with those of nuclear import (NLS) and export signals (NES) fused to GFP. When expressed in dysgenic myotubes NLS-GFP showed a clear nuclear distribution, whereas NES-GFP was excluded from the nucleus (Suppl. Fig. 3). A mutated, inactive NES-GFP construct was evenly distributed in the nucleus and the cytoplasm. $\beta_{4b}$-NT-GFP was not targeted into the nucleus but showed a distribution similar to that of NES-GFP. This indicates that in addition to the N-terminus other structural features of $\beta$ subunits contribute to the nuclear targeting of $\beta_{4b}$.

$\beta_{4b}$ nuclear targeting is developmentally regulated and influenced by the coexpressed calcium channel $\alpha_{1c}$ subunit isoform. Dysgenic ($\alpha_{1c}$-null) myotubes can be reconstituted with the native skeletal muscle $\alpha_{1c}$ subunit or with other $\alpha_{1}$ subunit isoforms. Both GFP-$\alpha_{1c}$ and GFP-$\alpha_{1c}$ are correctly targeted into triads and support excitation-contraction coupling.22 However, L-type calcium currents of GFP-$\alpha_{1c}$ transfected myotubes

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**Figure 3.** Mapping of a nuclear localization signal to the N-terminus of $\beta_{4b}$. Dysgenic myotubes were co-transfected with GFP-$\alpha_{1c}$ and different mutated $\beta_{4b}$-V5 constructs. (A) Domain architecture of $\beta_{4b}$-V5 indicating the positions of N-terminal truncations. (B and C) Deletion of the first 27 residues of $\beta_{4b}$ ($\Delta$1-27) did not reduce nuclear targeting, but deleting amino acids 1–38 ($\Delta$1-38) or the entire N-terminus ($\Delta$1-48) of $\beta_{4b}$ caused a significant reduction of nuclear targeting. (D) Schematic of the fusion protein $\beta_{4b}$-NT-$\beta_{2a}$-V5, consisting of the N-terminus of $\beta_{4b}$ attached to a NLS, and sequence of the $\beta_{4b}$-NT-GFP construct with residues mutated in $\beta_{4b}$-AAA-GFP in bold. (E and F) Mutation of residues RRS(28-30) in $\beta_{4b}$ significantly reduced nuclear targeting. Adding the N-terminus of $\beta_{4b}$ onto full-length $\beta_{2a}$ conferred nuclear targeting properties to this non-targeted $\beta$ subunit. Bars (B and E), 10 μm; quantification (C and F), 300 cells, 5 experiments, *p < 0.01, **p < 0.001.
activate faster and at less positive potentials, and they are many times larger than those of GFP-α1 (Fig. 4B). When β4b-V5 was cotransfected with GFP-α1, it associated with this channel in the junctional clusters and it also showed nuclear localization (Fig. 4A). However, the degree of β4b-V5 nuclear targeting in GFP-α1-transfected myotubes was reduced to about half of the level observed in myotubes transfected with the skeletal muscle GFP-α1s (Fig. 4C). This suggested that β4b nuclear targeting is a regulated process and that the regulatory mechanism involves the calcium channel α1 subunit. Interestingly, in mononucleated myoblasts β4b nuclear targeting was very pronounced and equally abundant in both myoblasts coexpressing GFP-α1s and GFP-α1c (Suppl. Fig. 4). This observation suggests that the differential efficiency in β4b nuclear targeting is not determined by the molecular entity of the α1 subunits but by the differences in their calcium currents, which occur only in differentiated myotubes firing spontaneous action potentials, but not in electrically silent myoblasts.

β4b nuclear targeting is regulated by electrical activity and calcium influx. To examine whether calcium influx through the L-type channels causes the observed reduction of β4b nuclear targeting in GFP-α1c expressing myotubes, we analyzed the effects of dihydropyridines on β4b nuclear targeting. Incubating the cultures with 1 μM of the L-type calcium channel activator BayK8644 for 24 hours decreased nuclear localization of β4b-V5 in GFP-α1s and in GFP-α1c expressing cells (Fig. 4D). This effect was more prominent in myotubes expressing GFP-α1s, which have an intrinsically smaller current than GFP-α1c expressing cells. Conversely, blocking L-type calcium currents for 24 hours with 1 μM nifedipine showed no effect on nuclear targeting in GFP-α1s-transfected myotubes, but increased β4b nuclear localization in GFP-α1c-transfected myotubes from 38% in the control to 66% (Fig. 4D). These experiments demonstrate that β4b nuclear targeting is downregulated by L-type calcium currents, and that the different efficacy of GFP-α1s and GFP-α1c in β4b nuclear targeting reflects their distinct current properties.

If β4b nuclear targeting is negatively regulated by calcium currents activated by the spontaneous electrical activity of dysgenic myotubes, blocking sodium channels with TTX should counteract this downregulation. In myotubes cotransfected with GFP-α1s, which intrinsically show a high degree of nuclear β4b, application of TTX for one day had no effect (Fig. 4E). However, in myotubes cotransfected with GFP-α1c, which normally show only 38% β4b nuclear targeting, TTX treatment significantly increased the fraction of cells showing β4b nuclear targeting to 58% (Fig. 4E). Thus, the decreased β4b nuclear targeting in cells expressing GFP-α1c depends on the spontaneous electrical activity of the myotubes. Consequently, depolarization of the myotubes with high potassium (K+) should have the opposite effect.
Leptomycin B (LMB) specifically inhibits the CRM-1 (Exportin1)-dependent nuclear export mechanism. To examine its possible involvement, we incubated dysgenic myotubes transfected with β4b-V5 and GFP-α1C for 12 h in 3.5 nM LMB. In variance with control cultures, LMB treated cultures showed a high degree of β4b nuclear targeting (Fig. 4F), indicating that the reduced β4b nuclear targeting normally seen in combination with GFP-α1C depends on the activity of CRM-1 dependent nuclear export. Consistent with this interpretation, LMB treatment also blocked the high K+-induced decrease of β4b nuclear targeting in myotubes cotransfected with GFP-α1S (Fig. 4F).

β4b is shuttled between nuclei in PEG-induced heterokaryons. In order to examine whether β4b is actively shuttled into and out of nuclei, we transfected dysgenic mouse myoblasts with β4b-V5 and subsequently cocultured and fused them with untransfected pig LLC-PK1 cells using polyethylene glycol (PEG). Nuclei of these two cell types have a characteristic morphology that allows the distinction of the donor- and the potential acceptor nuclei in the heterokaryons. In preliminary experiments we verified that β4b-V5 but not β2a-V5 was targeted into the nuclei of LLC-PK1 cells (Suppl. Fig. 2). Prior to cell fusion, transfected myoblasts expressed β4b-V5 in the nucleus. Immediately after induction of cell fusion with PEG, β4b-V5 was redistributed into the cytoplasm of the myoblasts (Fig. 5B). Fifteen minutes after PEG treatment the first signs of cell fusion were observed within groups of myoblasts, and β4b-V5 diffused throughout the cytoplasm of the syncyta. Thirty minutes after PEG treatment the first heterokaryons were recognized, and β4b-V5 had entered the cytoplasm around the LLC-PK1 nuclei. Sixty minutes after PEG treatment β4b-V5 was again found in the nuclei of the heterokaryons, including those of the LLC-PK1 cells. Estimating the percentage of cells showing β4b nuclear targeting (Fig. 5B) indicated the

Indeed, incubation of myotubes expressing GFP-α1S with 80 mM K+ for 5 minutes caused a significant 20% decrease of β4b nuclear targeting (Fig. 4E). This decrease could be observed as early as 5 minutes after K+ treatment and was only partially reversed within one hour (not shown). In myotubes expressing GFP-α1C, which already had a low degree of β4b nuclear targeting, depolarization with high K+ had no additional effect.

The depolarization-induced decrease of β4b nuclear targeting is dependent on CRM-1-dependent nuclear export. The rapid decline of β4b nuclear targeting in response to K+ depolarization suggests the involvement of an active nuclear export process. Leptomycin B (LMB) specifically inhibits the CRM-1 (Exportin1)-dependent nuclear export mechanism. To examine its possible involvement, we incubated dysgenic myotubes transfected with β4b-V5 and GFP-α1C for 12 h in 3.5 nM LMB. In variance with control cultures, LMB treated cultures showed a high degree of β4b nuclear targeting (Fig. 4F), indicating that the reduced β4b nuclear targeting normally seen in combination with GFP-α1C depends on the activity of CRM-1 dependent nuclear export. Consistent with this interpretation, LMB treatment also blocked the high K+-induced decrease of β4b nuclear targeting in myotubes cotransfected with GFP-α1C (Fig. 4F).

Figure 5. Dynamic nuclear shuttling of β4b between nuclei of heterokaryons. Fusion of mouse myoblasts transfected with β4b-V5 and untransfected pig LLC-PK1 cells was induced with PEG at t = 0 and cells were fixed and immunostained with anti-V5 at the indicated time points. (A) Control: Nuclei from pig (above line) and mouse cells (below line) can be distinguished by their typical appearance in phase contrast and Hoechst staining. β4b-V5 nuclear localization was only found in mouse cells. 2 and 5 minutes after PEG treatment substantial nuclear export was observed in the majority of transfected cells (arrows). 15 minutes after PEG treatment fusion between mouse cells can be observed. β4b-V5 was predominantly localized in the cytoplasm but not in the nuclei (arrows). 30 min after PEG treatment the first heterokaryons of transfected mouse cells (arrows) and pig cells (arrowhead) were observed. β4b-V5 was first localized in pig nuclei (arrowheads) and again in mouse nuclei (arrows) of the heterokaryons. Bar 10 μm. (B) Quantification showed that after PEG treatment β4b-V5 nuclear targeting dropped from above 90% to below 30% and within one hour gradually returned to 70%. Preincubation with LMB inhibited the rapid PEG-induced export of β4b-V5 from the nuclei (control: n = 180 cells, 3 experiments; LMB treated: n = 120, 2 experiments).
rapid nuclear export of $\beta_{4b}$ in response to PEG treatment and a fairly slow recovery to about 70% of original levels one hour after PEG treatment. Nevertheless, recovery of nuclear targeting within less than an hour is much faster than expression of $\beta_{4b}$-V5, which takes about one day after transfection to reach appreciable amounts. Therefore it is unlikely that de novo synthesis of $\beta_{4b}$-V5 significantly contributed to the observed recovery of $\beta_{4b}$ nuclear targeting in heterokaryon cells. This experiment demonstrates that $\beta_{4b}$-V5 is rapidly transported out of the nuclei by a CRM-1-dependent nuclear export mechanism and subsequently taken up by all nuclei, including those of the LLC-PK1 cells, which had not previously expressed $\beta_{4b}$-V5.

In hippocampal neurons $\beta_{4b}$ nuclear targeting is developmentally regulated, dependent on electrical activity, and on CRM-1-dependent nuclear export. Finally, we examined whether this mechanism of $\beta_{4b}$ nuclear targeting is also found in neurons, which naturally express this channel subunit isoform (Suppl. Fig. 6). Mouse low-density hippocampal neurons were infected with lentiviral expression plasmids coding for eGFP and either $\beta_{4b}$-V5 or $\beta_{2b}$-V5, and the subcellular distribution of the $\beta$ subunits was analyzed by immunofluorescence microscopy. In well-differentiated neurons (DIV24) both $\beta$ subunits showed a punctuate distribution pattern in the soma and along the processes, corresponding to the calcium channel clusters in the pre- and postsynaptic compartments (not shown). In addition, the $\beta$ subunits showed cytoplasmic and nuclear staining in the soma (Fig. 6B). Nuclear staining intensity was higher for $\beta_{4b}$-V5 than for $\beta_{2b}$-V5, although this was not pronounced in differentiated hippocampal neurons. Because these neurons are electrically active and our results from the dysgenic myotubes (see Fig. 4E) indicated that electrical activity opposes $\beta_{4b}$ nuclear targeting, we blocked spontaneous activity with TTX. As expected, 12 hours treatment with 1 $\mu$M TTX significantly increased nuclear targeting of $\beta_{4b}$-V5, but not of $\beta_{2b}$-V5 (Fig. 6B and C). This indicated that $\beta_{4b}$ nuclear targeting also occurs in cortical neurons, but is downregulated when they are electrically active. Consistent with this idea, we detected prominent $\beta_{4b}$ nuclear targeting in immature hippocampal neurons (DIV1, Fig. 6A) before they form synaptic contacts, suggesting that at least in hippocampal neurons $\beta_{4b}$ nuclear targeting is found in immature, electrically inactive neurons, and that it is downregulated when they start to fire action potentials.

If this activity-dependent downregulation of $\beta_{4b}$ nuclear targeting utilizes the same mechanisms as described in the dysgenic myotubes, blocking CRM-1 should cause the accumulation of $\beta_{4b}$-V5 also in nuclei of electrically active neurons. Indeed, over night application of LMB caused a substantial increase of $\beta_{4b}$ nuclear targeting in DIV17 hippocampal neurons. In contrast, $\beta_{2b}$-V5 showed little to no nuclear localization that did not increase on LMB treatment, indicating that nuclear targeting in hippocampal neurons is also specific for $\beta_{4b}$. Thus, in addition to its localization in calcium channel complexes in the neuronal processes, $\beta_{4b}$-V5 is specifically shuttled into and out of the nuclei of hippocampal neurons. Its accumulation in the nucleus is regulated in an activity-dependent manner and the export from the nuclei depends on the exportin CRM-1.

Discussion

This is the first report demonstrating the presence of a $\beta$ subunit of voltage-gated calcium channels in nuclei of neurons. Endogenous $\beta_{4}$ was localized in the nuclei of cerebellar granule and Purkinje cells. Of seven examined $\beta$ subunit isoforms and splice variants, $\beta_{4b}$ was the only one that showed predominant nuclear targeting when expressed in various neuronal and non-neuronal cells. This specific nuclear targeting property was mapped to a sequence in the N-terminus of $\beta_{4b}$, which was both necessary and sufficient for the nuclear targeting of $\beta$ subunits. Finally, we showed that electrical activity and calcium influx negatively regulated predominant nuclear targeting of $\beta_{4b}$ in nerve and muscle cells by activating CRM-1-dependent nuclear export. Thus, the concentration of $\beta_{4b}$ in the nucleus is inversely related to the activity of the voltage-gated calcium channels in the plasma membrane.

Transport of proteins larger than ~50 kD into and out of the nucleus is active processes that depend on specific nuclear import and export signals (NLS and NES), respectively. Sequence analysis did not reveal any canonical NLS in $\beta_{4b}$. However, exon 2 of $\beta_{4b}$ codes for a cluster of arginines and lysines, which is characteristic for NLS. Deletion of this sequence in $\beta_{4b}$-V5(D1-38), or mutation of two of the arginines and the adjacent serine in $\beta_{4b}$-V5(AAA) abolished $\beta_{4b}$ nuclear targeting. Conversely, fusing the N-terminus of $\beta_{4b}$ onto $\beta_{2b}$ conferred nuclear targeting properties onto that $\beta$ subunit. These experiments provide compelling evidence for the importance of this N-terminal sequence in the isoform-specific nuclear targeting of $\beta_{4b}$. Interestingly however, the cluster of positively charged amino acids encoded in exon 2 is conserved in $\beta_{4b}$ (Suppl. Fig. 7), which showed little to no nuclear targeting. Thus, either the small differences between these sequences determine the efficacy of $\beta_{4b}$ in nuclear targeting, or other structural features of $\beta$ subunits also contribute to their nuclear targeting properties. This notion is further supported by our observation that the N-terminus of $\beta_{4b}$ alone was not sufficient to accomplish nuclear targeting of a heterologous protein like GFP. Nevertheless, the N-terminus of $\beta_{4b}$ was sufficient to confer nuclear targeting properties onto another $\beta$ subunit. Thus, the critical sequence in the N-terminus of $\beta_{4b}$ is no independent NLS, but is the key determinant for the specific targeting of $\beta_{4b}$ into the nucleus.

Export of $\beta_{4b}$-V5 was induced by calcium influx and was shown to be dependent on the exportin CRM-1. Indeed, sequence analysis using the NetNES motif prediction tool identified a typical NES in the GuK domain of all $\beta$ subunits. This conserved leucine-rich motif is probably involved in the regulated export of $\beta_{4b}$ from the nucleus. However, blocking CRM-1-dependent nuclear export with LMB did not result in the accumulation of $\beta_{4b}$-V5 in hippocampal neurons. This finding shows that the isoform-specificity of $\beta_{4b}$ nuclear targeting is not determined by a reduced nuclear export of $\beta_{4b}$ compared to other $\beta$ subunits, but by its preferential import, which in turn critically depends on the N-terminal nuclear targeting sequence.

Nuclear export controls the calcium-dependent decline of $\beta_{4b}$ nuclear targeting. In quiescent cells nuclear export of $\beta_{4b}$ appears to be inhibited, resulting in the accumulation of $\beta_{4b}$ in the
Figure 6. Nuclear export of $\beta_{4b}$ is regulated by electrical activity of hippocampal neurons. Cultured mouse hippocampal neurons were transfected with $\beta_{4b}$-V5 or $\beta_{2b}$-V5 and immunostained on day 1, 3 or 24 in vitro (DIV). (A) On the first day in culture $\beta_{4b}$-V5 was concentrated in the nuclei (arrows), whereas beginning with three days in culture $\beta_{4b}$-V5 staining was primarily cytoplasmic. Bar, 10 $\mu$m. (B) Representative images of control, TTX- and Leptomycin-B (LMB) treated mature hippocampal neurons show that in the soma $\beta_{2b}$-V5 label is cytoplasmic and in no condition labels the nuclei (nuclei counterstained with Hoechst dye). In contrast, $\beta_{4b}$-V5 is localized in the cytoplasm and nuclei even in control neurons. After blocking electrical activity with TTX (left), or blocking CRM-1 dependent nuclear export with LMB (right) $\beta_{4b}$-V5 accumulates in the nuclei. Bar 25 $\mu$m. (C) Quantification of the labeling intensity in the nucleus and in the soma shows that TTX and LMB treatment caused a marked increase of the nucleus/soma ratio of $\beta_{4b}$-V5, while $\beta_{2b}$-V5 did not change (mean ± SE; n = 73–111 cells, 3 experiments, ANOVA with Tukey posthoc analysis).
nucleus. Upon calcium influx β4-V5 was rapidly released from the nuclei into the cytoplasm, whereas recovery of β4a nuclear targeting took between 30 to 60 minutes. Inhibition of the relatively slow nuclear import in response to calcium influx could hardly account for the rapid loss of β4a from the nuclei, suggesting that calcium regulates β4a nuclear targeting by unblocking its export from the nucleus.

Regulation of β4a nuclear export was modulated by dihydropyridines demonstrating that L-type calcium channels are critically involved in this process. Interestingly, basal β4a nuclear targeting was lower in myotubes transfected with Cαa,1.1 than in those transfected with Cαa,1.2. These L-type calcium channel isoforms produce currents with greatly different current densities. However, in myotubes both Cαa,1 isoforms give rise to similar robust action potential-induced calcium transients, although by different activation mechanisms. This suggests that in electrically active myotubes regulation of β4a nuclear export is more sensitive to the calcium influx through the Cαa,1 channels than to calcium released from the SR. In brain β4a nuclear targeting was particularly prominent in cerebellar granule cells but not in other brain regions. Because granule cells are under strong inhibitory control of Golgi neurons, the difference in β4a nuclear targeting could at least in part reflect the differences in spontaneous electrical activity of β4a expressing neurons. Alternatively, differential nuclear targeting of β4 in different populations of neurons might reflect differences in the expression of the non-targeted β4a and the targeted β4a, which were not distinguished by our β antibody.

The activity- and calcium-dependent translocation of β4a from the nucleus into the cytoplasm might participate in regulating the number of calcium channels on the cell surface. β subunits have a prominent effect on the membrane expression of α subunits, and the amount of available β appears to be limiting for functional expression of calcium channel complexes. Therefore, additional β4a exported from the nucleus into the cytoplasm is expected to promote the incorporation of calcium channels into the plasma membrane. Consequently, in quiescent neurons the accumulation of β4a in the nucleus would limit the number of voltage-gated calcium channels in the membrane, whereas upon the onset of electrical activity the extra available β4a subunit in the cytoplasm would mobilize additional calcium channels, thus generating a positive feedback loop by which calcium currents are facilitated.

In addition, the β4a subunit may serve an independent function in the nucleus. Hibino et al. identified a short version of β4a (termed β4aγ) in the chicken cochlea that interacts directly with the chromo shadow domain of chromobox protein 2/heterochromatin protein 1γ (HP1γ). Coexpression of these two proteins in mammalian cells caused the translocation of β4a into the nucleus and attenuated the gene silencing activity of HP1γ. It would be tempting to speculate that β4a has a similar function in mammalian neurons. However, in Hibino’s study the full-length β4a failed to interact with HP1γ, was not localized in the nucleus, and had no effect on transcriptional regulation. Moreover, β4a and β4c do not contain the sequence, which we showed to be essential for the targeting of β4a into the nucleus, and in our hands β4a was not targeted into the nucleus. Therefore, β4a and β4c have in common that they are both found in the nucleus; yet they differ with respect to their nuclear targeting mechanism and probably also with respect to their nuclear binding partners.

Whatever function β4a performs in the nuclei of neurons, its activity-dependent regulation indicates a role during development and/or in the activity-dependent modulation of neuronal properties. When a developing neuron forms synaptic contacts with other neurons and starts to fire action potentials, the expression and distribution patterns of many channels and receptors undergo change. Similarly, in mature neurons changes in activity patterns lead to short- and long-term adaptations of synaptic strength that require altered membrane expression of existing channels and the biosynthesis of new channels, respectively. In development as well as in synaptic plasticity, the activity-induced translocation of β4a from the nucleus into the cytoplasm could have a rapid effect on calcium channel density in the membrane. In parallel, a possible nuclear role of β4a in gene regulation may exert long-term effects.

The β subunit is not the only component of voltage-gated calcium channels that has been found in the nucleus. In 2006 two independent studies reported C-terminal fragments of Cαa,1.2 and of Cαa,2.1 in the nucleus. Gomez-Ospina et al. demonstrated that CCAT, a C-terminal fragment of Cαa,1.2 translocates into the nucleus, where it associates with promoters and regulates the expression of a variety of genes. Interestingly, the nuclear export of CCAT is regulated by activity and calcium in much the same way as shown here for the β4a subunit. This suggests that voltage-gated calcium channels utilize multiple pathways to communicate their own activity status to the nucleus. Kordasievicz et al. demonstrated that a C-terminal cleavage product of Cαa,2.1 is localized in the nucleus of cerebellar neurons. It has been demonstrated that β4a specifically interacts with the C-terminus of Cαa,2.1. Since both are heavily expressed in the nuclei of cerebellar granule and Purkinje cells, it is conceivable that they interact and cooperate with one another in the nuclei of these neurons.

The C-terminal fragment of Cαa,2.1 bears a polyglutamine tract which, when expanded as in spinocerebellar ataxia type 6 (SCA6), is toxic to cells. Importantly, this cytotoxicity was dependent on the import of the C-terminal fragment into the nuclei, suggesting that the nuclear targeting of this calcium channel fragment is involved in the pathogenesis of SCA6. Mutations in the β4 gene cause severe ataxia in the mouse and have been linked to epilepsy and ataxia in humans. It has been suggested that these disease phenotypes are caused by the lack of β4 effects on neuronal calcium channels. However, neurons express multiple β subunits and most of their effects on α subunits are redundant, so that loss of one β isoform is likely compensated by the activity of other β subunits. This appears to be the case for β4, β3, and β2 because null-mutations of these β isoforms do not produce a prominent neuronal phenotype. In contrast, the neuronal phenotypes of β4 mutants indicate that β4 is not functionally equivalent with the other β subunits. In light of our present findings, it is possible that the loss or alteration of β4a’s specific nuclear function may be casually related to the pathogenesis of neurological disease. Our observation that β4a is highly expressed in cerebellar granule and Purkinje cells nuclei is certainly consistent with...
the involvement of β4b nuclear targeting in these motor disease phenotypes.

Finding an auxiliary calcium channel subunit in the nuclei of neurons was unexpected. However, the facts that nuclear targeting of β4b is highly specific, dependent on nuclear import and export signals and regulated in response to neuronal activity indicate a function of β4b apart from the calcium channel complex. Apparently the β4b subunit communicates the activity status of calcium channels to the nucleus. How this novel signaling pathway affects neuronal functions and whether its lack causes neuronal disease are important questions to be addressed in the future.

Materials and Methods

Immunohistochemistry. Eight to 10-week-old male Wistar rats, and 10 to 12-week-old male C57BL/6 mice (Dept. Lab Animals and Genetics, Medical University, Vienna, Austria) were deeply anaesthetized by an intraperitoneal injection of thiopental (12 mg/100 g body weight). Animals were perfused first with phosphate-buffered saline (PBS) (25 mM, 0.9% NaCl, pH 7.4) and then with chilled fixative (PB 0.1 M pH 7.4 with 4% pF and 15% of a saturated solution of picric acid). Immediately after fixation brains were removed from the skull, washed in 0.1 M PB and stored in 0.1 M PB with 0.05% sodium azide at 4°C. Free-floating serial sagittal sections of 10 μm were sliced on a Vibrorslicer (Leica Microsystems VT1000S, Vienna Austria) and immunostained using the avidin-biotin-immunoperoxidase method adapted from Hsu and Raine.3 Briefly, sections were washed overnight in Tris-buffered saline (TBS) containing 0.1% Triton X-100 (T-TBS). Free aldehyde groups were quenched with 50 mM glycine (Sigma) in T-TBS for 60 min followed by washing in T-TBS, then with chilled fixative (PB 0.1 M pH 7.4 with 4% pF and 15% of a saturated solution of picric acid). Immediately after washing sections were treated with 0.5 mg/ml 3,3′-diaminobenzidine peroxidase complex (1:100, Vector labs) in T-TBS at RT. By a 90 min incubation with the avidin biotinylated horseradish peroxidase complex (both Neuromab, Davis, CA, USA), were incubated at 4°C for 60 hr in T-TBS (both Neuromab, Davis, CA, USA), were incubated at 4°C for 60 hr in T-TBS. After washing in T-TBS, sections were incubated with biotinylated goat anti-mouse IgG (1:400, Vector labs, Burlingame, CA, USA) in T-TBS overnight at 4°C followed by a 90 min incubation with the avidin biotinylated horseradish peroxidase complex (1:100, Vector labs) in T-TBS at RT. Finally, sections were treated with 0.5 mg/ml 3,3′-diaminobenzidine (Sigma) and 0.003% H2O2 for 5 min, rinsed in TBS and mounted on chromaluna gelatin-coated glass slides. Prior to their use in immunohistochemistry, the specificity of the primary antibodies was tested in transfected GLT myotubes and in western blot analysis (see Suppl. Methods).

Cell culture and transfection. Skeletal muscle cell line GLT. Myotubes of the homozygous dysgenic (mdg/mdg) cell line GLT were cultured and transfected as previously described.20,21 At the onset of myoblast fusion, GLT cell cultures were transfected with different plasmids using FuGene transfection reagent (Roche Diagnostics, Basel, Switzerland) according to the manufacturer’s instructions. A total of 2 μg of plasmid DNA was used per 35 mm culture dish. Three to five days after transfection the cells were fixed with 4% paraformaldehyde, 4% sucrose (pF) in PBS at room temperature (RT).

Cultured mouse hippocampal neurons. Low-density cultures of hippocampal neurons were prepared from 16.5-day-old embryonic BALB/c mice as previously described.30 Briefly, pregnant mice were decapitated, hippocampi were isolated from embryonic mice and subsequently dissociated by trypsin treatment and trituration. Neurons were plated on poly-l-lysine-coated glass coverslips in 60-mm culture dishes at a density of 3,500 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 GmbH, Karlsruhe, Germany) supplemented with glutamax and B27 supplements (Invitrogen GmbH). Ara-C (5 μm) was added 3 days after plating to stop proliferation of non-neuronal cells. For lentiviral transfection of β4b-V5 or β2b-V5 1 ml of HEK-cell medium supernatant (see below) was added to the neurons (60 mm culture dish) at plating. The virus-containing medium was removed by transferring the coverslips into the new dish with the glial feeder layer after 3–4 h (see above). 24 days after plating and transfection neurons were pF fixed and processed for immunocytochemistry (see below).

Drug treatments. Tetrodotoxin (TTX; 1 μM in citrate buffer; Sigma, St. Louis, MO, USA) was applied to the cultured cells for 24 h (GLTs) or 12 h (hippocampal neurons) prior to fixation. The calcium channel agonist BayK8644 (Sigma) and antagonist nifedipine (Sigma) were applied at a final concentration of 1 μM (in DMSO) for 24 h. Leptomycin B (LMB; 3.5 nM in absolute ethanol; Sigma) was applied for 12 h. Depolarization of myotubes was induced by applying a high K+ tyrode solution (in mM: 65 NaCl, 80 KCl, 2 CaCl2, 2 MgCl2, 10 Hepes, 30 glucose, pH 7.4) for 5 min prior to fixation.

Immunocytochemistry and image processing. Cells were immunostained as described in Flucher et al.21 for myotubes and in Obermaier et al.30 for neurons. Briefly, fixed cells were 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 rabbit polyclonal anti-GFP (1:5,000; Molecular Probes, Eugene, OR, USA) and/or mouse monoclonal anti-V5 (1:200; Invitrogen, CA, USA) were applied to the cells 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 (1:4,000, Molecular Probes) for 1 h at RT. Where relevant, Hoechst 33342 dye (~5 μg/ml) was applied to the immunostained cells for 1 min in PBS/BSA/Triton. After staining coverslips were washed and mounted in p-phenylene-diamine-glycerol to retard photobleaching. Preparations were analyzed either on an Axiohot microscope (Carl Zeiss, Inc.,) using 40x, 1.3 NA and 63x 1.4 NA objectives or on an Axiosimager microscope (Carl Zeiss, Inc.,) using 63x, 1.4 NA and 25x, 0.8 NA objectives. 12- or 14-bit images were recorded with cooled CCD cameras (SPOT; Diagnostic Instruments, Stirling Heights, MI, USA) and Metaview image processing software (Universal Imaging, Corp., West Chester, PA, USA). Image composites were arranged in Adobe Photoshop 9 (Adobe Systems...
Inc.) and, where necessary, linear adjustments were performed to correct black level and contrast.

Expression plasmids. Calcium channel subunit cDNAs and constructs used in this study are listed in Tables 1 and 2. For details about sources and cloning strategies see Supplementary Methods.

Analysis of β nuclear targeting. Skeletal myotubes. Cultures double-labeled with anti-GFP (GFP-α/α/Alexa488, green channel) and anti-V5 (β-V5/Alexa594, red channel) were systematically screened for transfected, well differentiated myotubes based on the GFP-α staining (green channel) using the 63x 1.4 NA objective. Selection criteria were: (1) multinucleated myotubes, (2) moderate expression levels of heterologous channel proteins, and (3) clustered distribution (correct targeting) of GFP-α subunits. In myotubes that met these criteria nuclear staining of the β-V5 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, or negative, when nuclear fluorescence intensity was below that of the cytoplasm (for example see Fig. 4A). Cells in which nuclear targeting could not be assessed (e.g., because the expression level of β-V5 was too low) were excluded from the analysis. The incidence of these cases was always below 10% of analyzed cells and did not differ between β-V5 isoforms. Each experiment was repeated between 5 and 15 times using different cell passages, two coverslips were analyzed per experimental condition and 60–70 myotubes were rated per coverslip. In at least 3 experiments of each condition the experimental paradigm (β isoform transfected and treatment) was blinded to the experimenter.

Cultured hippocampal neurons. The degree of nuclear targeting of heterologously expressed β-V5 and β-V5 under different experimental conditions was determined by calculating the nucleus/soma ratio of V5 fluorescence intensity. To this end 14-bit image pairs of the V5 fluorescence and corresponding Hoechst stain were acquired using the 25x, 0.8 NA objective. The Hoechst stain image was thresholded to trace the displayed nuclei and regions of interest (ROI) were automatically drawn around the nuclei (nucleus ROI). The corresponding soma ROI was generated by dilating the nucleus region by 20 pixels (morphological filter dilate) yielding a circular ring with 5 μm radius. Both ROIs were transferred onto the corresponding V5 fluorescence image and their fluorescence intensities were measured. The intensities of the corresponding nucleus and soma ROIs were background subtracted and the nucleus/soma ratio was determined. Nucleus/soma ratio analyses were performed with the help of a semi-automated procedure using a custom designed Metamorph Journal (macro). Both experiments (TTX treatment and leptomycin B treatment) were repeated three times with different culture preparations and 15–20 cells were analyzed per coverslip with two coverslips per experimental condition. The experimental condition (β isoform transfected and treatment) was blinded to the experimenter.

Nuclear shuttling assay. One day after transfection with β-V5 and before the onset of myoblast fusion, GLT cells were trypsinized and plated together with untransfected LLC-PK1 cells.34 Four days later cell fusion was initiated by incubating the cells in 50% polyethylene glycol 1500 (PEG1500, Roche diagnostics) for 1 min. Then, cells were washed three times in serum-free medium and incubated in growth medium for the indicated time periods before fixation and immunostaining (see above).
**Subcellular fractionation and western blotting.** Subcellular fractionation of transfected myotubes was carried out as described by de Araújo and Huber. Briefly, GLT cells were plated into 100 mm culture dishes and transfected as described above. Eight days after plating myotubes were harvested and lysed in homogenization buffer (250 mM sucrose, 3 mM imidazole, 1 mM EDTA) by titration through a small-bore needle. Homogenization was stopped when the ratio of clear nuclei to whole cells was 80%. The homogenate was centrifuged at 600 xg to remove unbroken cells. Following this the supernatant was centrifuged at 3,000 xg to separate the nuclear pellet from the post-nuclear supernatant (PNS). The pellet was sonicated to release the contents of the nuclei. The PNS was centrifuged at 100,000 xg to yield the cytosolic fraction in the supernatant and the crude membrane fraction in the pellet. All solutions contained the following protease inhibitors: leupeptin (1 μM), pepstatin (130 μM), E64 (0.3 mM), aprotinin (0.3 μM) and PMSF (2 mM). Obtained cell fractions were aliquoted, snap-frozen in liquid N2, and stored at -80°C.

Subcellular fractions from mouse cerebellum were obtained as described by Cox and Emili. Briefly 6-week-old female mice were killed with carbon dioxide and immediately decapitated. Whole cerebellum was dissected and minced in cold PBS. The tissue was then transferred into homogenization buffer containing 250 mM sucrose, 1 mM DTT and spermine and spermidine (25 μg/ml) and homogenized at 1,000 rpm. Subcellular fractions were isolated by differential centrifugation. The tissue homogenate was first centrifuged at 800 g to isolate nuclei and the post-nuclear supernatant. The nuclear pellet was further purified by centrifugation at 80,000 g through a 2 M sucrose cushion for 35 min. The isolated pure nuclei were then extracted with a high salt buffer containing Triton-X-100. The microsomal and cytosolic fractions were separated from the post-nuclear supernatant by centrifugation at 100,000 g for 1 h. The pellet containing microsomes was resuspended in detergent-containing buffer. The subcellular fractions were aliquoted, snap frozen and stored at -80°C. All solutions contained a mixture of protease inhibitors (Sigma).

**Protein concentration was determined using a modified Bradford assay (BIO-RAD Laboratories, Hercules, CA, USA).**

**Statistical analysis.** Results are expressed as mean ± standard error (SE) and data were organized in MS Excel and analyzed using ANOVA with Tukey post-hoc analysis in SPSS statistical software (SPSS Inc., Chicago, IL, USA).

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**Note.** Supplementary materials can be found at: www.landesbioscience.com-supplement/SubramanyamCHAN3-5-Sup.pdf

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