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β Subunit Reshuffling Modifies N- and P/Q-Type Ca^{2+} Channel Subunit Compositions in Lethargic Mouse Brain

Daniel L. Burgess,*,[1] Gloria H. Biddlecome,†,[1] Stefan I. McDonough,‡ Maria E. Diaz,* Carolyn A. Zilinski,* Bruce P. Bean,‡ Kevin P. Campbell,† and Jeffrey L. Noebels*,§,[2]

**Department of Neurology, §Department of Molecular and Human Genetics, Baylor College of Medicine, Houston, Texas 77030; †Howard Hughes Medical Institute, Department of Physiology and Biophysics, Department of Neurology, University of Iowa College of Medicine, Iowa City, Iowa 52242; and ‡Department of Neurobiology, Harvard Medical School, Boston, Massachusetts 02115*

Neuronal voltage-dependent Ca^{2+} channels are heteromultimers of α_1, β, and $\alpha_2\delta$ subunits, and any one of five α_1 subunits (α_{1A-E}) may associate with one of four β subunits (β_{1-4}). The specific α_1-β combination assembled determines single-channel properties, while variation in the proportion of each combination contributes to the functional diversity of neurons. The mouse mutant lethargic (*lh*) exhibits severe neurological defects due to a mutation that deletes the α_1 subunit interaction domain of the β_4 subunit. Since β subunits regulate critical α_1 subunit properties in heterologous expression systems, loss of β_4 in lethargic could dramatically alter channel localization and behavior unless β_{1-3} subunits can be used as substitutes *in vivo*. Here we demonstrate increased steady-state associations of α_{1A} and α_{1B} with the remaining β_{1-3} subunits, without significant changes in β_{1-3} mRNA abundance. The immunolocalization of α_{1A} and α_{1B} protein in lethargic brain is indistinguishable from wild-type by light microscopy. Furthermore, the measurement of large-amplitude P-type currents in dissociated lethargic Purkinje neurons indicates that these α_{1A}-containing channels retain regulation by β subunits. We conclude that several properties of α_{1A} and α_{1B} proteins are not uniquely regulated by β_4 *in vivo* and may be rescued by β_{1-3} subunit reshuffling. The complex neurological manifestation of the lethargic mutation therefore emerges from loss of β_4 coupled with the widespread pairing of surrogate β subunits with multiple Ca^{2+} channel subtypes. The existence of β subunit reshuffling demonstrates that molecular plasticity of Ca^{2+} channel assembly, a normal feature of early brain development, is retained in the mature brain.

INTRODUCTION

Neuronal voltage-dependent Ca^{2+} channels are heteromultimeric complexes of α_1, $\alpha_2\delta$, and β subunits that exist in a 1:1:1 stoichiometry in highly purified preparations from brain (Witcher *et al.*, 1993). At least 10 genes have been identified that encode α_1 subunits, 4 that encode β subunits, and 3 that encode $\alpha_2\delta$ subunits (De Waard *et al.*, 1996; Talley *et al.*, 1999; Klugbauer *et al.*, 1999). A neuronal homolog of the muscle-specific γ subunit was recently identified, but its interactions have not yet been characterized (Letts *et al.*, 1998). The transmembrane α_1 subunit is principally responsible for channel gating, permeability, voltage dependence of activation and inactivation, and pharmacological sensitivity (Catterall, 1996; De Waard *et al.*, 1996). Consequently, the biophysical and pharmacological classifications of neuronal channels (L-, N-, P-, Q-, R-, and T-type) are generally correlated with specific α_1 isoforms (Zhang *et al.*, 1993; Catterall, 1996; De Waard *et al.*, 1996; Perez-Reyes *et al.*, 1998; Talley *et al.*, 1999). Analysis of channel behavior in heterologous coexpression systems, such as Xenopus oocytes and mammalian cultured cells, demonstrates that β subunits regulate the assembly and membrane localization of α_1 subunits (Nishimura *et al.*, 1993; Chien *et al.*, 1995; Krizanova *et al.*, 1995; Brice *et al.*,

[1] These authors contributed equally to this work.

[2] To whom correspondence and reprint requests should be addressed. Fax: (713) 798-7528. E-mail: jnoebels@bcm.tmc.edu.

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1997; Neuhuber *et al.*, 1998; Yamaguchi *et al.*, 1998), and strongly influence the current amplitude, rate and voltage-dependence of activation and inactivation, inhibition by G proteins, coupling of voltage-sensing to pore opening, and ligand-binding properties of Ca^{2+} channels (Castellano and Perez-Reyes, 1994; De Waard and Campbell, 1995; Moreno *et al.*, 1997; Birnbaumer *et al.*, 1998).

β subunits have distinct effects on channel biophysical properties depending on the particular α_1 and β isoforms coexpressed in heterologous systems. For example, the inactivation rates of α_{1A}, α_{1C}, and α_{1E} are all modulated by coexpression of β subunits. For α_{1A}, inactivation was converted from a biphasic to a monophasic process by coexpression with any of the four β subunits, although the rate varied, with the fastest to slowest inactivation occurring in the rank order $\beta_3 > \beta_{1b} = \beta_4 > \beta_{2a}$ (De Waard and Campbell, 1995). The inactivation rate of α_{1C} increased more upon coexpression with the β_4 subunit than with β_3 (Castellano *et al.*, 1993a, 1993b). Coexpression of α_{1E} with β_{1b} accelerated inactivation and shifted the half-maximal inactivation voltage toward more negative potentials, while β_{2a} slowed inactivation and shifted the voltage at half-maximal inactivation toward more positive potentials (Olcese *et al.*, 1994). Channel activation rates are likewise dependent on particular α_1–β isoform combinations. For instance, β subunits have no effect on the activation rate of α_{1A} (Stea *et al.*, 1994; De Waard and Campbell, 1995), but β_{1b} accelerates activation of α_{1B} (Stea *et al.*, 1993), and α_{1C} activation is differentially accelerated by β_1, β_2, and β_3 subunits (Perez-Reyes *et al.*, 1992; Castellano *et al.*, 1993b; Tomlinson *et al.*, 1993). Isoform-dependent variation has also been described for β subunit modulation of channel voltage-dependence (Cens *et al.*, 1996; Mangoni *et al.*, 1997), prepulse-induced facilitation (Qin *et al.*, 1998), G protein-mediated inhibition (Qin *et al.*, 1998; Roche and Treistman, 1998), average current decay of single channels (Lacerda *et al.*, 1994), and toxin sensitivity (Moreno *et al.*, 1997). Based on the evidence of isoform-dependent modulation of particular α_1 subunits, and because L-, P/Q-, and N-type channels in brain can bind each of the β subunits (Liu *et al.*, 1996; Scott *et al.*, 1996; Pichler *et al.*, 1997; Qin *et al.*, 1998), it has been postulated that regulating the proportion of different α_1-β subunit combinations could generate functional diversity *in vivo*.

The mouse mutant lethargic (*lh*) exhibits ataxia, episodic dyskinesia, and generalized spike-wave epilepsy due to a four base pair insertion into a splice donor site within the *Cacnb4* gene on chromosome 2 (Burgess *et al.*, 1997). The mutation results in aberrant pre-mRNA splicing and translational frameshift and is predicted to encode a severely truncated β_4 protein missing 60% of the C-terminus relative to wild type, including the essential α_1-β interaction domain. β subunits expressed with deletions of this domain are unable to modulate α_1 subunit function *in vitro* (De Waard *et al.*, 1994). Thus, the absence of detectable wild-type transcripts by RT-PCR of lethargic brain RNA suggests that the *lh* mutation represents a null allele of the *Cacnb4* gene. Recently, McEnery *et al.* (1998) confirmed this prediction by using β_4-specific antibodies to demonstrate a complete absence of β_4 protein in *lh/lh* cerebellum and forebrain. Lethargic is therefore an excellent model for investigating the contribution of the β_4 isoform to Ca^{2+} channel assembly and function *in vivo*.

To evaluate the molecular basis of the lethargic channelopathy, we hypothesized that loss of β_4 from the pool of β subunits in *lh/lh* brain could affect neuronal Ca^{2+} channels in two distinct ways. First, α_1–β_4 interactions could be replaced by other β subunits. This hypothesis is favored because all four β isoforms are associated with N-, P/Q-, and L-type Ca^{2+} channels in wild-type brain, and β subunit genes are widely coexpressed (Tanaka *et al.*, 1995). Additionally, *in vitro* studies indicate that all β subunits bind the α_1 interaction domain (AID) of α_{1A} and α_{1B} with similar affinity (Pragnell *et al.*, 1994; De Waard *et al.*, 1995; Scott *et al.*, 1996). Based on the heterologous expression data, the effect of β_4 loss on the biophysical properties of channels may be evident, or not, depending on the β subunit(s) substituting for β_4 in a particular neuron. For example, coexpression of either β_{1b} or β_4 with α_{1A} in Xenopus oocytes increased the average current amplitude of the resulting Ca^{2+} channels 18- to 19-fold over that obtained by expression of α_{1A} alone, while coexpression of β_{2a} or β_3 with α_{1A} produced only a 5-fold increase (De Waard *et al.*, 1994). If the Xenopus oocyte model system accurately predicts channel behavior in mammalian neurons, replacement of β_4 by β_{1b} in lethargic brain might not be detected by cellular recordings of Ca^{2+} current amplitude, but replacement of β_4 by β_{2a} or β_3 should produce measurable differences. However, the mechanisms governing the assembly of individual channel subtypes *in vivo* are unknown, and an alternative hypothesis is that α_1 subunits normally associated with β_4 in wild-type brain could remain unpaired with β subunits in lethargic brain. In this case, dramatically decreased current amplitude and altered α_1 protein concentration and localization would be expected (Chien *et al.*, 1995; Brice *et al.*, 1997; De Waard *et al.*, 1995; De Waard and Campbell, 1995). Since substantial proportions of brain L-type (~40%), P/Q-type (~50%), and N-type (~30%) Ca^{2+} channels contain β_4 subunits (Liu *et al.*, 1996; Scott *et al.*, 1996; Pichler *et al.*, 1997), these aberrations would be

readily detected and evident in more than one channel type.

To test these alternatives, we examined the effects of β_4 loss on the subunit composition of α_{1A} and α_{1B} channels in whole lethargic brain; on the cellular expression of α_{1A}- and α_{1B}-containing channels and β_{1-4} subunits in lethargic brain, and on whole-cell Ba^{2+} currents in acutely dissociated lethargic neurons. Cerebellar Purkinje cells were selected because they coexpress all four β subunits. Our data demonstrate that loss of β_4 results in increased steady-state association of the β_{1-3} subunits with α_{1A} and α_{1B}. No changes were evident in β_{1-3} subunit mRNA expression by *in situ* hybridization analysis, and the concentration of membrane-associated α_{1A}, α_{1B}, and total β subunit protein was similar to wild type. Patch-clamp analysis revealed that large amplitude P-type currents in lethargic Purkinje cells were intact. These findings demonstrate that the role of β_4 in the assembly and sorting of P/Q-type and N-type Ca^{2+} channels *in vivo* and, in the production of P-type currents in cerebellar Purkinje neurons, can be performed by alternative pairing with β_{1-3} subunits in mammalian brain.

RESULTS

α_{1A} and α_{1B} Subunits Increase Association with β_1, β_2, and β_3 in the Absence of β_4

We hypothesized that loss of Ca^{2+} channel β_4 subunits in lethargic mice may be compensated by changes in the steady-state association of α_1 with β_1, β_2, or β_3 subunits. To test this, we examined β subunit association with brain α_{1A} and α_{1B} using a coimmunoprecipitation assay. Channel proteins solubilized from lethargic (*lh/lh*) or wild-type (WT) brain microsomes were incubated with C-terminal antibodies specific for β_{1b}, β_2, β_3, or β_4 (Liu *et al.*, 1996) and antibody-bound proteins were bound with Protein G Sepharose. Due to high affinity interactions among channel subunits, β subunit antibodies coimmunoprecipitate α_1 subunits (Leung *et al.*, 1988; Sakamoto *et al.*, 1991; De Waard *et al.*, 1995). The amount of α_{1A} and α_{1B} present in the immunoprecipitated complex can then be determined by measuring SNX-230 and ω-CTX GVIA binding, respectively.

We initially attempted to assess changes in the concentrations of α_1 subunit associated with each β subunit in lethargic versus wild-type brains with crude antiserum, using rabbit and sheep preimmune serum as irrelevant antibody controls. However, preimmune serum precipitated up to one-third of the maximal ω-CTX GVIA binding activity precipitated with immune serum (data not shown). To obtain a more reliable signal over background, we affinity-purified the β subunit antibodies and used affinity-purified GST antiserum as our negative control. In this case, less than 8% of the maximal ω-CTX GVIA binding was immunoprecipitated by GST antibody, and the absolute value was very similar to the nonspecific binding with β subunit antibodies (data not shown). Although the specificity of these antibodies has been reported previously by Western blot analysis and immunoprecipitation of *in vitro*-translated β subunits (Liu *et al.*, 1996), we confirmed that the signal detected as immunoprecipitation by a particular β subunit in our assay was specific by performing antigen competition experiments. Addition of 5 μg of GST-fusion protein antigen blocked more than 95% of the α_{1B} immunoprecipitated from wild-type brain by β_{1b}, β_2, β_3, and β_4 antibodies (data not shown).

Comparison of lethargic and wild-type brains revealed novel proportions of α_1–β subunit complexes accompanied loss of β_4. As shown in Fig. 1, the steady-state concentration of α_{1A} associated with β_{1b} and β_3 subunits increased in lethargic mouse. The concentration of α_{1A} bound to β_{1b} increased 2.7-fold (WT = 9 ± 1 fmol/mg, *lh/lh* = 25 ± 2 fmol/mg; $n = 4$), while the concentration of α_{1A} bound to β_3 increased 2.1-fold (WT = 16 ± 2 fmol/mg, *lh/lh* = 33 ± 2 fmol/mg; $n = 4$). No significant increase in α_{1A} association with β_2 was observed. A small but statistically significant amount of SNX-230 was coimmunoprecipitated from lethargic mouse with β_4 subunit antibody (10% of that from WT). The steady-state concentration of α_{1B} associated with β_{1b}, β_2, and β_3 also increased in lethargic (Fig. 2). The concentration of α_{1B} bound to β_{1b} increased 2.7-fold (WT = 12 ± 1 fmol/mg, *lh/lh* = 31 ± 2 fmol/mg; $n = 3$). For β_2 the increase was 1.9-fold (WT = 4 ± 1 fmol/mg, *lh/lh* = 7 ± 1 fmol/mg; $n = 3$) and for β_3 it was 1.5-fold (WT = 31 ± 2 fmol/mg, *lh/lh* = 45 ± 1 fmol/mg; $n = 3$). β_4 subunit antibody did not coimmunoprecipitate any ω-CTX GVIA binding activity from lethargic brain. Together, these results confirm our hypothesis that the other β subunits modify their associations with α_{1A} and α_{1B} in the absence of β_4.

The detection of SNX-230, even at low levels, upon coimmunoprecipitation with β_4 subunit antibody was unexpected since no mRNA transcripts that are predicted to encode the C-terminus of β_4 were detected in lethargic brain by RT-PCR (Burgess *et al.*, 1997). Since ω-CTX GVIA did not coimmunoprecipitate with β_4 antibody, we suspect that the SNX-230 signal reflects residual, nonspecific ligand binding rather than antibody cross-reactivity or production of β_4 protein containing the C-terminal epitope recognized by our antibody.

Indeed, a pan-specific β antibody that recognizes an amino-terminal epitope of β_4 did not detect any β protein of the expected size from lethargic brain (McEnery *et al.*, 1998).

Significantly, the concentration of total β subunits associated with α_{1A} and α_{1B} is largely unaffected by loss of β_4 in the lethargic mouse. As shown in Table 1, the concentration of α_{1B} bound to β subunits was not different in lethargic and wild-type brain, whether measured by coimmunoprecipitation with the β_{com} antibody or by summation of the amount immunoprecipitated by each β subunit antibody (β_{sum}). This compares favorably to the concentration of α_{1B} measured directly in a soluble channel binding assay and to the α_{1B} immunoprecipitated with monoclonal antibody CC18 (Scott *et al.*, 1996), which recognizes the II–III cytoplasmic loop of this subunit. There is a slight, but statistically significant, decrease in *lh/lh* brain in the concentration of β subunits bound to α_{1A} and in the concentration of α_{1A} as measured by direct SNX-230 binding (Table 1). Since the α_{1A} subunit is most highly expressed in cerebellum (Tanaka *et al.*, 1995), and lethargic cerebellum is reduced in size relative to wild type (Dung and Swigart, 1971; D. L. Burgess, M. E. Diaz, and J. L. Noebels, unpublished observations), this could cause a reduction of α_{1A} in lethargic brain that is sufficient to explain the data. The overall congruence of these experimental values suggests that, for both wild-type and lethargic mouse, the α_{1A} and α_{1B} proteins retain their β subunit associations during extraction and immunoprecipitation. More importantly, these results indicate that loss of β_4 does not result in formation of CTX-reactive α_{1A}- and α_{1B}-containing channels that lack a β subunit.

Changes in β_{1-3} Subunit mRNA Expression Do Not Account for Altered α_1-β Interactions

Changes in lethargic brain of the steady-state levels of α_1 subunits associated with β_1, β_2, or β_3 could be explained by altered transcription of the β_{1-3} genes.

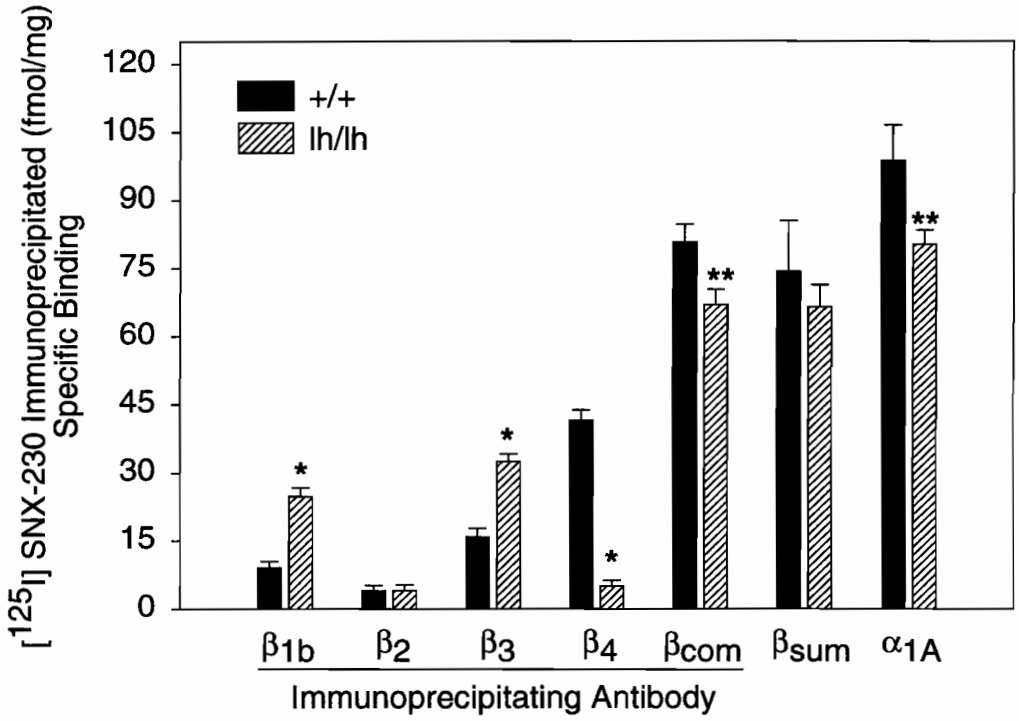

FIG. 1. Change in relative association of Ca^{2+} channel β subunits with α_{1A} in lethargic mouse brain. Ca^{2+} channels were solubilized from wild-type (+/+) or lethargic (*lh/lh*) mouse brain and immunoprecipitated. Shown is the concentration of α_{1A} that coimmunoprecipitated with each β subunit antibody (β_{1b}, β_2, β_3, β_4, β_{com}) and the total amount of α_{1A} present in the assay (α_{1A}), determined by direct binding using the centrifugal gel filtration binding assay as described under Experimental Methods. These data are the mean ± SEM of replicates from four independent experiments conducted with a single preparation of KCl-washed microsomes prepared from a pool of 20 brains each of *lh/lh* and wild-type mice. Similar results as those shown in Figs. 1 and 2 were obtained from a separate preparation of KCl-washed membranes prepared from a pool of 15 *lh/lh* or wild-type brains (data not shown). Also shown is the sum of α_{1A} coimmunoprecipitated by β_{1b}, β_2, β_3, and β_4 antisera (β_{sum}, mean of each experiment ± SEM). In lethargic brain, where β_4 protein is absent, there is a significant increase in the proportion of α_{1A} that associates with β_{1b} and with β_3. Unpaired Student's *t* test (*$P < 0.001$; **$P < 0.03$).

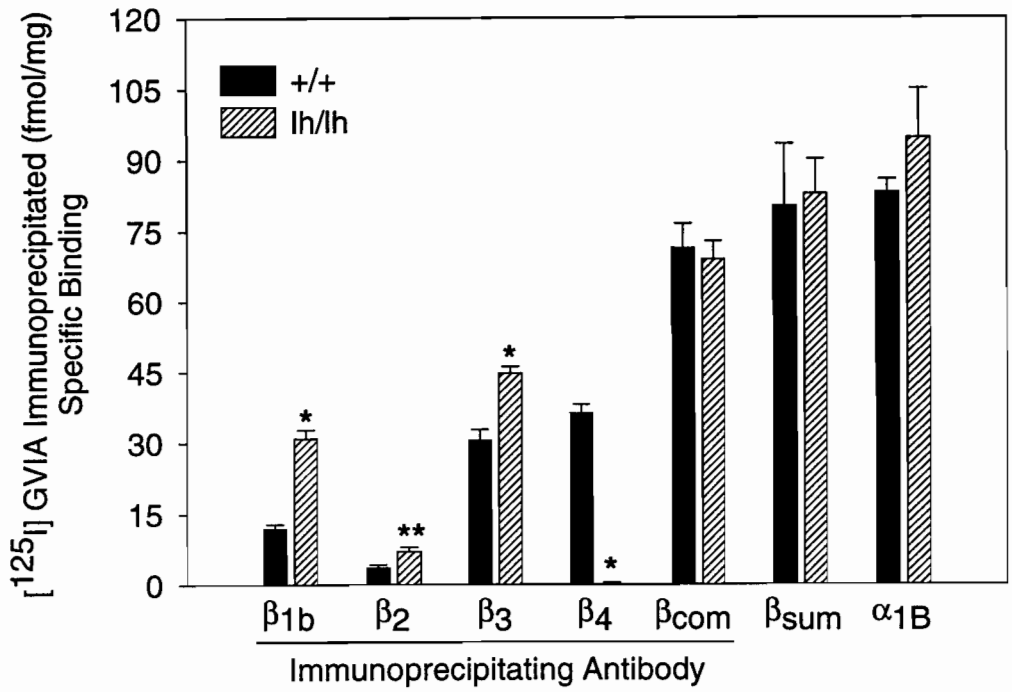

FIG. 2. Change in relative association of Ca^{2+} channel β subunits with α_{1B} in lethargic mouse brain. Samples were solubilized, immunoprecipitated, and analyzed as described in the legend of Fig. 1. Data are the mean ± SEM of replicates from three experiments. In lethargic mouse there is a significant increase in the proportion of α_{1B} that associates with β_{1b}, β_2, and β_3. Unpaired Student's *t* test (*$P < 0.001$; **$P = 0.002$).

Since analysis of whole brain mRNA by Northern blot or ribonuclease protection could conceal regionally limited, but functionally significant, changes in β subunit expression, we carried out *in situ* hybridization (ISH) of matched lethargic and wild-type serial brain sections with probes specific to each of the β subunit transcripts. The probes were of identical length and similar A + T:C + G ratios (see Experimental Methods).

TABLE 1

Comparison of the Concentrations of β Subunits and of α_{1A} and α_{1B} in Lethargic and Wild-Type Mouse Brain

		α_{1B} Concentration (fmol/mg)			α_{1A} Concentration (fmol/mg)		
Assay		lh/lh	WT	*P*	lh/lh	WT	*P*
β IP	β_{com}	69 ± 4†	71 ± 5	NS	67 ± 3*	81 ± 4**	0.013
β IP	β_{sum}	83 ± 7	80 ± 13	NS	67 ± 5	74 ± 11	NS
α_1 IP	CC18	63 ± 4†	68 ± 6††	NS			
α_1 DB		95 ± 11†	83 ± 3††	NS	80 ± 3*	99 ± 8**	0.027

Note. Mean ± SEM of three (α_{1B}) or four (α_{1A}) experiments pooled. IP, Immunoprecipitation; DB, direct binding assay. β_{com}, β_{sum}, and direct binding are described in the legend to Fig. 1. CC18, mAB to α_{1B} subunit; NS, difference in the concentration of subunits in *lh/lh* and WT is not significant by unpaired Student's *t* test. †$P < 0.03$; ††$P = 0.029$; *$P = 0.048$; **$P = 0.029$.

No differences between wild-type and *lh/lh* brain were observed in the levels or localization of β_1, β_2, or β_3 mRNA (Fig. 3, Table 2). In lethargic brain, ectopic expression of β_{1-3} subunit mRNA was not observed, making it unlikely that entirely novel Ca^{2+} channel subunit pairings arise in neurons as a result of β_4 subunit loss. The levels of β_4 mRNA in *lh/lh* brain are reduced compared to wild-type levels, consistent with the previously described Northern blot analysis, and may reflect a decrease in either β_4 transcription or mRNA stability due to the lethargic mutation. However, this mRNA reduction does not simply represent a proportionate decrease in the number of β_4 subunits that compete with β_1, β_2, or β_3 for α_1 subunit binding, since none of the mutant β_4 mRNA detected in lethargic brain encodes the essential α_1-β interaction domain (Burgess *et al.*, 1997). These data suggest that significant changes in transcriptional regulation of the β_{1-3} subunit genes are not required for the striking alterations in Ca^{2+} channel subunit composition detected by coimmunoprecipitation. Instead, the increases in lethargic brain in the β_{1b}, β_2, and β_3 protein bound to α_{1A} and α_{1B} (Figs. 1 and 2) are most likely accounted for by posttranscriptional mechanisms.

Coimmunoprecipitation studies in whole brain lack the spatial resolution necessary to determine which

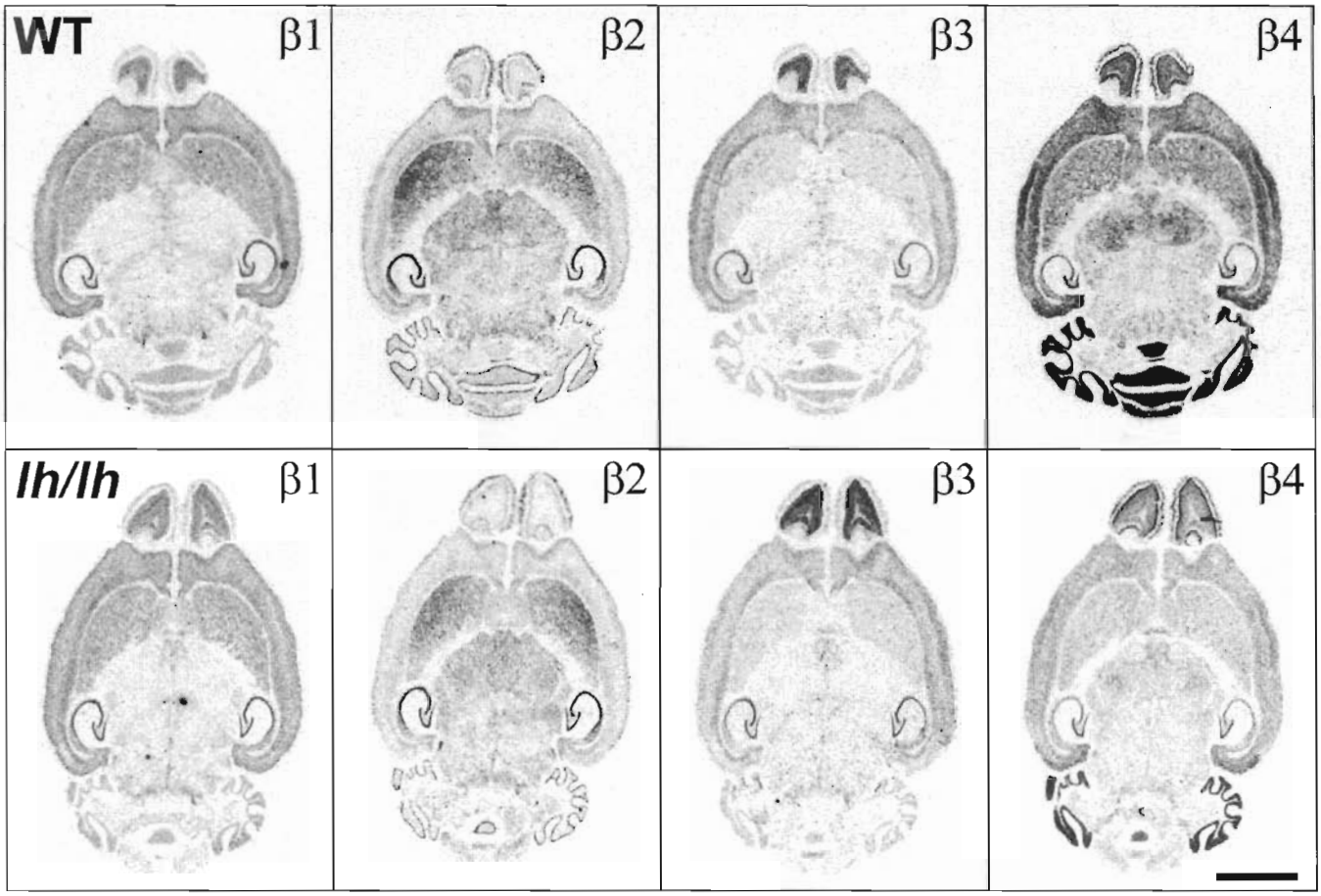

FIG. 3. β_1, β_2, and β_3 subunit mRNA expression is similar in wild-type and lethargic brain. *In situ* hybridization of serial horizontal brain sections from adult wild-type (WT) and lethargic (*lh/lh*) littermates was carried out with probes specific to the Ca^{2+} channel β_1, β_2, β_3, and β_4 subunit genes. Gene expression was quantified by densitometric analysis of the autoradiographs (see Table 2). No differences were detected between WT and *lh/lh* brain in the abundance or localization of the β_1, β_2, and β_3 mRNA. Apparent differences in cerebellar morphology are due to the decreased size of *lh/lh* cerebellum relative to WT. The lethargic β_4 gene is mutated and is associated with lower steady state brain mRNA levels than wild-type. The relative decrease in lethargic β_4 mRNA levels is less apparent in cerebellum than in other brain regions in this image due to saturation of the autoradiographic film emulsion, but is evident with shorter exposures (not shown) and in Fig. 4. All four genes exhibit extensive coexpression. Scale bar, 3 mm.

remaining β subunits replace β_4 in distinct neuronal populations. This information, which is likely to have functional significance, may be partially inferred from the ISH analysis. Our results indicate that most regions in both wild-type and lethargic mouse brain express at least two different β subunits. In cerebellum, β_4 mRNA is abundant in both Purkinje and granule cells. Purkinje cells also express β_2 at high levels and β_1 and β_3 at very low levels, any of which might be responsible for replacing missing β_4 subunits in lethargic. In contrast, β_4 expression in granule cells is complemented by β_1 and β_3, while β_2 mRNA is not detected (Fig. 4). A densitometric comparison of autoradiographs of the four β subunits indicates that β subunit mRNA levels vary considerably among wild-type mouse brain regions: cerebellar Purkinje cells ($\beta_4 = \beta_2 \gg \beta_1 = \beta_3$), cerebellar granule cells ($\beta_4 > \beta_1 = \beta_3 \gg \beta_2$), hippocampus ($\beta_2 = \beta_1 > \beta_3 = \beta_4$), dentate gyrus ($\beta_1 = \beta_2 = \beta_4 = \beta_3$), thalamus ($\beta_4 = \beta_2 \gg \beta_1 = \beta_3$), neocortex ($\beta_1 = \beta_4 > \beta_2 = \beta_3$), caudate putamen ($\beta_2 > \beta_1 = \beta_4 > \beta_3$), olfactory glomerular layer ($\beta_2 = \beta_4 > \beta_1 = \beta_3$), olfactory mitral cell layer ($\beta_4 = \beta_3 > \beta_1 = \beta_2$), and olfactory granule cell layer ($\beta_3 = \beta_4 > \beta_1$). We did not identify any mouse brain regions that expressed β_4 exclusively. These data are concordant with the previously described expression of Ca^{2+} channel β subunits in rat brain (Tanaka *et al.*, 1995; Ludwig *et al.*, 1997). It is clear from these results that loss of a single β subunit from whole brain would likely trigger different compensatory patterns in individual cell types, and that subsequent Ca^{2+} channel behavior in

TABLE 2
Comparison of β Subunit mRNA Expression Levels in Lethargic Relative to Wild-Type Mouse Brain

Region	β_1	β_2	β_3	β_4
Cerebellum				
+/+	97.54 ± 3.45	74.48 ± 2.24	61.05 ± 3.01	207.17 ± 3.25
lh/lh	98.24 ± 5.03	75.05 ± 2.90	62.42 ± 2.99	173.15 ± 4.29
	+0.7%, $P = 0.91$	+0.8%, $P = 0.88$	+2.2%, $P = 0.75$	−16.4%, $P < 0.001$
Neocortex				
+/+	131.31 ± 9.46	48.60 ± 3.18	81.49 ± 3.34	145.98 ± 3.09
lh/lh	119.27 ± 7.55	48.62 ± 2.51	79.05 ± 4.17	82.04 ± 3.58
	−9.2%, $P = 0.37$	0.0%, $P = 0.99$	−3.0%, $P = 0.68$	−43.8%, $P < 0.001$
Thalamus				
+/+	26.43 ± 2.80	85.54 ± 2.70	17.19 ± 1.57	95.95 ± 7.97
lh/lh	26.06 ± 3.85	77.48 ± 1.29	18.38 ± 1.62	38.95 ± 1.73
	−1.4%, $P = 0.95$	−9.4%, $P = 0.08$	+6.9%, $P = 0.67$	−59.4%, $P < 0.01$
Caudate				
+/+	94.21 ± 2.33	111.58 ± 3.74	37.50 ± 1.23	112.95 ± 0.92
lh/lh	87.53 ± 1.32	100.33 ± 2.32	38.50 ± 1.24	48.56 ± 2.08
	−7.1%, $P = 0.09$	−10.1%, $P = 0.08$	+2.7%, $P = 0.64$	−57.0%, $P < 0.001$
Hippocampus				
+/+	140.15 ± 2.55	154.61 ± 11.55	115.16 ± 2.95	131.44 ± 5.97
lh/lh	142.15 ± 1.55	158.19 ± 5.460	117.01 ± 2.62	102.52 ± 0.42
	+1.4%, $P = 0.59$	+2.3%, $P = 0.82$	+1.6%, $P = 0.70$	−22.0%, $P < 0.05$

Note. Autoradiographic data from *in situ* hybridization experiments were quantified by densitometry. Shown are mean grayscale values ± SEM for autoradiographs of each β subunit probe hybridized to lethargic or wild-type brain, followed by percentage change in the lethargic mean value relative to wild-type and the *P*-value of the difference (unpaired Student's *t* test). β_4 mRNA was significantly decreased ($P < 0.05$) in lethargic in all regions examined. β_{1-3} mRNA levels were not significantly different from wild-type ($P > 0.05$).

these cells would depend on the particular regulatory properties of the substituting β.

Localization of α_{1A} and α_{1B} in Lethargic Brain Appears Normal Despite Absence of β_4

Ca^{2+} channel α_1 subunits expressed without β subunits are not correctly immunolocalized in the plasma membrane, but exhibit perinuclear localization and decreased immunoreactivity (Nishimura *et al.*, 1993; Chien *et al.*, 1995; Gregg *et al.*, 1996; Brice *et al.*, 1997). The association of β_4 with large proportions of α_{1A} and α_{1B} in brain suggests that clear abnormalities in localization or protein levels could be apparent upon loss of β_4 from lethargic brain if it is not replaced by other β subunits. We therefore examined the distributions of α_{1A} and α_{1B} proteins in lethargic brain by immunohistochemistry to determine if they were altered by loss of β_4. Affinity-purified rabbit polyclonal anti-α_{1A} and anti-α_{1B} antibodies were used to compare α_1 subunit expression in wild-type and lethargic brain. These antibodies recognize the peptides CNA1 and CNB1, respectively, which correspond to the intracellular region between domains II and III of the α_{1A} and α_{1B} subunits (Westenbroek *et al.*, 1992, 1995).

In wild-type mouse brain, α_{1A} immunoreactivity was most abundant in cerebellar cortex, where it localized to Purkinje, granular, and molecular layers (Fig. 5). The cell bodies and proximal dendrites of Purkinje cells exhibited dense staining, while much less staining was observed in distal dendrites. Relatively intense, punctate staining of basket cell synapses onto the soma, axon hillock, and initial segment of Purkinje cells was occasionally seen at higher magnification (data not shown). In the molecular layer, moderate α_{1A} immunoreactivity was evenly distributed in a fine-grained pattern that may represent synapses of climbing or parallel fibers onto Purkinje cell dendritic arbors. The granular layer was moderately stained throughout, at levels comparable to the molecular layer, in a pattern consistent with α_{1A} localization within granule cells and related processes. Examination of α_{1A} immunoreactivity in lethargic cerebellum revealed no differences in localization compared to wild-type cerebellum. Further examination of α_{1A} immunoreactivity revealed no differences in staining patterns between wild-type and lethargic brain in neocortex, hippocampus, dentate gyrus, and other subcortical regions (data not shown). Although we observed a decrease in α_{1A} protein in lethargic brain by immunoprecipitation and direct SNX-230 binding (Table

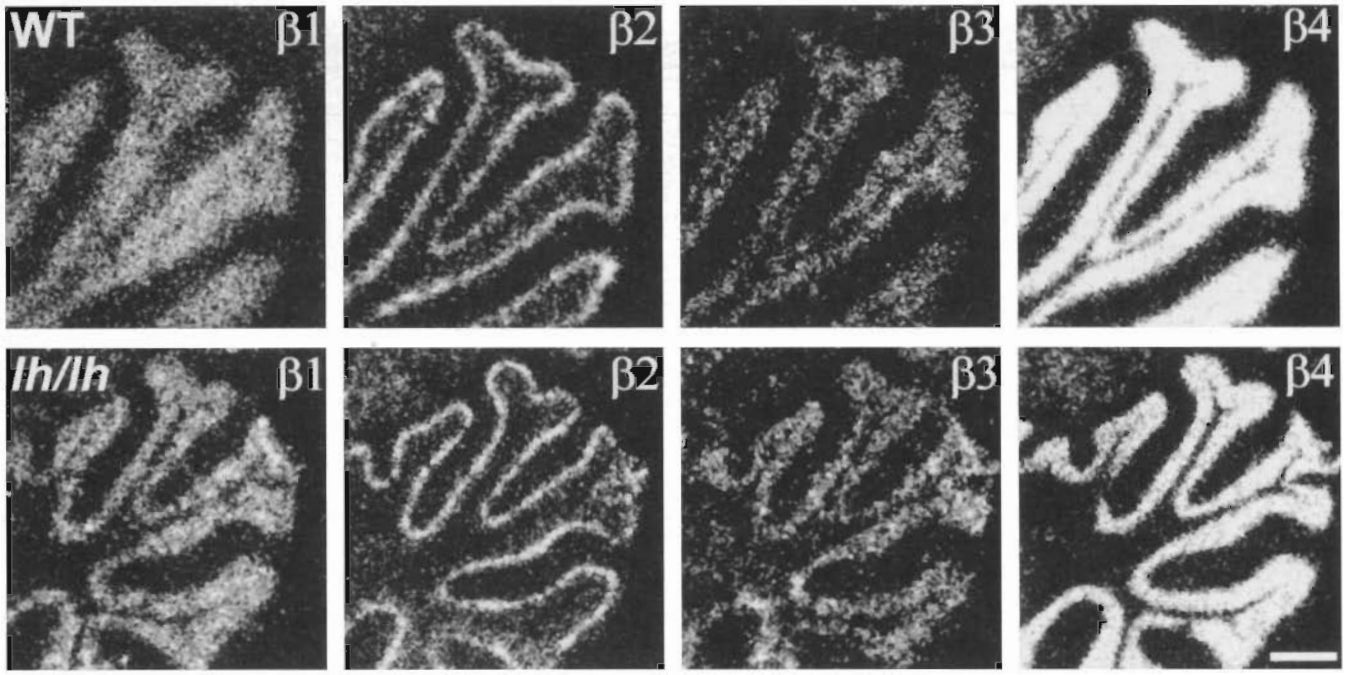

FIG. 4. Multiple β subunits are transcribed in cerebellar Purkinje and granule cells. *In situ* hybridization of serial horizontal cerebellar sections from wild-type (WT) and lethargic (*lh/lh*) adult littermates with β_1, β_2, β_3, and β_4-specific probes. Purkinje cells contain abundant β_4 and β_2 mRNA, but low levels of β_1 and β_3. Granule cells contain high levels of β_4, lower levels of β_1 and β_3, and nearly undetectable β_2 mRNA. No differences in β_{1-3} subunit expression were identified between WT and *lh/lh* Purkinje cells. Scale bar, 0.5 mm.

1), this decrease was not evident by immunohistochemistry. This might be explained in part by observations that the size and weight of lethargic cerebellum relative to forebrain is decreased compared to wild-type mice (D. L. Burgess and J. N. Noebels, unpublished). Although unproven, the decrease in α_{1A} protein measured by immunoprecipitation could therefore be accounted for by fewer neurons or synapses in the cerebellum, a region where α_{1A} is highly expressed.

Immunoreactivity to the α_{1B} antibody was also most intense in cerebellum, but in a pattern very distinct from α_{1A} (Fig. 5). In wild-type mice, no α_{1B} staining was detected in the Purkinje cell soma or dendrites, while intense punctate immunoreactivity was observed in the cerebellar glomeruli, which are large (~10–20 μm) synaptic complexes of mossy fiber terminals, Golgi cell terminals, and granule cell dendrites within the granular layer. This pattern is in contrast with that described by Westenbroek *et al.* (1992), who noted strong anti-CNB1 staining in rat Purkinje cell soma and dendrites and much weaker staining in the granular layer. The disparity may reflect differences in antibody specificity, in epitope sequence divergence between rat and mouse α_{1B} amino acid sequences (which differ at 2 of 17 residues contained within the CNB1 peptide antigen) (Westenbroek *et al.*, 1992; GenBank Accession Nos. M92905 and U04999) or in species differences in α_{1B} isoform expression due to alternative mRNA splicing. In contrast to the anti-CNB1 staining, however, our data are consistent with the absence of α_{1B} mRNA expression and N-type Ca^{2+} currents in rat Purkinje cells (Usowicz *et al.*, 1992; Bindokas *et al.*, 1993; Tanaka *et al.*, 1995) and with the near absence of N-type currents in mouse Purkinje cells (see below). The punctate α_{1B} staining observed in the molecular layer is likely to mark the terminals of climbing or parallel fibers onto Purkinje cell dendrites. As with α_{1A}, the pattern of α_{1B} immunostaining that we observed in lethargic was indistinguishable from that of wild-type in the cerebellum and other brain regions (data not shown).

Robust Whole-Cell P-Type Currents in Dissociated Lethargic Purkinje Neurons

The predominant Ca^{2+} current in cerebellar Purkinje cells is ω-Aga IVA-sensitive P-type current (Mintz *et al.*, 1992). P-type channels are composed of α_{1A}, $\alpha_2\delta$, and one of the four β subunits (Liu *et al.*, 1996; Gillard *et al.*, 1997). The ISH analysis presented here, and previous studies in rat and human, demonstrate that the β_4 subunit is abundant in Purkinje neurons (Tanaka *et al.*, 1995; Ludwig *et al.*, 1997; Volsen *et al.*, 1997), indicating

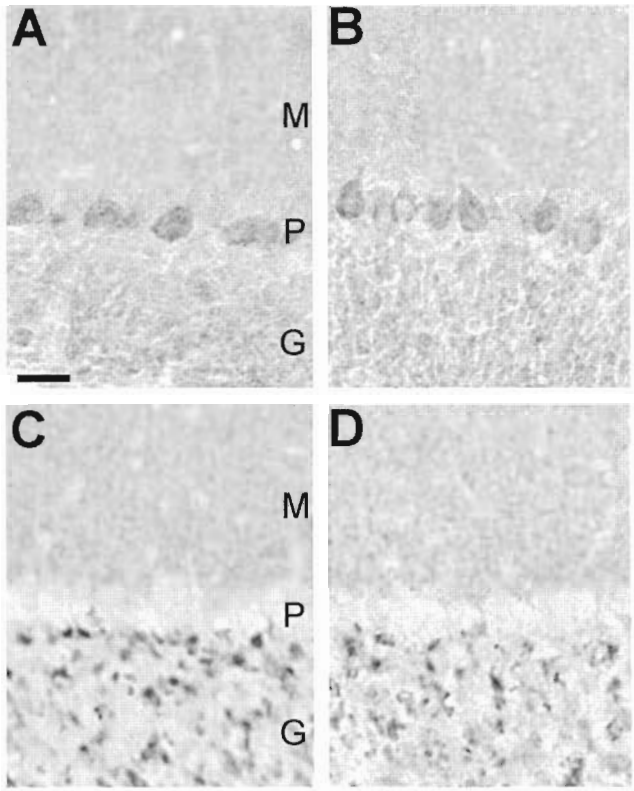

FIG. 5. Immunohistochemical localization of α_{1A} and α_{1B} subunits in wild-type and lethargic cerebellum. Sagittal sections from wild-type (A, C) and lethargic (B, D) adult cerebellum were stained with affinity purified antibodies to Ca^{2+} channel α_{1A} (A, B) and α_{1B} (C, D) subunits. α_{1A} immunoreactivity is most abundant in Purkinje cell soma and proximal dendritic segments and is present at lower levels in the granular and molecular layers. Intense punctate α_{1B} staining is present in glomeruli in the granular layer, with moderate immunoreactivity in the molecular layer. Purkinje cells did not exhibit α_{1B} staining. No differences between wild-type and *lh/lh* were observed in the abundance or localization of either α_1 subunit. M, molecular layer; P, Purkinje cell layer; G, granule cell layer. Scale bar, 20 μm.

that these cells are good candidates for evaluating the effects of β_4 loss on P-type channel function. In addition, tottering (*tg*) and leaner (tg^{la}) mice, which have mutations in the α_{1A} subunit that result in phenotypes similar to lethargic (Fletcher *et al.*, 1996; Doyle *et al.*, 1997), show defects in whole-cell P-type currents in Purkinje neurons (Dove *et al.*, 1998; Lorenzon *et al.*, 1998; Wakamori *et al.*, 1998).

Ba^{2+} currents through voltage-dependent Ca^{2+} channels were recorded from acutely dissociated cerebellar Purkinje cells from 14- to 19-day-old lethargic (*lh/lh*) and wild-type (WT) mice using the whole-cell patch clamp technique. Ataxia is observable in *lh/lh* mice by 14 days of age. We examined the voltage dependence of activation, the voltage dependence of inactivation, and the current amplitude, but found no significant difference between *lh/lh* and WT mice in these respects. The voltage dependence of activation, shown for representative cells in Fig. 6, was measured by depolarizations from a holding potential of −80 mV, with tail currents measured at −60 mV. Measurable currents were first detected from both *lh/lh* and WT cells at depolarizations of approximately −45 to −50 mV, while peak tail currents (best measured at 12°C) occurred at depolarizations of approximately 0 mV. The voltage dependence of activation could be fit well by a Boltzmann function, $1/(1 + \exp(-(V - V_h)/k))$, where V is the test potential, V_h is the midpoint in mV, and k is the slope factor in mV. The midpoint and slope factor were indistinguishable in neurons from WT mice ($V_h = -18 \pm 4$ mV, $k = 5.4 \pm 0.5$ mV, mean ± standard deviation, $n = 8$ cells) and *lh/lh* mice ($V_h = -17 \pm 5$ mV, $k = 5.4 \pm 0.1$, $n = 10$). Although the magnitude of current varied considerably from cell to cell, we found no systematic difference in peak tail current amplitude between WT and *lh/lh* mice (3.3 ± 1.5 nA ($n = 8$) and 4.7 ± 3.3 nA ($n = 10$), respectively). The voltage dependence of inactivation, shown for representative cells in Fig. 7, was also similar in *lh/lh* and WT cells. With 4-s conditioning pulses to different holding potentials, the voltage of half-maximal (V_h) inactivation was −25 ± 8 mV for WT ($n = 4$) and −26 ± 6 mV for *lh/lh* ($n = 4$), while the inactivation slope (k) was 4.3 ± 1.2 mV for WT ($n = 4$) and 4.3 ± 1.4 mV for *lh/lh* ($n = 4$). These results indicate that despite loss of β_4, and altered proportions of β_{1b} and β_3 associated with α_{1A}-containing channels in lethargic whole brain, whole-cell P-type current is present at normal amplitude in lethargic cerebellar Purkinje neurons.

In cerebellar Purkinje neurons from rats, ~95% of the voltage-dependent Ca^{2+} channel current is carried by P-type channels sensitive to block by ω-Aga IVA (Mintz *et al.*, 1992). We examined the contribution of such channels to overall Ca^{2+} channel current in Purkinje cells from both wild-type and lethargic mice by testing the sensitivity of the current to saturating concentrations (200 or 500 nM) of ω-Aga IVA. Cells were depolarized to −10 mV from a holding potential of −80 mV, with tail currents measured at −60 mV (Fig. 8). The percentage of Ba^{2+} current remaining after exposure to ω-Aga IVA was 5 ± 2% for WT ($n = 3$) and 6 ± 3% for *lh/lh* ($n = 4$). Thus, as in rat neurons, P-type channels underlie ~95% of the calcium channel current in Purkinje cells of normal mice, and there was no detectable difference in lethargic mice.

DISCUSSION

In this study we used coimmunoprecipitation to demonstrate that loss of β_4 from lethargic mouse brain

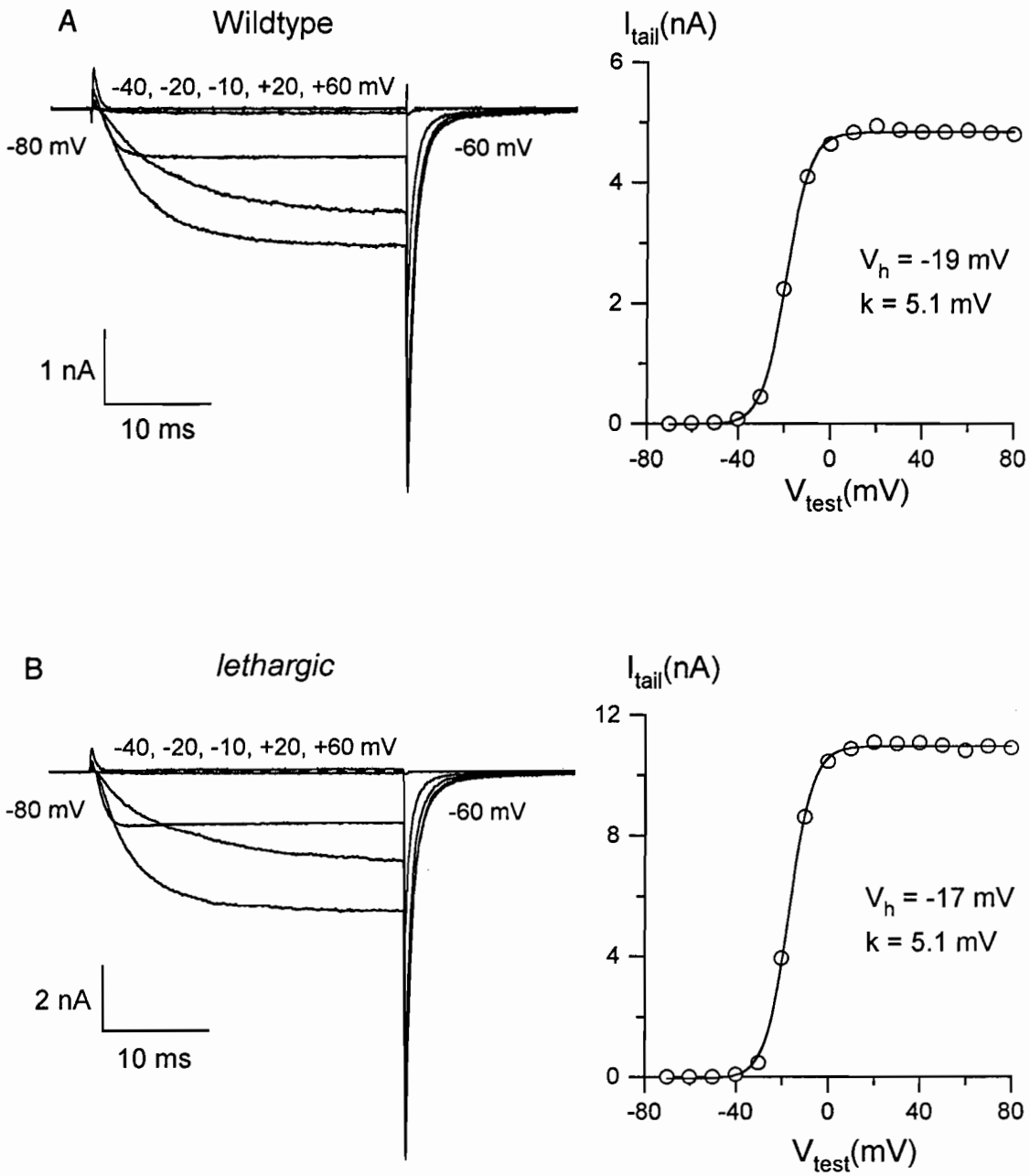

FIG. 6. Ba^{2+} currents recorded from representative wild-type and lethargic Purkinje neurons. Whole-cell Ba^{2+} currents recorded from acutely dissociated (A) wild-type and (B) lethargic Purkinje neurons were not significantly different. Traces at left are currents in response to 30-ms test depolarizations to −40, −20, −10, +20, and +60 mV, with tail currents measured at −60 mV. The dashed line represents the zero current level. Graphs at right show peak inward tail currents evoked by test depolarizations from −70 to +80 mV. Tail current activation curves are from the same trial from which the raw traces are shown. Solid lines are Boltzmann functions of the form $I_{tail} = I_{max}/(1 + \exp(-(V_{test} - V_h)/k))$, where I_{max} is peak current, V_{test} is the test voltage, V_h is the half-maximal activation voltage, and k is the slope factor.

results in increased steady-state association of the β_{1b}, β_2, and β_3 subunits with α_{1A} and α_{1B}, with minimal changes in the abundance of either α_1 subunit. Furthermore, since the proportion of total α_1 protein that was coimmunoprecipitated by β subunit antibodies from lethargic mouse brain was not less than the proportion coimmunoprecipitated from wild-type brain, it is clear that all α_{1A}- and α_{1B}-containing channels in lethargic brain are associated with β subunits. We found that loss of β_4 resulted in no significant changes in α_{1A} or α_{1B}

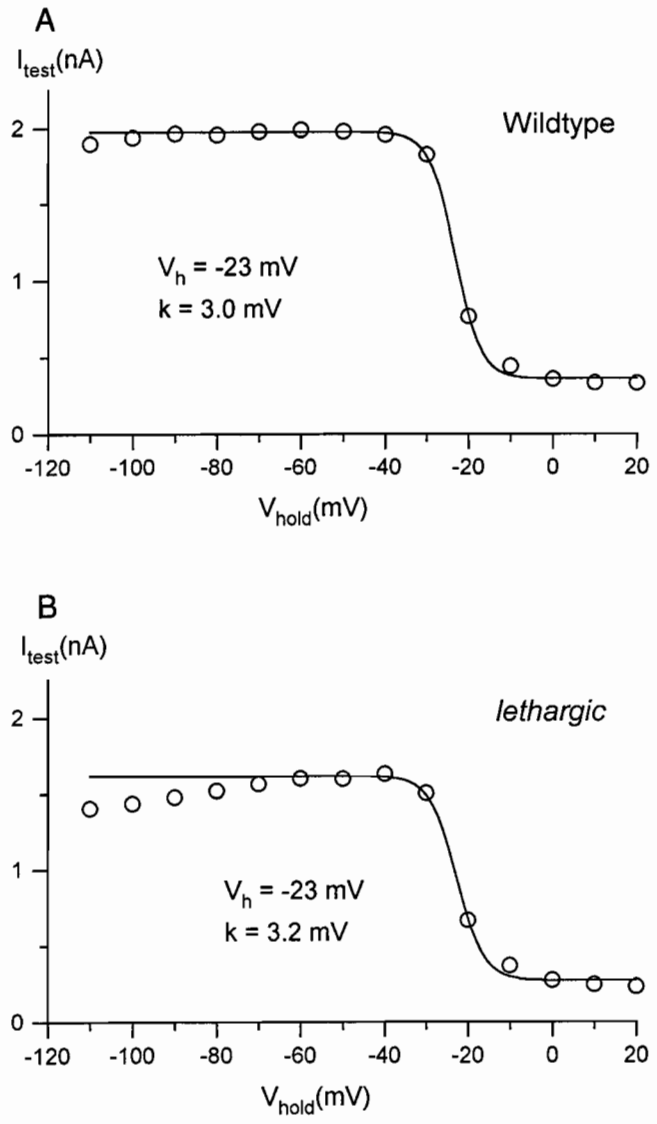

FIG. 7. Voltage-dependent inactivation of Ba^{2+} currents from representative wild-type and lethargic Purkinje neurons. The voltage-dependence of Ba^{2+} current inactivation from dissociated (A) wild-type and (B) lethargic Purkinje neurons was indistinguishable. Currents plotted are inward currents evoked by a test pulse to −10 mV after a 4-s conditioning pulse to the indicated holding voltage. Solid lines are Boltzmann functions of the form $I_{test} = I_{max} \cdot (1 - 1/(1 + \exp \cdot (-(V_{hold} - V_h))/k))) + C$, where I_{max} is peak current, V_{hold} is the holding voltage, V_h is the half-maximal inactivation voltage, k is the slope factor, and C is a constant.

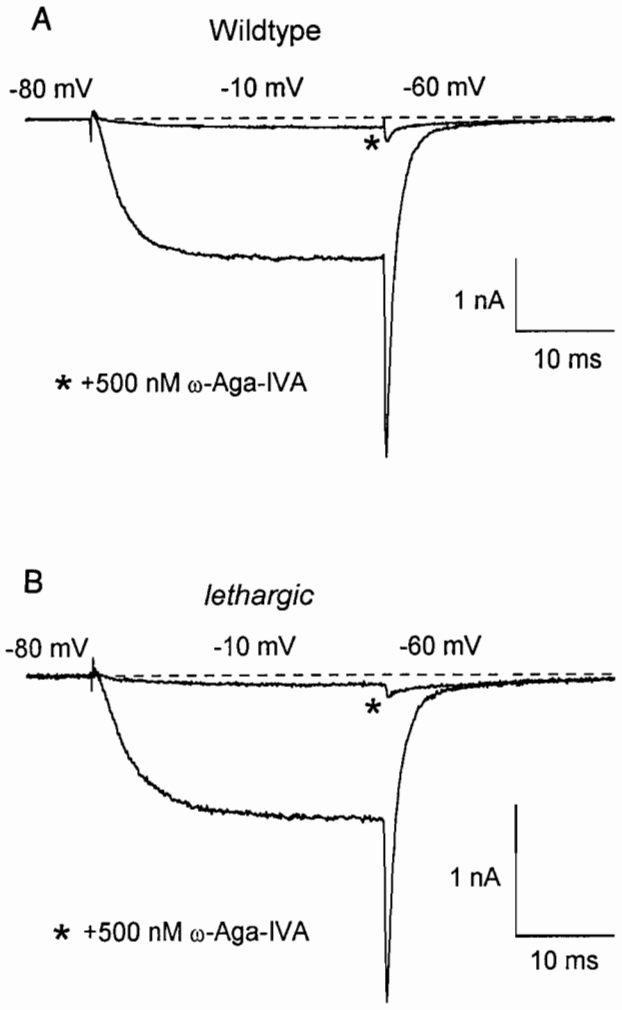

FIG. 8. ω-Aga IVA inhibition of inward Ba^{2+} currents from representative wild-type and lethargic Purkinje neurons. Ba^{2+} currents recorded from (A) wild-type and (B) lethargic dissociated Purkinje neurons were inhibited by saturating concentrations of ω-Aga IVA to a similar extent, indicating the equivalent contributions of P-type Ca^{2+} channels in both cells. Traces shown are currents in response to a step to −10 mV from a holding potential of −80 mV, followed by a tail current at −60 mV. Traces marked with an asterisk were taken after addition of 500 nM ω-Aga IVA for both wild-type and lethargic. The dashed line represents the zero current level.

protein localization at the resolution of light microscopy and that the amplitude and voltage-dependence of whole-cell P-type current in dissociated lethargic Purkinje neurons was indistinguishable from normal. These results strongly support the hypothesis that β_4 is replaced by the remaining coexpressed β subunits in lethargic brain, a compensatory process we term "subunit reshuffling." This process demonstrates that molecular plasticity of calcium channel assembly, a normal feature of early brain development (Vance *et al.*, 1998), is retained in the mature brain.

During the preparation of this manuscript, McEnery *et al.* (1998) reported altered β subunit composition of N-type Ca^{2+} channels by coimmunoprecipitation assays in forebrain and cerebellum of adult lethargic mice, as we report here for both N- and P/Q-type channels. Although both studies demonstrate β subunit reshuf-

fling among N-type channels in lethargic, the isoform ratios were disimilar. While we determined that the concentration of β_2 and β_3 associated with α_{1B} in whole brain increased significantly, the study by McEnery *et al.* did not find a difference in β_2 or β_3 associations between lethargic and wild-type mice in forebrain or cerebellum. In addition, we did not identify a significant difference between wild-type and lethargic mice in the concentration of ω-CTX GVIA-reactive α_{1B} immunoprecipitated from whole brain (Table 1) or in the concentration of α_{1B} in KCl-washed microsomes prepared from whole brain (data not shown). McEnery *et al.* reported a 50% decrease in ω-CTX GVIA binding to α_{1B} in total particulate preparations of lethargic forebrain and a 25% decrease in cerebellum. Differences in the specificity of the antibodies used in the two studies, or other methodological variations, may account for this disparity.

The Lethargic β_4 Subunit Mutation Disrupts Multiple Ca^{2+} Channel Types

The lethargic phenotype illustrates an important distinction between Ca^{2+}-channelopathies caused by mutations in α_1 subunits and those arising from β subunit mutations. Since the cytoplasmic β_4 subunit associates with more than one type of α_1 subunit in wild-type brain, loss of this subunit in the lethargic mutant could produce simultaneous changes in the subunit composition of multiple Ca^{2+} channel types. As shown here (Figs. 1 and 2), alterations in the subunit composition of both P/Q- and N-type channels occur in lethargic brain as a result of β subunit reshuffling. In contrast, mutation of individual α_1 subunit genes would be expected to result in defects restricted to a single class of pharmacologically distinct channels. For example, mutations in the α_{1A} isoform gene (the tg locus) should exclusively affect the structure of ω-Aga IVA-sensitive P/Q-type channels, while mutations in the α_{1B} subunit gene would theoretically result in specific structural alterations in ω-CTX GVIA-sensitive N-type channels.

In addition, β subunit mutations may be predicted to fall into one of two possible classes: those that prevent binding of the mutated β subunit (β_m) to α_1 subunits and allow β subunit reshuffling to occur (as in lethargic) or, conversely, those that do not disrupt α_1-β_m associations and preclude reshuffling. Examples of the second class have not been demonstrated *in vivo;* however, they are simulated *in vitro* by expression of mutant β subunits that retain α_1–β_m interactions but abnormally regulate channel currents (De Waard *et al.*, 1994; Qin *et al.*, 1996; Walker *et al.*, 1998). Either class of mutation could potentially affect multiple channel types, as discussed above. However, the effect of β subunit reshuffling on the pathogenesis of a Ca^{2+}-channelopathy introduces additional complexity since the channelopathy would consist not only of the loss of one β subunit and its unique functions, but also of the altered channel properties introduced by the substitution of other β subunits. The subunit composition of the channel complex, and hence the behavior of channels after reshuffling should depend on the proportion of different α_1 and β subunit isoforms expressed in each individual neuron, their relative binding affinities, and mechanisms governing α_1-β association.

Abnormal α_1-β Subunit Associations in Lethargic Brain Involve Posttranscriptional Mechanisms

Coimmunoprecipitation detected a steady-state elevation in α_1-associated β_{1b}, β_2, and β_3 protein in lethargic brain (Figs. 1 and 2; Table 1). It was important to determine the source of this increase since it could illuminate the mechanisms regulating β subunit reshuffling. No significant differences in β_1, β_2, or β_3 mRNA abundance or localization in brain were observed between wild-type and lethargic mice (Fig. 3; Table 2), arguing against the primary involvement of transcriptional mechanisms as a basis for the altered β subunit associations. Our results suggest that the subunits replacing β_4 are derived from a preexisting β subunit pool. Two lines of reasoning support the presence of β subunits in brain that are not tightly associated components of existing channel complexes. First, unpaired β subunits can be affinity purified from tissue homogenates extracted under very mild conditions that are not expected to interrupt α_1–β binding (Witcher *et al.*, 1995). Second, the lack of a detectable neurological phenotype in mice heterozygous for either the β_4 mutation (*lh*/+) or a targeted inactivation of the β_1 gene (Gregg *et al.*, 1996) suggests that neurons produce more β subunits than are required for normal function. It has been hypothesized that a pool of unassembled β subunits could facilitate neuronal regulation of Ca^{2+} channel properties in response to electrical activity or chemical signal transduction (Witcher *et al.*, 1995). Although no changes were evident in mRNA levels that correspond to the scale of the increases we see at the protein level (50–100%; Figs. 1 and 2), we cannot exclude the possibility that insignificant changes in steady-state mRNA levels, as measured by *in situ* hybridization, could result in substantial changes in protein levels. Enhanced stability of β subunits, or increased translation of existing mRNA, could also contribute to elevated steady-state levels of α_{1A}- and α_{1B}-associated β_{1-3} subunits in lethargic brain.

The mechanisms governing subunit reshuffling and altered α_1–β associations in adult lethargic brain are distinct from the β subunit switching of α_{1B} (N-type) channels during postnatal rat brain development described by Vance *et al.* (1998). At early postnatal stages in rat, β_4 protein is not expressed and α_{1B} channels contain only β_{1b} and β_3 subunits. However, β_4 protein expression increases 10-fold from P0 to adult, and β_4 is associated with 25% of total α_{1B} channels in adult brain. During this same period, total β_3 association with α_{1B} channels doubles despite constant β_3 protein expression. Thus, developmental *increases* in β_4 protein expression in rat brain correlate with increased β_4 and β_3 association with α_{1B} channels. This is in contrast to our observations in lethargic brain, where *loss* of β_4 is correlated with increased β_{1b} and β_3 association with α_{1B} channels.

Loss of β_4 Reveals a Limited Functional Redundancy among Ca^{2+} Channel β Subunits in Vivo

The role of the β subunit in channel assembly, stability, and membrane localization was shown by the failure of α_{1A} and $\alpha_2\delta$ subunits to localize in the plasma membrane when expressed either alone or together in COS-7 cells, and the restoration of normal α_{1A} and $\alpha_2\delta$ localization upon coexpression with any of the four β subunits (Brice *et al.*, 1997). These data agree with the demonstration that targeted inactivation of the Ca^{2+} channel β_1 gene, the only β subunit expressed at appreciable levels in mouse skeletal muscle, also resulted in a loss of α_{1S} immunoreactivity from the skeletal muscle membrane and the absence of excitation–contraction coupling (Gregg *et al.*, 1996). The importance of β subunits in regulating the biophysical properties of Ca^{2+} currents was established by expression in oocytes or cultured cells of α_1 subunits without β subunits, which leads to decreased current amplitudes, and altered voltage-dependences of current activation and inactivation (Berrow *et al.*, 1995; De Waard and Campbell, 1995; De Waard *et al.*, 1996). Based on these observations, loss of β_4 from lethargic mouse brain would be expected to affect channel properties in similar ways, unless β subunit reshuffling allowed $\beta_{1\text{-}3}$ to physically replace β_4 and provide at least partial functional compensation. In support of the latter, we did not observe differences in lethargic brain in α_{1A} or α_{1B} abundance or localization, and robust P-type currents in lethargic cerebellar Purkinje cells (measured at 12°C to optimally resolve tail currents) behaved identically to those in wild-type cells. The same mechanism may also explain the surprising absence of abnormal clinical phenotype resulting from targeted inactivation of the abundantly expressed mouse β_3 subunit gene (Namkung *et al.*, 1998). Together, these findings indicate a partial functional redundancy among the Ca^{2+} channel β subunits that is consistent with their common evolutionary origin.

The study of Ca^{2+} channel regulation in heterologous expression systems provides valuable insights into isoform-dependent β subunit effects on individual α_1 subunits. For example, coexpression of α_{1A} and $\alpha_2\delta$ with each β subunit in Xenopus oocytes differentially modulated the biophysical properties of Ba^{2+} currents (De Waard and Campbell, 1995). However, β_{1b} and β_4 showed very similar effects on the voltage-dependence of activation of α_{1A}, causing 15 and 17 mV hyperpolarizing shifts, respectively. Likewise, β_{1b} and β_4 had similar effects on the rate of inactivation of α_{1A}, converting the biphasic rate to a single monophasic rate of $\tau = 214$ and 243 ms, respectively. β_{1b} and β_4 also increased α_{1A} current amplitude similarly (19- and 18-fold, respectively). In contrast, β_2 shifted the voltage-dependence of activation by only 7 mV, produced an inactivation of $\tau =$ 533 ms, and increased current amplitude only fivefold. The effects of the β_3 subunit were also notably dissimilar to those of β_{1b} and β_4. Based on these results, and the indistinguishable P-type currents recorded from lethargic and wild-type mice cerebellar Purkinje neurons in this study, we postulate that β_4 is predominantly replaced by β_{1b} in these cells. Although β_2 mRNA is detected in Purkinje cells by ISH at higher levels than β_1 and β_3 mRNA (Fig. 3; Table 2; Tanaka *et al.*, 1995; Volsen *et al.*, 1997), steady-state mRNA levels are not a reliable indicator of steady-state protein levels. Direct evidence of which particular β subunit(s) are responsible for the maintainence of P-type currents in lethargic cerebellar Purkinje neurons would require the individual depletion of the remaining subunits, β_1, β_2, and β_3, perhaps using antisense oligonucleotide injections. Alternatively, alleles of these subunits that have been inactivated by homologous recombination (Gregg *et al.*, 1996; Namkung *et al.*, 1998) could be introduced into the lethargic genome by breeding.

Subunit Reshuffling and the Lethargic Neurological Phenotype

Mutation of the β_4 subunit gene clearly alters the subunit composition of both N- and P-type Ca^{2+} channels (and possibly other subtypes) and it will be important to determine the functional contribution of each to the neurological disorder in lethargic mice. The pheno-

types of the mouse mutants tottering (*tg*) and leaner (*tg^{la}*) are nearly indistinguishable from that of lethargic, and are caused by mutations in the α_{1A} subunit gene which encodes the P-type Ca^{2+} channel (Fletcher *et al.*, 1996; Doyle *et al.*, 1997). Recent studies have revealed reduced P-type currents (~40 and ~60% decreased, respectively) in acutely dissociated Purkinje cells from *tg* and *tg^{la}* mice (Dove *et al.*, 1998; Lorenzon *et al.*, 1998; Wakamori *et al.*, 1998). Thus, it would be surprising if abnormal P-type currents were not also the primary electrophysical defect underlying the lethargic neurological phenotype. Despite the demonstration of normal amplitude whole-cell P-type currents in 14- to 19-day-old lethargic Purkinje cells under optimized recording conditions in this study (functional evidence of β subunit reshuffling in these cells), further investigation will be required to determine whether the underlying channels behave normally at all stages of development, under more physiologically relevant temperatures, or in response to modulation by second messengers. Another possibility is that Purkinje cells, which express all four β subunit isoforms, exhibit intact P-type currents, whereas other cells, with fewer β isoforms available for reshuffling, may not.

The presence of a complex neurological disorder in lethargic brain clearly indicates that β subunit reshuffling is insufficient to entirely replace β_4 function in critical brain pathways. Although β_4 has not yet been demonstrated to mediate any Ca^{2+} channel function that is categorically distinct from that of the other β subunits, a second α_1–β interaction site, specific to the β_4 subunit, was recently identified in the carboxyl-terminal region of the α_{1A} subunit (Walker *et al.*, 1998). It is tempting to speculate that this α_{1A}–β_4 specific interaction mediates a unique regulatory function of α_{1A}–β_4-containing channels that cannot be replaced by paralogous α_{1A}–β_{1-3} subunit interactions and could explain the similarities between the tottering and lethargic phenotypes. The remaining differences between the two phenotypes, particularly the earlier onset of ataxia in lethargic, could potentially be explained by non-P-type defects present only in this mutant.

EXPERIMENTAL METHODS

Solubilization of Ca^{2+} channels from brain microsomes. Microsomes were prepared as described (Witcher *et al.*, 1994) with modification. Brains were dissected from wild-type or *lh/lh* mice (aged 2–6 months) and immediately frozen in liquid nitrogen. For all experiments shown here, a single pool of 20 frozen *lh/lh* or wild-type brains were thawed on ice with 10 vol of cold buffer (20 mM Na pyrophosphate plus 20 mM Na phosphate monobasic, pH 7.1, 0.3 M sucrose, 1 mM EDTA, 1 mM EGTA, and 5 protease inhibitors (0.75 mM benzamidine, 5 μg/ml leupeptin and aprotinin, 1 μg/ml pepstatin A, and 0.1 mM PMSF)). Brains were homogenized by polytron and five strokes of a mechanical dounce then centrifuged (20 min at 35,000 *g*) at 4°C. The supernatant was discarded and the pellet resuspended in the same volume of KCl wash buffer (50 mM Tris–Cl, pH 7.4, 0.3 M sucrose, 0.6 M KCl, 1 mM EDTA, 1 mM EGTA, and five protease inhibitors). After stirring for 20 min at 4°C, the suspension was filtered through cheesecloth to remove viscous DNA and centrifuged (60 min at 186,000*g*) at 4°C. The supernatant was discarded; the pellet was rewashed in KCl wash buffer and centrifuged at 73,000*g* for 35 min at 4°C. The pellet was resuspended in 20 mM Tris-maleate, pH 7.0, 0.3 M sucrose, 1 mM EDTA, 1 mM EGTA, and five protease inhibitors, flash frozen in liquid nitrogen, and stored at −80°C. The protein concentration was determined by the BCA assay (Pierce, Rockford, IL) using BSA as the standard. Membrane proteins were extracted by incubating 4.5 mg/ml microsomes in assay buffer (20 mM Tris-maleate, pH 7.0, 0.1 mM EDTA, 0.1 mM EGTA) that contained 1% purified digitonin (Striessnig and Glossman, 1991), 1 M NaCl, and five protease inhibitors. The mixture was rotated end-over-end at 4°C for 1–1.5 h and then centrifuged (20 min at 147,000*g*). The supernatant was diluted 10-fold with assay buffer containing 1 mg/ml protease-free BSA to yield a final concentration of 0.1% digitonin and 0.1 M NaCl. The concentration of solubilized protein was measured prior to dilution.

Immunoprecipitation of Ca^{2+} channel subunits. Development of antibodies to the C-terminus of the neuronal Ca^{2+} channel β subunits, and to the α_{1B} II–III loop, has been described (Lui *et al.*, 1996; Scott *et al.*, 1996). Antibodies specific for β_{1b}, β_2, β_4, and β_3 were affinity-purified (Sharp and Campbell, 1989) from rabbit 142, 143, 145, and sheep 49 antisera, respectively. Monoclonal antibody $VD2_1$ (β_{com}), which recognizes all β subunits (Sakamoto and Campbell, 1991), was purified on Protein G Sepharose (Amersham-Pharmacia, Arlington Heights, IL). Affinity-purified anti-GST antiserum was used as a background control. For each assay tube, a saturating concentration of affinity-purified antisera was rotated end-over-end at 4°C for 2–3 h with 25 μl packed volume protein G–Sepharose in buffer that contained 0.1% digitonin (final volume 165 μl). Diluted, solubilized channel (250 μl) was added and rotation continued for an additional 4–6 h at 4°C. During the final hour, the samples were brought to room tempera-

ture for binding of conotoxin (CTX) ligands. Specific binding was defined as total binding minus nonspecific binding. For ω-CTX GVIA binding to α_{1B} channels, 10 μl of assay buffer that lacked or contained 20 μM ω-CTX GVIA (Bachem, King of Prussia, PA; total or nonspecific binding, respectively) was added prior to 10 μl of assay buffer that contained ~5 nM [^{125}I]-ω-CTX GVIA (NEN, Boston). For SNX-230 (ω-CTX MVIIC) binding to α_{1A} channels, 10 μl of assay buffer that contained 20 μM ω-CTX GVIA alone or with 20 μM SNX-230 (Neurex, Menlo Park, CA; total and nonspecific binding, respectively) was added prior to 10 μl of ~7.5 nM [^{125}I] SNX-230 (NEN). ω-CTX GVIA was added here to block the low affinity binding of SNX-230 to α_{1B} (Liu *et al.*, 1996). The final concentrations of ligands in the assay were 0.5 nM ω-CTX GVIA, 0.5 nM SNX-230, ~125 pM [^{125}I] ω-CTX GVIA, and ~200 pM [^{125}I] SNX-230. Following the 1-h binding incubation, the samples were chilled. Free and bound ligand were separated by washing the beads three (ω-CTX GVIA) or two times (SNX-230) by repeated centrifugation (3 s at 7000 rpm, benchtop microfuge), aspiration of the supernatant, and addition of 1 ml ice-cold wash buffer (assay buffer containing 75 mM NaCl and 0.1% digitonin). Bound radioligand was measured by gamma counting. For each antibody, three samples of total binding and two of nonspecific were prepared. Specifically bound radioligand precipitated by GST antibody was deducted from each sample. To determine the total amount of α_{1A} or α_{1B} available for immunoprecipitation, parallel samples without antibody and protein G beads were prepared and 100 μl was applied to columns of Sephadex G-50 fine that had been equilibrated with ice-cold wash buffer. The columns were centrifuged (1 min at 1200*g*) to separate bound from free ligand (Levinson *et al.*, 1979). To ensure that our experiments were conducted with saturating concentrations of antibody at equilibrium for antigen–antibody binding and CTX binding, we performed time courses and volume titrations. Data were analyzed and graphed using SigmaPlot for Windows (SPSS, Chicago). Data are presented as mean ± SEM. Significance was determined using Unpaired Student's *t* test.

Oligonucleotide probes. Antisense DNA probes contained 45 nucleotides (nt) corresponding to nonhomologous portions of each mouse calcium channel β subunit cDNA sequence: β_1 [5′-GCTTCTCCGAGGCTGCTATTTGGACATTGAGGTGTTTGGATTGAG-3′], GenBank W36837 (nts 212–168); β_2 [5′-CAGTAGCGATCCTTAGATTTATGCCGGCTTCGTTGTTTGATGCAC-3′], GenBank L20343 (nts 1813–1769); β_3 [5′-AAGTGATCCTGGCCTGAGATTGTTGCCCACGGGGGCGGCAGCTTA-3′], GenBank U20372 (nts 1811–1767); β_4 [5′-TAACGGGTTGCACATACAGTCAAGACAGTGGTTTGGTGGGATTCA-3′], GenBank AF061330 (nts 909–865). Probes were end-labeled using terminal deoxynucleotidyl transferase (Promega, Madison, WI) and [α-^{35}S]dATP (1250 Ci/mmol; NEN) to a specific activity of ~10^9 dpm/μg. Unincorporated nucleotides were removed using Bio-Spin 6 chromatography columns (Bio-Rad, Hercules, CA) and dithiothreitol (DTT) was added to a final concentration of 20 mM.

In situ hybridization Five month old *lh/lh* and wild-type male littermates were sacrificed by cervical dislocation and the brains removed, immediately frozen on dry ice, and embedded in Tissue-Tek O.C.T. Compound (Ted Pella, Redding, CA). Horizontal sections (12 μm) were cut using a cryostat microtome and thaw-mounted onto Superfrost/Plus glass slides (Fisher). Sections were fixed in 4% paraformaldehyde (PFA) in PBS, rinsed in PBS, and dehydrated in an ascending ethanol series. Hybridization solution contained 50% (v/v) formamide, 4× SSC, 25 mM sodium phosphate, 1 mM sodium pyrophosphate, 10% dextran sulfate (w/v), 5× Denhardt's solution, 200 μg/ml sonicated (100–600 bp) herring sperm DNA (Promega), 100 μg/ml polyadenylic acid [5′] (Sigma-Aldrich, St. Louis, MO), and 5 × 10^2 dpm of [α-^{35}S] dATP-labeled oligonucleotide probe. Control sections were hybridized in the presence of an additional 100-fold unlabeled oligonucleotide. Sections were hybridized under parafilm coverslips overnight at 42°C. After hybridization, sections were washed in 1× SSC at room temperature for 20 min, 0.3× SSC at 55°C for 40 min, and 2× SSC at room temperature for 5 min. All wash solutions contained 10 mM DTT. Sections were dehydrated in an ascending ethanol series and exposed to Kodak (Rochester, NY) BioMax MR film for 10 (β_4), 20 (β_1, β_2), or 40 (β_3) days. Exposure times were varied to facilitate comparisons between lethargic and wild-type brains rather than between β_{1-4} genes. Most notably, short exposures of β_3-probed sections indicated high mRNA abundance in medial habenula and olfactory granule cells, but much longer exposures were required to visualize β_3 mRNA in other brain regions. Autoradiographs were digitalized using SprintScan 35 (Polaroid, Cambridge, MA) and arranged using Photoshop 4.0.1 (Adobe Systems, San Jose, CA). Densitometry was performed using Scion Image, a modified version of NIH Image (Scion Corp., Frederick, MD). Individual grayscale measurements of selected brain regions (0–255) were converted to mean uncalibrated OD to identify exposure times below film saturation levels (except for measurements of β_4 in +/+ cerebellum, all measurements of mean uncalibrated OD were <0.55; uncalibrated OD = $\log_{10}$ (255/(255 − grayscale value)). Aver-

age pixel grayscale values were compared, with background (mean local grayscale values of film regions exposed to the glass slide but not to the section) subtracted independently for each section. All comparisons were between regions of similar cross-sectional area on matched brain sections from at least two pairs of +/+ and *lh/lh* male littermates. At least two sections were analyzed from each brain, for a minimum comparison of 4 +/+ and 4 *lh/lh* sections/probe ($n = 4$): thalamus, caudate, and hippocampus (4 +/+ and 4 *lh/lh* sections/probe, $n = 4$); neocortex (8 +/+ and 8 *lh/lh* sections/probe, $n = 8$); cerebellum (23 +/+ and 23 *lh/lh* sections/probe, $n = 23$). The entire anatomical region(s) was measured on each section. Average grayscale values from wild-type sections only were used to estimate the rank order of expression of β_{1-4} subunit genes for different brain regions as presented in the text.

Immunohistochemistry. Wild-type and *lh/lh* littermates ($n = 5$ in each group) were anesthetized with a solution of ketamine, xylazine, and acepromazine (0.5–1.0 ml/kg body weight, i.m. injection) and were transcardially perfused with PBS followed by 4% PFA in PBS. Brains were immediately removed and postfixed overnight before cryoprotection in 30% (w/v) sucrose in PBS at 4°C for 48 h. Brains were immobilized in O.C.T. Compound and 40 μm coronal or parasaggital sections cut using a cryostat microtome. Free-floating sections were stored in Tris-buffered saline (TBS) (0.1 M Tris–HCl, 0.15 M NaCl, pH 7.4) until processing using the avidin–biotin complex (ABC) immunoperoxidase method and the Vectastain ABC kit (Vector Laboratories, Burlingame, CA). All incubations and washes were at room temperature unless indicated. Sections were rinsed in TBS for 1 h (3 changes/20 min), incubated in TBS, 1% H_2O_2 for 1 h to block endogenous peroxidase activity, rinsed in TBS for 1 h, and incubated in blocking serum (TBS, 0.25% Triton X-100, 1% normal goat serum) for 1 h. Sections were rinsed in TBS for 1 h and incubated with affinity-purified rabbit polyclonal Ca^{2+} channel antibodies (Alamone, Israel; anti-α_{1A} (1:90 dilution) or anti-α_{1B} (1:500 dilution)) in TBS, 0.25% Triton X-100, 1% normal goat serum for 1 hr at room temperature and then for 48 h at 4°C. Sections were rinsed in TBS for 1 h, then incubated in biotinylated goat anti-rabbit IgG (1:200 dilution, 7.5 μg/ml IgG) in TBS, 1.5% normal goat serum for 1 h. Avidin DH-biotinylated horseradish peroxidase H (1:50 dilution) in TBS was added for 30 min, and the sections were then rinsed in TBS for 1 h. Sections were next incubated in 3,3′-diaminobenzidine (DAB Substrate kit, Vector Laboratories) for 2–5 min, and the reactions stopped by rinsing in TBS for 30 min. Sections were float-mounted onto Superfrost/Plus slides (Fisher), air-dried, cleared in Histo-Clear (National Diagnostics, Atlanta, GA), and coverslipped with D.P.X. mounting media (Sigma-Aldrich). Incubation of control sections, carried out by omitting primary antibody or preincubating primary antibody with a 1:1 (w/w) ratio of antibody to the antigen peptide for 5 h at room temperature, abolished staining. Slides were photographed and film negatives processed as described above.

Cell preparation. Cerebellar Purkinje neurons were isolated from the brains of 14- to 19-day-old mice. Mice were the progeny of genotyped *lh/+* mice (background strain B6EiC3H-a/A) obtained from The Jackson Laboratories (Bar Harbor, ME). Lethargic homozygotes were recognized on the basis of ataxic gait and lethargic behavior, and the genotypes were confirmed by PCR of tail or liver DNA as described in Burgess *et al.* (1997). Dissociated Purkinje neurons were prepared with modifications of the procedure of Mintz *et al.* (1992). Mice were anesthetized with methoxyflurane and decapitated. Cerebellar pieces were cut off from the brain with fine scissors and put into oxygenated ice-cold dissociation solution, consisting of 82 mM Na_2SO_4, 30 mM K_2SO_4, 5 mM $MgCl_2$, 10 mM Hepes, 10 mM glucose, and 0.001% phenol red indicator (pH 7.4, adjusted with NaOH). Tissue pieces were chopped into ~1 mm^3 pieces with a razor blade and transferred into dissociation solution with 3 mg/ml protease XXIII (Sigma-Aldrich) and incubated at 35°C for 7–8 min under a continual stream of oxygen. The tissue was then transferred to dissociation solution with added 1 mg/ml trypsin inhibitor (Sigma-Aldrich; pH 7.4, adjusted with NaOH) and cooled to room temperature under an oxygen stream. Cells were kept at room temperature for up to 8 h. Tissue pieces were transferred as needed to Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 10 mM glucose, 10 mM Hepes, pH 7.4, adjusted with NaOH). Purkinje neurons were released by gentle trituration (approximately 10 passages) through the tip of a fire-polished Pasteur pipette and then allowed to settle to the bottom of the recording chamber. Purkinje neurons were identified morphologically by their large cell bodies (15–25 μm diameter) and single dendritic stump. Purkinje neurons from lethargic mice were morphologically indistinguishable from Purkinje neurons from wild-type mice and readily formed gigaohm seals in Tyrode's solution.

Electrophysiological recordings. Barium currents through voltage-dependent Ca^{2+} channels were recorded using the whole-cell configuration of the patch-clamp technique (Hamill *et al.*, 1981). Patch pipettes were made from borosilicate glass tubing (Boralex;

Dynalab, Rochester, NY) and coated with Sylgard (Dow Corning, Auburn, MI). Pipettes had resistances of 1–2 MΩ when filled with internal solution (56 mM CsCl, 68 mM CsF, 2.2 mM $MgCl_2$, 4.5 mM EGTA, 9 mM Hepes, 4 mM MgATP, 14 mM creatine phosphate (Tris salt), 0.3 mM GTP (Tris salt), pH 7.4, adjusted with CsOH). External solution contained 2 mM $BaCl_2$, 160 mM tetraethylammonium (TEA) Cl, 10 mM Hepes, pH 7.4, adjusted with TEAOH, with 0.6 μM tetrodotoxin to block Na^+ channels and 1 mg/ml cytochrome-*c* to prevent adsorption of toxins to reservoirs or tubing. After establishment of the whole-cell recording configuration, the cell was lifted off the bottom of the dish and positioned in front of an array of 12 perfusion tubes made of 250-μm internal diameter quartz tubing connected by Teflon tubing to glass reservoirs. External solutions were exchanged in less than a second by moving the cell between continuously flowing solutions from the perfusion tubes. Currents were recorded with an Axopatch 200A amplifier (Axon Instruments, Foster City, CA), filtered with a corner frequency of 5 kHz (4-pole Bessel filter), digitized at 10 kHz using a Digidata 1200 interface and pClamp 6 software (Axon Instruments), and stored on a computer. Compensation (typically 80–95%) for series resistance was employed. Only data from cells with uncompensated series resistance and current small enough to give a maximum voltage error of less than 5 mV were analyzed. Ba^{2+} currents were corrected for leak and capacitive currents by subtracting a scaled current elicited by a 10 mV hyperpolarization from the holding potential of −80 mV. For better resolution of tail current kinetics, experiments were done at 12°C. The temperature was measured using a thermistor in the bath. Potentials reported are uncorrected for a junction potential of −2 mV between the pipette solution and the Tyrode's solution in which the offset potential was zeroed before seal formation. Synthetic ω-Aga IVA was stored as a 100 μM solution in water. Statistics are given as mean ± standard deviation.

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