Altered Expression and Assembly of N-type Calcium Channel \(\alpha_{1B}\) and \(\beta\) Subunits in Epileptic lethargic (lh/lh) Mouse*

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Voltage-dependent calcium channels (VDCC) are multisubunit complexes whose expression and targeting require the assembly of the pore-forming \(\alpha\) with auxiliary \(\beta\) and \(\alpha_{2}\beta\) subunits. The developmentally regulated expression and differential assembly of \(\beta\) isoforms with the \(\alpha_{2}\beta\) subunit form N-type VDCC suggested a unique role for the \(\beta 4\) isoform in VDCC maturation (Vance, C. L., Begg, C. M., Lee, W.-L., Haase, H., Copeland, T. D., and McEnery, M. W. (1998) J. Biol. Chem. 273, 14495–14502). The focus of this study is the expression and assembly of \(\alpha_{1B}\) and \(\beta\) isoforms in the epileptic mouse, lethargic (lh/lh), a mutant anticipated to produce a truncated \(\beta 4\) subunit (Burgess, D. L., Jones, J. M., Meisler, M. H., and Noebels, J. L. (1997) Cell 88, 385–392). In this report, we demonstrate that neither full-length nor truncated \(\beta 4\) protein is expressed in lh/lh mice. The absence of \(\beta 4\) in lh/lh mice is associated with decreased expression of N-type VDCC in forebrain and cerebellum. The most surprising characteristic of the lh/lh mouse is increased expression of \(\beta 1B\) protein. This result suggests a previously unidentified cellular mechanism wherein expression of the total pool of available \(\beta\) subunits is under tight metabolic regulation. As a consequence of increased \(\beta 1B\) expression, the \(\beta 1B\) is incorporated into \(\alpha_{1B}\) complexes relative to wild type. Thus, in striking similarity to the population of N-type VDCC present in immature rat brain, the population of N-type VDCC present in adult lh/lh mice is characterized by the absence of \(\beta 4\) with increased \(\beta 1B\) expression and assembly into N-type VDCC. It is intriguing to speculate that the increased excitability and susceptibility to seizures observed in the lh/lh mouse arises from the inappropriate expression of an immature population of N-type VDCC throughout neuronal development.

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expression and assembly of N-type VDCC in the lh/lh mouse with emphasis upon identifying possible compensatory mechanisms that occur from altered β4 expression.

EXPERIMENTAL PROCEDURES

Lethargic (B6EiC3H-a-A-lh) and wild-type mice (strain B6EiC3H) were obtained from Jackson Labs. All reagents were obtained from sources previously cited (17). Adult mice were euthanized in accordance with accepted university guidelines, and the brains were removed and immediately placed in 50 mM HEPES, pH 7.4, 1 mM EGTA plus protease inhibitor mixture (17). The tissues were homogenized with a Polytron homogenizer for 10 s and centrifuged at 18,000 rpm (48,000 g) for 15 min. The membranes were resuspended in 50 mM HEPES, pH 7.4, plus protease inhibitors at a resulting protein concentration of 50 mg/ml.

The N-type VDCC was solubilized from forebrain and cerebellar membranes from wild-type and lh/lh mice as described previously (18). For Western blot analysis, all homogenates were stored at −20 °C at concentrations of 2 mg/ml in sample buffer (5× sample buffer: 325 mM Tris, pH 7.0, glycerol (25% v/v), mercaptoethanol (25% v/v), SDS (10%)) in 100-μl aliquots. The samples were not freeze-thawed. The production of anti-peptide polyclonal antibodies to VDCC subunit epitopes has been described previously (16, 17, 19, 20). Methods for 125I-CTX binding, Scatchard analysis (21, 22), quantitative Western blot analysis using 125I-goat anti-rabbit IgG, immunoprecipitation of N-type VDCC, and all other general methods have been described in detail (17, 19). The results are expressed as mean ± S.D. Statistical analysis was performed by a paired t test or Mann-Whitney Rank Sum test. p values less than 0.05 were considered significant.

RESULTS AND DISCUSSION

β4 Isoform Is Not Expressed in lh/lh Mice—The lh gene mutation was anticipated to lead to truncation of β4 to an N-terminal fragment predicted to have a mass of 21 kDa (9). The lh mRNA was detected at levels 20% of the wild-type β4 message (9), suggesting the possibility that the lh gene product may be expressed in lh/lh mice. Using β4-specific antibodies, we probed forebrain and cerebellar homogenates from lh/lh mice to evaluate the level of expression of full-length β4. In contrast to wild-type mice where we detected full-length β4 (62 kDa), there was no β4 detected in either forebrain or cerebellum from lh/lh mice (Fig. 1). To further investigate expression of the lh gene product, we used an antibody (Ab CW24) raised to amino acids 53–70 in the β4 which are also present in all β isoforms (16, 17). Ab CW24 identified two populations of high (β1b and β2) and low (β3 and β4) molecular weight β isoforms previously characterized in rat brain (17). The relative intensity of these bands clearly differed among the lh/lh versus wild-type mouse samples (Fig. 1). However, with the exception of 42–40-kDa proteolytic β fragments, there were no detectable Ab CW24-immunoreactive proteins that could be attributed to the predicted 21-kDa product of the lh gene in either lh/lh mouse forebrain or cerebellum. These results suggest that the lh mutation causes a complete loss of β4 protein.

The Pool of Available β Subunits Is Decreased in lh/lh Mice—To investigate the pool of available β isoforms in forebrain and cerebellum of lh/lh and wild-type samples, the level of expression of all β isoforms was quantified using a pan-specific anti-β antibody (Ab CW24) and a panel of β isoform-specific antibodies. There are regional differences in expression of β isoforms with increased expression of all β isoforms (with the exception of the β4) in forebrain samples. Significantly, we observed differences in expression among specific β isoforms in lh/lh mice compared with wild-type mice. The level of expression of all β isoforms as detected by the anti-β pan-specific antibody is lower in lh/lh forebrain and cerebellum than in wild-type samples (Fig. 2), indicating that the level of total β isoforms is not maintained in the lh/lh samples. In both lh/lh forebrain (p < 0.001) and cerebellum (p < 0.05), the level of expression of β1b was increased compared with wild-type mice (Fig. 2). In forebrain, the increase in β1b expression was greater than 50%. These results are consistent with our previous characterization of β1b as an inducible and regulated protein (16, 17). In contrast, differences in the levels of expression of β2 and β3 in lh/lh versus wild-type mice were not statistically significant in either forebrain or cerebellar samples.

Decreased Expression of N-type VDCC and α1b in lh/lh Compared with Wild-type Mice—The density of N-type VDCC has been previously shown to be higher in forebrain versus cerebellar samples (21, 23, 24). Furthermore, β4 is the predominant

![Fig. 1. β4 isomorf is not detected in forebrain or cerebellum of lh/lh mice. Forebrains and cerebella from lh/lh and wild-type mice were removed, resuspended in 50 mM HEPES, 1 mM EGTA, and protease inhibitors, and homogenized. The samples (150 μg/lane) were resolved by SDS-PAGE on a 12% gel (19), transferred to nitrocellulose, and incubated with affinity-purified antibodies to β4 (1/100 dilution) or Ab CW24 (a pan-specific anti-β antibody, 1/200 dilution) and visualized with enhanced chemiluminescence. Lane 1, lh/lh cerebellum; lane 2, wild-type cerebellum; lane 3, lh/lh forebrain; lane 4, wild-type forebrain.](image)

![Fig. 2. Altered level of expression of β isoforms in wild-type and lh/lh brain. Forebrains (FB) and cerebella (CB) from lh/lh and wild-type mice were removed, resuspended in 50 mM HEPES, 1 mM EGTA, and protease inhibitors and homogenized. The samples (150 μg/lane) were resolved by SDS-PAGE, transferred to nitrocellulose, and incubated with affinity-purified antibodies pan-specific for all β and isoform-specific antibodies to β1b, β2, β3, and β4. The amount of β was quantified using 125I-IgG. Results obtained were from duplicate blots representing n = 3 wild-type (■) and 3 lh/lh (■) animals for forebrain samples and n = 2 wild-type and 3 lh/lh animals for cerebellar samples; **, p < 0.001 and *, p < 0.05 as determined by a paired t test.](image)
isoform associated with VDCC from cerebellum, and β3 is the predominant isoform associated with VDCC from forebrain (5, 17, 20, 25). Therefore, these patterns of VDCC subunit expression suggested regional differences in acquisition of functional N-type VDCC in cerebellum and forebrain from lh/lh versus wild-type mice. Using 125I-CTX radioligand binding assays and Scatchard analyses (18) (Fig. 3), we observed a significant decrease (p < 0.05) in expression of N-type VDCC in lh/lh forebrain (1.49 ± 0.41 pmol/mg) compared with wild-type forebrain (2.70 ± 0.63 pmol/mg). There was a single 125I-CTX binding site detected in the forebrain samples with Kd values of approximately 28 pm for both the lh/lh and wild-type samples. The level of α1B expressed in forebrain samples was also quantified (Fig. 3) to examine possible discrepancies between expression of 125I-CTX binding sites and α1B protein (17). Despite the decrease in 125I-CTX binding sites in lh/lh forebrain, similar levels of α1B protein are expressed in forebrain of lh/lh and wild-type mice. These data strongly suggest that expression of α1B protein in forebrains of lh/lh mice is maintained at wild-type levels, while the assembly of α1B into a complex that can support 125I-CTX binding is compromised. The decreased expression of 125I-CTX binding sites in lh/lh cerebellum (0.32 ± 0.05 pmol/mg) compared with wild-type cerebellum (0.53 ± 0.10 pmol/mg). However, in contrast to the forebrain samples, radioligand binding experiments detected two 125I-CTX binding sites in cerebellum. The high affinity site (Kd values of approximately 70 and 67 pM for the lh/lh and wild-type cerebellar samples, respectively) is characteristic of the N-type VDCC. The low affinity site for 125I-CTX detected in cerebellar samples is likely because of the low affinity binding of 125I-CTX for the P/Q-type VDCC (5) and was not pursued further in these studies. In contrast to lh/lh forebrain, decreased α1B protein is expressed in lh/lh mouse cerebellum, suggesting that expression of α1B protein is not maintained at wild-type levels. It seems reasonable to consider that the loss of the β4 from lh/lh cerebellum cannot be entirely compensated despite the increased level of expression of β1b (Fig. 2). The decreased expression of N-type VDCC or altered expression of other VDCC in the cerebellum of the lh/lh mouse may be the molecular basis of ataxia associated with the lh/lh phenotype. It should be stated that the level of expression of functional N-type VDCC in sympathetic neurons was also decreased in the lh/lh mouse (27). However, in contrast to the lh/lh mouse, the “β3 knock-out” mouse is phenotypically normal (27). The expression of other β isoforms in response to the elimination of β3 has not yet been reported.

**Increased Incorporation of 1b Subunit into N-type VDCC of the lh/lh Mouse**—To determine the structural consequences of abnormal β isofrom expression in lh/lh mice upon N-type VDCC assembly, the endogenous α1b/β1 subunit complexes were evaluated in immunoprecipitation assays using anti-α1b, anti-β generic (Ab CW24), and β isofrom-specific antibodies (17). The assay conditions were defined such that the pan-specific anti-β antibody immunoprecipitated a similar fraction of N-type VDCC in all
samples (Fig. 4). The relative contribution of β isoforms to the N-type VDCC present in forebrain and cerebellum of lh/lh is clearly altered compared with the wild-type mice (Fig. 4). As anticipated from the lack of detectable β4 in forebrains from lh/lh mice (Fig. 1), the disparity in the association of β4 with N-type VDCC in lh/lh versus wild-type mice was quite dramatic, as antibodies to β4 immunoprecipitated less than 10% of the total N-type VDCC solubilized from the cerebellum of lh/lh mouse (Fig. 4). With regard to the N-type VDCC extracted from forebrain, the fraction of N-type VDCC associated with β1b was statistically increased in lh/lh versus wild type mice (Fig. 4). In contrast, neither the association of β2 nor β3 with the N-type VDCC was affected in forebrains of lh/lh versus wild-type mice (Fig. 4). However, although β1b was increased in expression in lh/lh cerebellum (Fig. 2), there was no statistically significant increase in incorporation of any β isoform into cerebellar N-type VDCC (Fig. 4). It is reasonable to suggest that down-regulation of α1b expression (Fig. 3), rather than increased incorporation of β1b into assembled N-type VDCC, is the primary mechanism of compensation in lh/lh cerebellum. Additional studies are required to determine whether the compensatory mechanisms that alter β subunit composition of the N-type VDCC in lh/lh mice influence the expression and assembly of other VDCC.

Although the specific biophysical properties derived from the population of N-type VDCC present in wild-type and lh/lh forebrain have yet to be determined, it is interesting to note that β1b and β4 have similar effects upon closed state inactivation of recombinant N-type VDCC (8). Similar kinetic effects of β1b and β4 suggest tolerance of the β1b assembled into N-type VDCC, and this atypical channel composition may explain the absence of the neurodegeneration frequently observed in other epileptic mouse strains (3, 4).

These results are the first to indicate that assembly of the high voltage-activated N-type VDCC is altered in the lh/lh mouse. These findings do not exclude the possibility that the expression of other high voltage-activated VDCC is also affected as β4 is associated with mature L-type, N-type, and P/Q-type VDCC (17, 20, 25). However, it is interesting to point out that although low voltage-activated T-type channels have been implicated in the initiation of thalamic seizures in absence epilepsies (28, 29), the T-type α10 and α11 isoforms do not contain consensus β binding domains (14, 30), suggesting that T-type VDCC expression, unlike the high voltage-activated VDCC, may not be directly regulated by β subunits.

Differential modulation of the N-type VDCC by protein kinases in lh/lh mice is another property that may result from the assembly of β1b in place of β4. The β1b (31) contains consensus sites for protein kinase A modification; conversely in the β4, the protein kinase A consensus sites are absent (32). Thus, the inappropriate inclusion of β1b into the N-type VDCC complex in the lh/lh mouse in lieu of β4 may alter protein kinase-mediated modulation of the channel and thus effect calcium entry and calcium-dependent signaling.

β Subunit Composition of N-type VDCC Expressed in lh/lh Mice Resembles N-type VDCC Population of Immature (P2) Neurons—In earlier studies, β4 was discriminated from the other β isoforms by virtue of its striking increase in expression during development (17, 33). The importance of β4 to neuronal functioning is reflected in the epileptic and ataxic phenotype of the lh/lh mice, which stands in contrast to the “β3 knock-out” mouse that is phenotypically normal (27). The phenotype of lh/lh mice is evident at postnatal day 15 (10, 11), which is consistent with the loss of β4 that is normally increased in expression after P7 in developing rat brain (17). The question now arises as to whether the phenotype of lh/lh mice arises primarily because of the loss of β4 or as a result of the increased fractional contribution of β1b to N-type VDCC complexes. Our recent report that identifies β4 as a marker for N-type VDCC maturation unifies these two possibilities (17). The increased fractional contribution of β1b to N-type VDCC complexes and the absence of β4 assembled into adult lh/lh N-type VDCC result in a population of N-type VDCC that is strikingly similar to immature (P2) N-type VDCC in β subunit composition (17). We propose that the mechanism that promotes absence seizures in lh/lh mice, a form of epilepsy more commonly associated with immature brain (28), may be a consequence of prolonged and inappropriate expression of immature α1b/β3 complexes.

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