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Differential Expression and Association of Calcium Channel Subunits in Development and Disease

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Voltage-gated calcium channels (VDCC) are essential to neuronal maturation and differentiation. It is believed that important signaling information is encoded by VDCC-mediated calcium influx that has both spatial and temporal components. VDCC are multimeric complexes comprised of a pore-forming α_1 subunit and auxiliary β and α_2/δ subunits. Changes in the fractional contribution of distinct calcium conductances to the total calcium current have been noted in developing and differentiating neurons. These changes are anticipated to reflect the differential expression and localization of the pore-forming α_1 subunits. However, as *in vitro* studies have established that β regulates the channel properties and targeting of α_1, attention has been directed toward the developmental expression and assembly of β isoforms. Recently, changes in the β component of the omega-conotoxin GVIA (CTX)-sensitive N-type VDCC have indicated differential assembly of α_{1B} with β in postnatal rat brain. In addition, unique properties of $\beta 4$ have been noted with respect to its temporal pattern of expression and incorporation into N-type VDCC complexes. Therefore, the expression and assembly of specific α_1/β complexes may reflect an elaborate cellular strategy for regulating VDCC diversity. The importance of these developmental findings is bolstered by a recent study which identified mutations in the $\beta 4$ as the molecular defect in the mutant epileptic mouse (*lethargic; lh/lh*). As $\beta 4$ is normally expressed in both forebrain and cerebellum, one may consider the impact of the loss of $\beta 4$ upon VDCC assembly and activity. The importance of the $\beta 1b$ and $\beta 4$ isoforms to calcium channel maturation and assembly is discussed.

KEY WORDS: Neurons; VDCC; beta subunits; expression; assembly; development; differentiation; epilepsy.

INTRODUCTION

The dynamic expression and targeting of specific voltage-dependent calcium channels (VDCC) play key roles in neuronal development and plasticity. N-type and P/Q-type VDCC, for example, are localized specifically to neurons and neuroendocrine cells (Sakurai *et al.*, 1996; Westenbroek *et al.*, 1992; Westenbroek *et al.*, 1995) where they are essential components of the presynaptic excitation–secretion machinery (Rettig et al., 1997; Sheng *et al.*, 1994). It is intriguing that in immature neurons, where synaptic mechanisms are underdeveloped, calcium entry may be coupled to other intracellular events early in development (Brosenitsch *et al.*, 1998; Calakos and Scheller, 1996; Doherty *et al.*, 1993) including neurite outgrowth (Kater and Mills, 1991; Manivannan and Terakawa, 1994; Moorman and Hume, 1993), neuronal migration (Komuro and Rakic, 1992), and gene activation (Brosenitsch *et al.*, 1998). Interestingly, in many systems, the rise in intracellular calcium mediated by calcium ionophores does not itself elicit a response, suggesting that the important event is the fluctuation of calcium within a discrete domain localized to the vicinity of VDCC (Haydon *et al.*, 1994; Llinas *et al.*, 1992; Moorman and Hume, 1993). In addition to the spatial component,

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the temporal signal encoded by the flux of calcium through a specific VDCC complex may hold important information.

MULTIMERIC STRUCTURE OF VDCC SUGGESTS DIFFERENTIAL ASSEMBLY OF ISOFORMS AS A MECHANISM FOR ESTABLISHING DIVERSITY

Calcium channels are multimeric complexes comprised of a channel-forming α_1 subunit, a glycosylated α_2/δ subunit, and β subunit (Isom *et al.*, 1994). It is now widely appreciated that all three subunits are required for the optimal expression of functional VDCC *in vitro* (Brust *et al.*, 1993). The α_1 subunit determines the overall electrophysiological and pharmacological properties of the VDCC. There are currently nine different genes which encode the α_1 (termed α_{1sk} and α_{1A} through α_{1H}) with multiple splice variants (Lin *et al.*, 1997; Pereverzev *et al.*, 1998; Rettig *et al.*, 1996; Snutch *et al.*, 1991; Williams *et al.*, 1992b). The resolution of VDCC can be made based upon their biophysical properties as well as their sensitivity to specific inhibitors (for review see De Waard *et al.*, 1996, Reuter, 1996, and papers included in this minireview series). Thus, it has been possible using detailed biophysical analyses and the specific classes of VDCC inhibitors to determine the relative contribution of the various VDCC subtypes to the composite calcium conductance present in developing neurons. Superimposed upon the pharmacological specificity imparted by the α_1 subunit, coexpression with a β subunit (termed β1, β2, β3, and β4) *in vitro* influences the time course of activation and inactivation of the α_1 in an isoform-specific manner. In addition to its impact upon channel activity, β influences the overall conformation (Zamponi *et al.*, 1996) and membrane distribution of the α_1/β complex (Brice *et al.*, 1997). Thus, heterogeneity in the β subunit component is anticipated to increase the complexity of the endogenous VDCC complex severalfold.

Changes in calcium channel currents have been observed at different stages of embryogenesis (Hilaire *et al.*, 1996), during synapse formation in cultured neurons (Basarsky *et al.*, 1994; Scholz and Miller, 1995; Verderio *et al.*, 1995), and during neuronal maturation in cerebellar Purkinje cells (Gruol *et al.*, 1992) and cerebellar granule cells (D'Angelo *et al.*, 1997). Relative to the question of heterogeneity within a specific subtype of VDCC, there have been reports of omega-conotoxin GVIA (CTX) sensitive N-type VDCC expressed in developing and differentiating cells (Rossi *et al.*, 1994; Seward and Henderson, 1990) that display two components of inactivation. These results suggest that the biophysical heterogeneity in N-type VDCC may be a consequence of the structural heterogeneity of the complex.

TEMPORAL AND SPATIAL PATTERNS OF EXPRESSION OF VDCC SUBUNITS IN ADULT AND DEVELOPING RAT BRAIN

Localization of VDCC mRNA by *in situ* Hybridization

A landmark study investigated the distribution of all α_1 and β subunit mRNAs in developing and adult rat brain using *in situ* hybridization (Tanaka *et al.*, 1995). There were two important findings of this study. The first was to address the question of which subunits comprised an endogenous VDCC by correlating the pattern and density of the mRNA for four α_1 subunits (α_{1A}–α_{1D}) with the four β subunits. The results of this study indicated that the N-type α_{1B} subunit (class B) was often expressed in regions where there was an absence of β3 subunit mRNA, demonstrating that the co-expression of the N-type α_{1B} and β3 was not obligatory. Interestingly, the pattern of expression of the N-type α_{1B} subunit mRNA could not be correlated with the expression of a single β subunit isoform. Another study using similar techniques was able to draw conclusions regarding the distribution of α_1 and β subunits (Ludwig *et al.*, 1997) and supported a complex formed between the α_{1B} and the β3. The developmental time course for the expression of the α_{1B} and β subunits was also investigated (Tanaka *et al.*, 1995). The authors present evidence which demonstrates an increase in level of α_{1A} and α_{1B} early in development until adulthood, where levels decrease. The pattern and level of expression of the β mRNA was very intriguing and demonstrated spatial heterogeneity in expression as early as embryonic day 18 (E18) (Tanaka *et al.*, 1995).

Localization of VDCC by Ligand Binding

It is well known that the level of expression of mRNA does not always correlate with the level of expressed protein. Furthermore, the use of *in situ* hydridization to localize mRNA does not identify

where the protein is ultimately expressed. Using autoradiographic techniques, the distribution of a functional, high-affinity binding site can be determined. Localization of N-type VDCC in mature and developing brain has been determined using autoradiographic techniques with [^{125}I] Tyr-22-omega-conotoxin GVIA ([^{125}I]CTX) (Filloux *et al.*, 1994; Kerr *et al.*, 1988). In the cerebral cortex there is a gradual increase in expression of N-type VDCC as the rat matures, while in the hind brain (e.g., medulla) there is a gradual disappearance of the binding site. Furthermore, substructural differentiation within the cerebellum is evident, as well as a transient expression in the brainstem region (e.g., pons), indicating that expression of N-type channels throughout brain development is highly regulated (Filloux *et al.*, 1994; Kerr *et al.*, 1988).

Utilizing fluorescently labeled CTX to monitor surface expression of N-type calcium channels during development in the rat hippocampus, labeling was found initially on cell bodies (Jones *et al.*, 1997). In P7 hippocampal CA1 neurons, N-type channels are confined to the somata and very proximal dendrites even though there are extensive dendritic formations. However, in adult a punctate staining pattern is observed along the entire neuron. These results suggested that expression of N-type VDCC in mature neurites proceeds subsequent to the appropriate processing and targeting of the complex

Immunohistochemical Localization of VDCC

Immunostaining of rat adult neurons using a peptide-specific antibody to the α_{1B} subunit revealed patchy distribution of N-type calcium channels with a high density in presynaptic nerve terminals and a lower density seen in dendrites (Westenbroek *et al.*, 1992). With respect to regional distribution, α_{1B} was detected in cell bodies of some pyramidal neurons in the dorsal cortex, granule cells of the granular cell layer, and most of the Purkinje cells in the cerebellum, suggesting that α_{1B} is not exclusively a "presynaptic" calcium channel (Westenbroek *et al.*, 1992). In fact, the presence of N-type VDCCs on dendrites and spines of hippocampal neurons suggests the possibility of postsynaptic localization (Jones *et al.*, 1997; Mills *et al.*, 1994). Interestingly, it was observed that N-type VDCC binding sites were present on cultured CA1 neurons which did not label with an antibody directed towards the N-type α_{1B} subunit (Westenbroek *et al.*, 1992), suggesting possible subtypes of N-type VDCC (Mills *et al.*, 1994).

There are several studies that have demonstrated differential localization of structurally and functionally similar α_1 subunits. The differential localization of α_{1A} isoforms was demonstrated in rat hippocampal pyramidal cells (Sakurai *et al.*, 1996). These two α_{1A} isoforms, which differ in their II–III intracellular binding domain for SNARE proteins, had previously been shown to differ in their ability to support high-affinity syntaxin binding in recombinant studies (Rettig *et al.*, 1996). The α_{1A} isoform most similar to the rabbit sequence (Mori *et al.*, 1991) which bound to both SNAP-25 and syntaxin (Rettig *et al.*, 1996) was found on dendrites and nerve terminals, while the rat isoform (termed rbA-1) which bound only SNAP-25 (Rettig *et al.*, 1996) was found on cell bodies and in nerve terminals, suggesting a distinct role for these two isoforms (Sakurai *et al.*, 1996). Differential patterns of localization of the α_1 subunits which comprise neuronal L-type VDCC (α_{1C} and α_{1D}) were observed in mature rat neurons (Hell *et al.*, 1993). The α_{1D} was observed to be generally distributed on the surface of cell bodies and proximal dendrites with little or no staining of the distal dendrites. In comparison, the α_{1C} was localized in clusters on the cell bodies and proximal dendrites with significant staining of distal dendrites (Hell *et al.*, 1993). It is difficult to envision the complexity of the developmental processes which contribute to this highly localized distribution of VDCC isoforms in mature neurons.

SUBUNIT COMPOSITION OF NEURONAL VDCC IN ADULT AND POST-NATAL BRAIN

The *in situ* hybridization and immunohistochemical studies described above suggested potential VDCC subunit combinations based upon correlations in the distribution of the different isoforms. These results prompted the direct assessment of the subunit composition of adult L-, N-, and P/Q-type VDCC. Structural heterogeneity has been noted in the β subunit component of mature, biochemically purified L-type, N-type, and P/Q-type VDCC from rat and rabbit brain. Using [^{125}I]CTX binding as the *in vitro* assay for the CTX-sensitive N-type VDCC, the adult N-type VDCC complex has been purified from rat brain (Leveque *et al.*, 1994; McEnery, 1993; McEnery *et al.*, 1991) and rabbit brain (Witcher *et al.*, 1993).

The N-type VDCC is comprised of a 230-kDa α_{1B} subunit which binds [^{125}I]CTX, a 140-kDa α_2/δ subunit, and β subunit(s). The rabbit brain preparation was purified by immunoaffinity chromatography using an immobilized monoclonal antibody to the β subunit (Witcher *et al.*, 1993). In earlier communication from this group, two β subunits were observed to be eluted from the affinity column with apparent molecular weights of 78 and 58 kDa (Sakamoto and Campbell, 1991). However, the 78-kDa β subunit was separated from the peak of [^{125}I]CTX binding activity on a sucrose gradient and the purified N-type VDCC was reported to be comprised of a stoichiometric complex of a 230-kDa α_{1B} subunit, an 140-kDa α_2/δ subunit, a 90-kDa protein, and a single β subunit of 57 kDa, verified by antibodies as a β3 isoform (Witcher *et al.*, 1993). The 90-kDa subunit was recently identified as the N-terminal portion of the α_{1A} subunit (Scott *et al.*, 1998). Other groups have identified a 90-kDa subunit in their preparations as the Na/K-ATPase α subunit (Choi *et al.*, 1997; Leveque *et al.*, 1994).

The idea of a single β isoform associated with the mature N-type VDCC gave way to β subunit heterogeneity in the assembled complex (Pichler *et al.*, 1997; Scott *et al.*, 1996; Vance *et al.*, 1998), consistent with the protein staining pattern of the rat brain N-type VDCC (McEnery *et al.*, 1991; McEnery, 1993; Leveque *et al.*, 1994). The β subunit composition of the L-type and P/Q-type VDCC were similarly determined using [^{3}H]isradipine and [^{125}I]MVIIC binding, respectively, in *in vitro* radioligand binding assays. Interestingly, while the subunit composition of the L-VDCC solubilized from forebrain was very similar to the subunit composition of the N-type VDCC (Pichler *et al.*, 1997), the subunit composition of the P/Q-type VDCC was different from that of the N-type VDCC with the most obvious difference being the large contribution of the β4 isoform to the mature P/Q-type VDCC (Table I). However, a point to be considered in the comparison of these VDCC is their tissue of origin. The P/Q-type VDCC was solubilized from rabbit cerebellum (Liu *et al.*, 1996a), whereas the N-type VDCC was from rat forebrain (Leveque *et al.*, 1994; McEnery *et al.*, 1991). The L-type VDCC solubilized from cerebellum was more similar to the P/Q-type VDCC than the L-type VDCC extracted from forebrain (Pichler *et al.*, 1997). These studies suggest that the population of β subunits that drives VDCC complex assembly may significantly differ among tissues.

Table I. Subunit Composition of Adult L-, N-, and P/Q-Type VDCC

VDCC Tissue	Fractional contribution of individual β isoforms (%)			
	β1b	β2	β3	β4
L-type VDCC				
rat forebrain[a]	20	12	42	42
rat cerebellum[a]	6	0	12	45
N-type VDCC				
rat forebrain[a]	8	3	40	42
rat cerebellum[a]	2	2	8	25
rat forebrain[b]	32	8	55	25
rabbit forebrain[c]	10	<3	56	31
P/Q-type VDCC				
rabbit cerebellum[d]	8	7	36	48

[a] Pichler *et al.* (1997).
[b] Vance *et al.* (1998).
[c] Scott *et al.* (1996).
[d] Liu *et al.* (1996a).

CHANGES IN THE β SUBUNIT COMPOSITION OF THE N-TYPE VDCC DURING RAT BRAIN DEVELOPMENT

An interesting trend to note is the correlation between the expression and assembly of specific VDCC and the acquisition of functional synapses during specific intervals of development (Aghajanian and Bloom, 1967). For example, one might anticipate a "burst" in N-type α_{1B} protein expression with subsequent acquisition of active N-type conductances during a well-defined period of axonal outgrowth, infiltration, and synapse formation in the rat neocortex which occurs in the first two weeks of postnatal life (Aghajanian and Bloom, 1967; Mahalik *et al.*, 1992; Scheinman *et al.*, 1989). Increases in the level of expression of the sodium channel alpha subunit protein in rat forebrain have been previously observed to increase dramatically between P1 and P6 (Scheinman *et al.*, 1989).

As shown in Fig. 1A, the level of expression of α_{1B} as detected by CW14 increased dramatically from embryonic day 14 (E14) to adult. This increase in expression of α_{1B} subunits which occurs during rat brain development parallels the increase in expression of [^{125}I]CTX binding sites as determined by radioli-

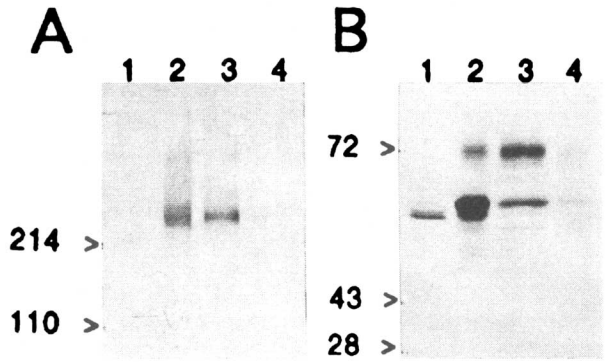

Fig. 1. Detection of α1B and β subunits in adult skeletal muscle and rat brain. Tissue homogenates were prepared from adult skeletal muscle, adult rat brain, newborn rat brain, and embryonic rat brain day 14 (E14) and resolved on a 4–17% gradient gel. The amount of protein present in each lane was as follows: lane 1: skeletal muscle (25 μg); lane 2: adult rat forebrain (50 μg); lane 3: P0 rat brain (100 μg); and lane 4: embryonic rat brain (E14) (150 μg). After transfer to nitrocellulose, the filter was cut horizontally and probed as follows: Panel A: anti-α_{1B} (1/200 dilution) and Panel B: anti-β generic antibody (1/200 dilution), incubated with goat anti-rabbit secondary coupled to HRP and visualized with ECL.

gand binding and Scatchard analysis (neonatal (P0) membranes K_d = 15.8 pM with a B_{max} of 200 fmol/mg, adult membranes K_d = 12.5 pM with a B_{max} of 1,050 fmol/mg). There was no detectable [^{125}I]CTX binding at E14. However, a high-affinity [^{125}I]CTX binding site could be detected in E18 rat brain samples, suggesting that the assembly of N-type VDCC occurs late in gestation (Vance *et al.*, 1998).

When similar samples were probed with an antibody which reacts with the four β subunits identified in brain as well as the β subunit present in skeletal muscle (Fig. 1B, lane 1), changes were observed in both the level and isoform pattern expressed in adult rat brain (lane 2), postnatal day 0 (P0) rat brain (lane 3), and embryonic day 14 brain (lane 4). The concentration of total β subunits in adult brain appeared to be dramatically higher than the population of β subunits in embryonic brain. When P0 rat brain samples were compared to the adult sample, a redistribution of β isoforms was apparent, with a much higher level of expression of the larger β isoforms (β1b and β2) in P0 and a greater amount of the smaller β subunits (β3 and β4) present in the adult sample. In contrast, embryonic tissue (Fig. 1A lane 4) shows only barely detectable levels of all β subunits and α_{1B} subunit.

Using β isoform-specific antibodies to probe western blots of developing rat brain samples, major trends were observed in β subunit content at different ages (Fig. 2). The interaction of specific β isoforms with the α_{1B} during development was also determined (Vance *et al.*, 1998). The results are summarized in Table II. There are significant changes in the pattern of association of the specific β isoforms with the N-type α_{1B} in postnatal rat brain. There are three interesting consequences of this study: first, the predominant β subunit incorporated into the N-type VDCC changes at different ages. The immature N-type VDCC is comprised of primarily the β1b, while the mature N-type is comprised of the β3>β1b>β4. Second, returning to the data presented in Fig. 2, it is clear that the abundance of a particular β isoform does not, of itself, drive the assembly of β1b, β2, or β3 with the α_{1B} to form N-type VDCC. The level of expression of the β1b is the lowest of all β isoforms at P2, yet is the most highly represented β isoform in the immunoprecipitated N-type VDCC from P2 animals. In contrast, the β2 isoform, which is constitutively expressed throughout rat brain postnatal development, appears to be actively excluded from the N-type complex at all ages. Lastly, the β3 subunit, also constitutively expressed, appears to be recruited into N-type VDCC complex in P14-adult. In striking contrast, the β4 is upregulated approximately 10-fold (Figs. 2D and 3) The incorporation of the β4 isoform with the α_{1B} subunit during rat brain development is unique, as its level of expression parallels its fractional contribution to N-type VDCC (Vance *et al.*, 1998). It is therefore clear that there are several processes at play which regulate the incorporation of specific β isoforms into VDCC.

α_2/δ Expression During Development

There is a paucity of information concerning the role of the α_2/δ in VDCC expression in the development of the central nervous system. The α_2/δ subunit is a highly glycosylated protein component of all VDCC complexes. The product of a single gene, a post-translational cleavage results in a disulfide-linked α_2/δ complex (De Jongh *et al.*, 1990; Jay *et al.*, 1991). Several splice variants have been identified, some of which appear to be expressed primarily within brain tissue (Angelotti and Hofmann, 1996; Ellis *et al.*, 1988; Kim *et al.*, 1992). Recently, a novel α_2/δ gene has been identified on a region of chromosome 3 (3p21.3) as being involved in a human lung cancer (accession numbers AF042792, 2935326). Although this novel gene product shares a great deal of homology with the

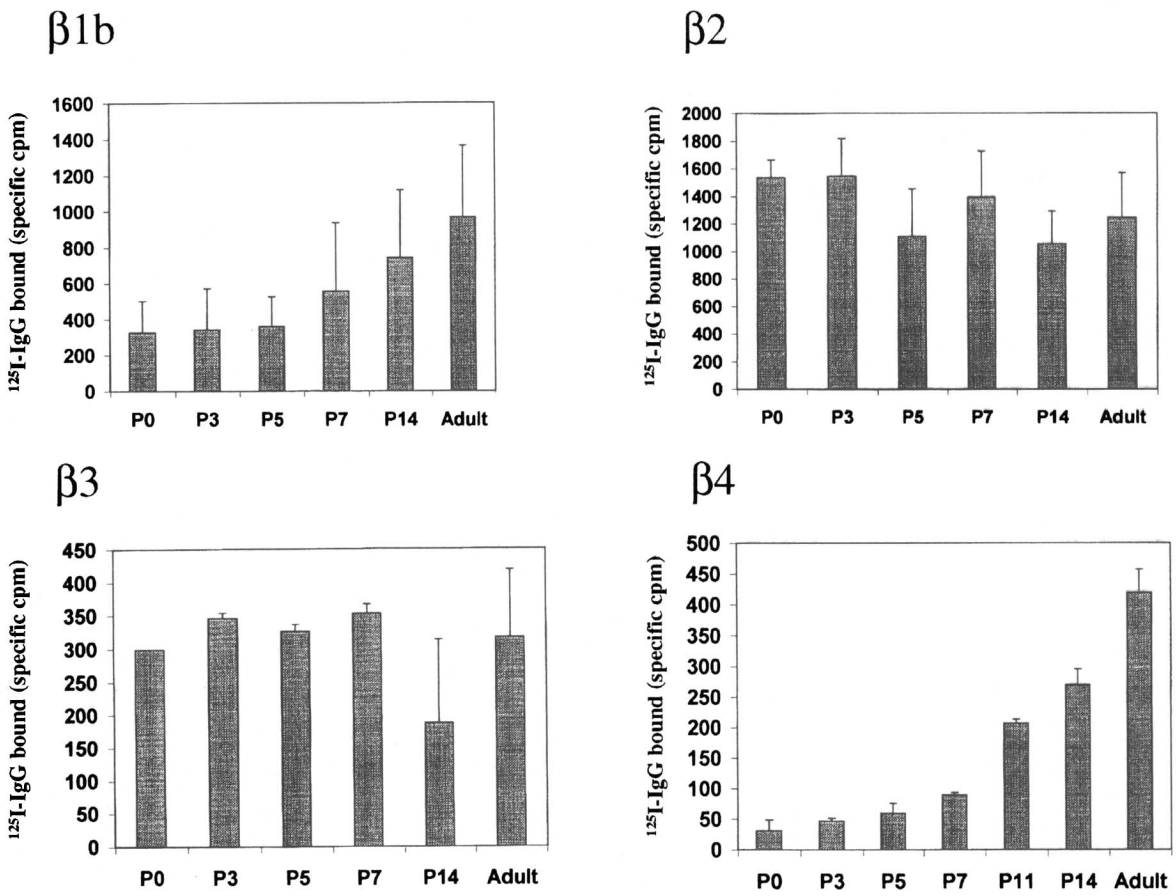

Fig. 2. Expression of specific β subunit isoforms during development: The protein samples obtained from rats aged PO to adult were transferred to nitrocellulose and probed with β-isoform-specific antibodies. Panel A: anti-β1b (1/60 dilution); panel B: anti-β2 antibody (1/60 dilution); panel C: anti-β3 antibody (1/200 dilution); panel D: β4 antibody (1/100 dilution). Analysis was carried out using [^{125}I]goat anti-rabbit IgG to detect the antigens; the filter was exposed to film, autoradiographed, and the single immunoreactive bands excised and counted in a gamma counter.

previously characterized α_2/δ, it is not known whether it associates with an α_1.

Recent studies have tried to address the functional role of the domains of the α_2/δ protein. The small, transmembrane-spanning δ subunit appears to modulate the gating properties of both the L-type and P/Q-type channels, while the extracellular α_2 domain appears to be important in both determining the level of expression of the VDCC and the stabilization of the complex as determined by drug binding assays

Table II. Subunit Composition of Immature and Mature N-Type VDCC (Vance *et al.*, 1998)

	Fractional contribution of individual β isoforms (%)			
Age	β1b	β2	β3	β4
P2	37	1	27	0
P14	13	0	32	14
Adult	32	8	55	25

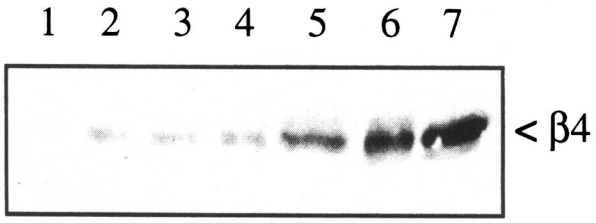

Fig. 3. Expression of β4 isoform during rat brain development. Brain homogenates from postnatal rat were analyzed for β4 content by Western blot analysis using a β4-specific antibody and visualized by ECL. Lane 1: PO; lane 2: P3; lane 3: P5; lane 4: P7; lane 5: P11; lane 6: P14; lane 7: adult. The protein concentration was 150 μg/lane.

(Felix *et al.*, 1997). In the case of the human N-type VDCC, the coexpression of the α_2/δ subunit affects the affinity of [^{125}I]CTX binding without altering the kinetic properties of the channel (Brust *et al.*, 1993). The expression of both [^{125}I]CTX binding and calcium channel activity is optimized when the N-type α_{1B} subunit is co-expressed with α_2/δ and β subunits (Brust *et al.*, 1993; Williams *et al.*, 1992a). However, in the absence of α_2/δ and β subunits, a small number of [^{125}I]CTX binding sites are expressed, and under these circumstances it is difficult to discount the possibility that endogenous α_2/δ or β subunits have been recruited into the complex.

Few reports exist in examining $\alpha 2/\delta$ subunit expression in development. In developing rat hippocampus, the $\alpha 2/\delta$ subunit was detected at E18 (~45% of adult levels) and rose to a plateau at P10 (Jones *et al.*, 1997). However, no correlation can be made with a single VDCC isoform as $\alpha 2/\delta$ would be anticipated to be associated with L-, N-, and P/Q-type VDCC. In addition, attempts to immunoprecipitate CTX binding using a similar anti-skeletal $\alpha 2/\delta$ antibody only recovered a small percentage of CTX binding (Westenbroek *et al.*, 1992), suggesting that nonskeletal isoforms of $\alpha 2/\delta$ may be associated with the N-type VDCC.

A comparison of the level of expression of VDCC subunits in developing tissues (Morton and Froehner, 1989) noted that the level of expression of L-type VDCC in developing skeletal muscle was best correlated with the level of expression of the α_{1sk} subunit, rather than the steady-state expression of the highly conserved α_2/δ subunit (Morton and Froehner, 1989). Therefore, it is not the level of expression of the α_2/δ driving the assembly of mature VDCC subunits. Another study focused upon the localization of the α_2/δ during the development of T-tubules in rabbit muscle cell cultures (Vandaele and Rieger, 1994). These results suggested that during myogenesis there was a strong co-localization of α_2/δ with N-CAM, inferring a role of the α_2/δ in early morphogenesis.

EXPRESSION OF VDCC α_B AND β SUBUNITS IN *IN VITRO* MODELS OF NEURODIFFERENTIATION (PC12 CELLS AND IMR32 CELLS)

The observation that levels of α_1 and β mRNA change during development (Tanaka *et al.*, 1995) and our results on the expression and association of VDCC in postnatal rat brain (Vance *et al.*, 1998) suggest exquisite differential regulation of β isoform expression. The addition of neurotrophic agents to neurotypic cells in culture results in changes in calcium currents (Plummer *et al.*, 1989; Streit and Lux, 1987; Usowicz *et al.*, 1990). The *in vitro* models of neuronal differentiation offer the opportunity to examine VDCC subunit expression under controlled conditions. Following differentiation of PC12 cells, two pharmacologically distinct components of N-type calcium current were detected which differed in their sensitivity to CTX (Plummer *et al.*, 1989). The CTX-resistant component observed in undifferentiated cell bodies decreased upon differentiation, whereas the CTX-sensitive component increased upon differentiation (Plummer *et al.*, 1989; Reber and Reuter, 1991). Changes in the expression of VDCC subunits in PC12 cells have been examined by western blot analysis and immunoprecipitation of N-type VDCC (Lin *et al.*, 1997). As shown in Fig. 4A, there is upregulation of the α_{1B} upon differentiation with NGF. This increase in expression of α_{1B} parallels an increase in CTX binding with a B_{max} of 100 fmol/mg in undifferentiated PC12 cells and a B_{max} of 300 fmol/mg in NGF-treated PC12 cells with no change in affinity. Furthermore, there are changes in the level of expression of the $\beta 2$ and $\beta 3$ isoforms (Fig. 4B and 4C, respectively). These results suggest control of

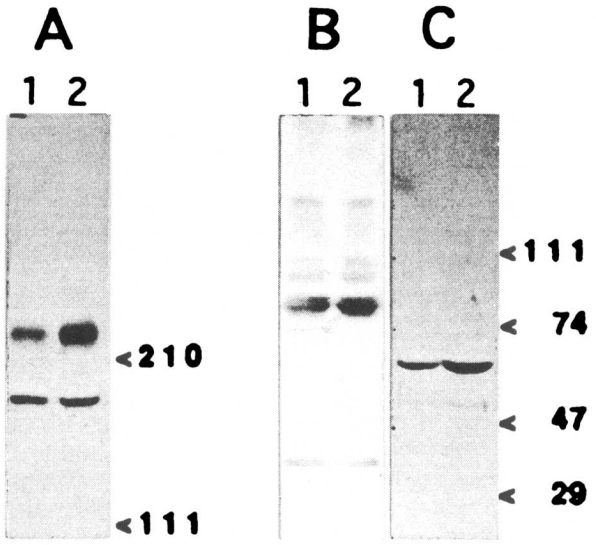

Fig. 4. Increased expression of N-type α_{1B} and β subunits following NGF-stimulation of PC12 cells. PC12 cells were treated with NGF and harvested as described (Streit and Lux, 1987) and analyzed by SDS-PAGE. Following transfer to nitrocellulose, the filters were probed with anti-α_{1B} antibodies (1/200 dilution, Panel A) or anti-β antibodies (1/50 dilution, Panels B and C) and visualized by ECL. The samples are as follows: lane 1: PC12 cells differentiated with NGF; lane 2: undifferentiated PC12 cells. The protein concentration was 150 μg/lane.

VDCC subunit expression by neurotrophic factors in PC12 cells.

There have been very few studies on the expression and assembly of N-type VDCC in differentiating cells. Human neuroblastoma cells (IMR32 cells), which possess N-type and L-type VDCC (Carbone *et al.*, 1990), exhibit an increased expression of N-type VDCC at the cell surface following differentiation (Passafaro *et al.*, 1992). Evidence from using antisense oligonucleotides (Tarroni *et al.*, 1994) suggested that changes in the total β subunit content influence the acquisition of N-type VDCC at the plasma membrane. Upon analysis of the VDCC content of control and differentiated IMR32 cells, a dramatic increase in the expression of the N-type α_{1B} subunit was observed that correlated with the appearance of increased N-type VDCC at the plasma membrane (McEnery *et al.*, 1997; Passafaro *et al.*, 1992). More striking were the results on the expression of β isoforms during IMR32 cell differentiation. In contrast to the pattern of expression of multiple β isoforms seen in PC12 cells, only the β1b was expressed in IMR32 cells. The level of expression of β1b increased dramatically and in parallel to the expression of the α_{1B} subunit (McEnery *et al.*, 1997).

The abbreviated pattern of expression of the β1b in IMR32 cells highlights its importance to the process of neuronal differentiation. These results are consistent with previous studies that reported changes in the surface expression of [^{125}I]CTX receptors upon introducing anti-sense β1b oligonucleotides into IMR32 cells (Tarroni *et al.*, 1994). As IMR32 cells are reported to express several subtypes of VDCC, the contribution of β1b to the subunit composition of the other VDCC requires additional attention. The basis for the specific disposition of N-type VDCC in the neuron remains unknown, but one may consider the possibility that subtle changes in the structure of N-type VDCC may influence the interaction among proteins which associate with the N-type VDCC, consequently altering the structure and properties of the presynaptic membrane itself.

THE ROLE OF THE β4 ISOFORM IN THE EPILEPTIC *LETHARGIC (LH/LH)* MOUSE

The dramatic increase in the expression of the β4 isoform between P0 and adult and its parallel association with the α_{1B} through development is in striking contrast to the other β isoforms and identifies a property unique to the β4 (Vance *et al.*, 1998). Interestingly, there has been a report which demonstrates the importance of the β4 isoform. Analyses of the mutations which underlie the mouse *lethargic* phenotype (Dung and Swigart, 1971; Dung and Swigart, 1972), a model of human epilepsy (Hosford and Wang, 1997), have identified an insert in the β4 gene that leads to a truncated gene product (Burgess *et al.*, 1997). Specifically, the truncation of the β4 subunit protein eliminates the α_1 binding domain (De Waard *et al.*, 1994) as well as more than 60% of the C-terminus of the protein (Burgess *et al.*, 1997). The mRNA level for the *lh* gene product is 20% that of the normal β4 (Burgess *et al.*, 1997), and the question remains as to whether the truncated form of β4 is present in the *lh/lh* mouse or unstable and, hence, not detected.

The study by Burgess *et al.* is significant as it is the first to implicate VDCC auxiliary subunits as the basis for neurological diseases. While the β4 subunit is the predominant β isoform expressed in cerebellum, both mRNA and protein are expressed in cortex, hippocampus, olfactory bulb, and inferior colliculus (Ludwig *et al.*, 1997; Tanaka *et al.*, 1995), regions which also express α_{1B} and α_{1A} subunits. The co-localization of the α_{1B} and α_{1A} with the β4 isoform in normal rat forebrain and cerebellum (Tanaka *et al.*, 1995) and the identification of the β4 as a component of the adult P-type (Liu *et al.*, 1996b), N-type VDCC (Pichler *et al.*, 1997; Scott *et al.*, 1996), and L-type VDCC (Pichler *et al.*, 1997) suggest important lines of investigation toward understanding the role of the β4 truncation in epileptic *lh/lh* mice. Furthermore, the epileptic phenotype of *lh/lh* mice brought about by a defect in a single β subunit intimates a role for the β4 isoform which cannot be complemented by the expression of the other β isoforms.

The singular importance of another β isoform was demonstrated in a β1 "knockout" mouse (Gregg *et al.*, 1996). The β1 gene exists as three splice variants, the β1a is only expressed in skeletal muscle and is its predominant β subunit, and the β1b is expressed in brain, spleen, and heart (Powers *et al.*, 1992; Ruth *et al.*, 1989). The absence of the β1 is perinatal lethal for the mouse due to asphyxia (Gregg *et al.*, 1996), a consequence of the abolition of E–C coupling. The muscle is severely disorganized at all structural levels inclusive of the absence of T-tubule/SR-junctional complexes which normally contains the L-type VDCC and the ryanodine receptor. In fact, eliminating the β1 led to the loss of α_{1sk} expression, suggesting an essential role of the β1 in the targeting of the L-type VDCC

(Gregg *et al.*, 1996). The impact of the absence of the β1 in embryonic neuronal tissues has not yet been reported. In contrast, mouse genomic "knockouts" of the β3 subunit have a phenotype which cannot be distinguished from the wild-type mice and show statistically insignificant differences in the biophysical properties of N-type VDCC (Smith *et al.*, 1998). The absence of the β3, however, did lead to a reduction in the amount of N-type current expressed in superior cervical ganglion neurons. It is clear from recent studies in rat that the β4 isoform is unique among β subunits in its magnitude of induction and temporal pattern of expression (Vance *et al.*, 1998), whereas the β3 is constitutively expressed throughout development. Thus, returning to the epileptic phenotype of the *lh/lh* mouse, one might consider that alterations in the level of expression of full-length β4 may have profound effects upon the regulation of expression and localization of α_1 and other β isoforms and, hence, the excitability of the afflicted neurons (McEnery *et al.*, 1998).

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