Differential Expression and Association of Calcium Channel \(\alpha_{1B}\) and \(\beta\) Subunits during Rat Brain Ontogeny*

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Calcium functions as an essential second messenger during neuronal development and synapse acquisition. Voltage-dependent calcium channels (VDCC), which are critical to these processes, are heteromultimeric complexes composed of \(\alpha_1\), \(\alpha_2/\alpha_3\), and \(\beta\) subunits. \(\beta\) subunits function to direct the VDCC complex to the plasma membrane as well as regulate its channel properties. The importance of \(\beta\) to neuronal functioning was recently underscored by the identification of a truncated \(\beta4\) isoform in the epileptic mouse lethargic (lh) (Burgess, D. L., Jones, J. M., Meisler, M. H., and Noebels, J. L. (1997) Cell 88, 385–392). The goal of our study was to investigate the role of individual \(\beta\) isoforms (\(\beta1\), \(\beta2\), \(\beta3\), and \(\beta4\)) in the assembly of N-type VDCC during rat brain development. By using quantitative Western blot analysis with anti-\(\alpha_{1B}\)-directed antibodies and \(^{125}\text{I}-\text{Tyr}^{22}\) conotoxin GVIA (\(125\text{I}-\text{CTX}\)) radioligand binding assays, we observed that only a small fraction of the total \(\alpha_{1B}\) protein present in embryonic and early postnatal brain expressed high affinity \(^{125}\text{I}-\text{CTX}\)-binding sites. These results suggested that subsequent maturation of \(\alpha_{1B}\) or its assembly with auxiliary subunits was required to exhibit high affinity \(^{125}\text{I}-\text{CTX}\) binding. The temporal pattern of expression of \(\beta\) subunits and their assembly with \(\alpha_{1B}\) indicated a developmental pattern of expression of \(\beta\) isoforms: \(\beta1\) increased 3-fold from P0 to adult, \(\beta4\) increased 10-fold, and both \(\beta2\) and \(\beta3\) expression remained unchanged. As the \(\beta\) component of N-type VDCC changed during postnatal development, we were able to identify both immature and mature forms of N-type VDCC. At P2, the relative contribution of \(\beta\) is \(\beta1 > \beta3 > \beta2\), whereas at P14 and adult the distribution is \(\beta3 > \beta1 > \beta2\). Although we observed no \(\beta4\) associated with the \(\alpha_{1B}\) at P2, \(\beta4\) accounted for 14 and 25% of total \(\alpha_{1B}/\beta\) subunit complexes in P14 and adult, respectively. Thus, of the \(\beta\) isoforms analyzed, only \(\beta4\) was assembled with the rat \(\alpha_{1B}\) to form N-type VDCC with a time course that paralleled its level of expression during rat brain development. These results suggest a role for the \(\beta4\) isoform in the assembly and maturation of the N-type VDCC. Ca\(^{2+}\) channels play important roles in neuronal development. Both VDCCs and calcium entry have been implicated in many processes of immature neurons including neurite outgrowth (1–3), neuronal migration (4), and axon and dendrite extension (5, 6). Calcium entry through VDCCs has been shown to be essential for sculpting neuromuscular synapses (7–10). Calcium channels have also been implicated in the initiation of developmental gene expression (11) and are necessary for spinal cord motoneuron differentiation in rat (12).

In vitro studies indicate that functional VDCCs are composed of three subunits: \(\alpha_1\), \(\alpha_2/\alpha_3\), and \(\beta\). Multiple VDCCs (N-, P/Q-, L-, and R-type) are expressed in neuronal tissues (13, 14). The \(\alpha_1\) subunit, of which there are at least six genes, resides in the membrane and forms the pore of the channel (14). The \(\beta\) subunit, a putative hydrophilic protein, binds the \(\alpha_1\) (15) and acts to regulate channel gating and kinetics (16, 17). Four \(\beta\) subunit genes have been identified, several of which exist as splice variants (14). N-type VDCCs are localized to the plasma membrane of neuronal processes (18) and are essential for the generation of action potentials and the subsequent release of neurotransmitters (19, 20). Adult N-type as well as other neuronal VDCCs are heterogeneous in their \(\beta\) subunit component (21–24). Although the in vivo process which gives rise to the heterogeneity in \(\alpha_{1B/}\beta\) subunit complexes is not understood, it is anticipated to generate N-type VDCC with different channel properties based upon in vitro co-expression studies (25, 26).

Developing neurons offer the opportunity to investigate the underlying trends that contribute to subunit diversity. Several families of ligand-gated ion channels evidence developmental changes in their subunit composition (27–29). Whereas studies have identified alterations in the expression of N-type and P/Q-type VDCC during synapse formation in cultured neurons (30–32), few reports have investigated the developmental or differentiation-dependent expression of calcium channel subunits and their assembly (33, 34). Interestingly, there have been reports of N-type VDCC in developing cerebellar granule cells (35) and differentiated human neuroblastoma SH-SY5Y cells (36) which are inhibited by \(\omega\)-conotoxin GVIA (CTX) yet display two components of inactivation. The expression of N-type VDCC with different channel properties is a possible mechanism for controlling membrane excitability. As \(\beta\) sub-
units are known to influence the time course of channel inactivation, the diversity in N-type VDCC activity was suggested to reflect heterogeneity in the β subunit component. We undertook this study to test directly the hypothesis that N-type VDCCs are differentially associated with β subunits during rat brain development. The objectives of this investigation are 1) to identify possible trends in calcium channel subunit expression by evaluating changes in the expression of α1B and β isoforms throughout postnatal development and 2) to identify possible "immature" and "mature" forms of the N-type VDCC by characterizing the β subunit component of N-type VDCC at different stages of rat brain maturation.

EXPERIMENTAL PROCEDURES

Materials—Unless noted, all reagents were obtained from Sigma. Calpain inhibitors I and II were obtained from Calbiochem. Enhanced chemiluminescence kit (ECL) was purchased from Amersharm Pharmacia Biotech; unlabeled α-gVIA conotoxin was from Peninsula Laboratories; 125I-Tyr22-conotoxin GVIA (specific activity 2200 Ci/mmol) and 125I-protein A (specific activity 21.1 Ci/mg) were obtained from NEN Life Science Products. 125I-IgG was obtained from ICN (specific activity 12.9 Ci/mg). Nitrocellulose membranes were obtained from Schleicher & Schuell. Maleimide-activated keyhole limpet hemocyanin from Boehringer Mannheim. Sulforhodamine B columns were purchased from Pierce. Bovine serum albumin was from U. S. Biochemical Corp. Hesper was from Research Organics.

Preparation of Rat Homogenates and Membranes—Embryonic (E18), neonatal, and adult rats were euthanized and the brains removed and immediately placed in 50 mM Heps, pH 7.4, 1 mM EGTA plus protease inhibitors at a protein to volume ratio of 1.3 g/25 ml. The protease inhibitors were added from stock solutions prepared as follows: phenylmethanesulfonyl fluoride (1/1000 dilution from 200 mM stock in ethanol), calpain inhibitors I and II (1/1000 dilution from 4 mg/ml stock), benzamidine (1/600 dilution of 200 mM stock), aprotinin (1/500 dilution from 1 mg/ml stock), leupeptin (1/500 dilution from 1 mg/ml stock), pepstatin (1/500 dilution from 1 mg/ml stock in MeSO4), and DTT (1/1000 dilution from 1 mM stock). The tissue was homogenized with a Polytron for 10 s and centrifuged at 18,000 rpm (48,000 × g) for 15 min. The membranes were resuspended in 5 ml of 50 mM Heps, pH 7.4, plus protease inhibitors at a resulting protein concentration of 3–10 mg/ml. For subsequent use in Western blot analysis, all samples were stored in −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 100–200 μl aliquots. The samples were not freeze-thawed.

Production of Anti-peptide Polyclonal Antibodies to VDCC Subunit Epitopes—The peptide antigens were synthesized to include a unique cysteine residue to be used both in the unambiguous attachment of peptide to carrier protein (maleimide-derivatized keyhole limpet hemocyanin) and to the affinity column (Sulforhodamine B). The peptides were synthesized, purified, and coupled to keyhole limpet hemocyanin. The coupled peptide antigens were used in the production of polyclonal sera in rabbits under continued contractual agreement with Covance, Inc. The rabbits were bled twice per month (15–20 ml/blood) and tested for production of specific antibodies after 4 weeks as described previously (34).

Anti-α1B rat sequence-specific antibodies (Ab CW8) were raised to a sequence (ASTPAGGEEQDRTC) corresponding to amino acid residues 863–875 in the rat cDNA that is present in the rat brain and rat spinal cord α1B cDNA (37, 38). Anti-α1B subunit antiserum (Ab CW14) (34) was raised to a sequence (EQPEDADNQRVTRMGSQP corresponding to amino acid 1051–1069) in the rat cDNA (37) which was present in all N-type α1B subunit cDNAs cloned to date.

Preparation of Peptide Columns—The peptide antigens were synthesized to include a unique CYS as described above.

Production of Anti-peptide Polyclonal Antibodies to VDCC Subunit Composition—The α1B subunit cDNA (37, 38) was present in all N-type α1B subunit isoforms cloned to date that is not implicated in either the binding of the β subunit or the consensus sites for protein phosphorylation or ATP-binding in the β subunit (39). This peptide was coupled via a unique CYS as described above.

For the generation of β isoform-specific antibodies, the peptides were synthesized with a N-terminal monochloroacetyl-glycyl extension (BioTeZ, Berlin, Germany) and coupled to keyhole limpet hemocyanin after activation with 2-iminothiolane hydrochloride as described (40). Immunization was done in New Zealand White rabbits in accordance with internationally accepted principles concerning the care and use of laboratory animals.

Antigenic epitopes comprised the amino acid sequences 554–567 (WYHLRLELRNRRNGHRRN) and 918–937 (ENYNHARKRSNLRS) of the β subunit (25, 43), and 16 amino acids (ENYNHARKRSNLRS) of the β subunit (39). Polyclonal antibodies were affinity purified on antigen affinity columns as described (44). The β subunit isoform-specific antibodies have been characterized elsewhere (23, 34).

Methods for 125I-CTX Binding—125I-CTX ([125I-Tyr22]α1B-conotoxin GVIA (specific activity 2000 Ci (81.4 TBq/mmol)) binding was assayed by published procedures which use filtration over PEI-soaked glass fiber filters (Whatman GF/B) to separate bound from unbound ligand in the presence of bovine serum albumin (BSA). N-type VDCC fractions were routinely screened at several protein concentrations to determine the linear range for the binding. Individual assay tubes contained 100 μl of representative N-type VDCC fractions diluted into 50 mM Hepes, pH 7.4, 100 μl of 1% BSA (v/v), 100 μl of 125I-CTX stock solution diluted to correspond to approximately 20,000 cpm 125I-CTX (or approximately 4.2 fmol), 100 μl of 500 nm stock solution of unlabelled CTX (Peninsula Laboratories) or 50 mM Hepes, pH 7.4 buffer in 1 ml final volume. The samples were incubated at room temperature for 30 min, filtered over 0.45-μm GFL glass fiber filters, and rapidly washed as described. The filters were counted for 1 min in Packard Cobra autogamma counter. Scatchard analysis of 125I-CTX binding to membranes was carried out under similar conditions with protein assayed at dilutions that supported approximately 2,500 cpm of specific 125I-CTX bound (1–200 μg/ml). The amount of protein present in each assay was as follows: adult, 2 μg; P0, 25 μg; and E18 rat brain, 200 μg. Unlabeled 1 μM pepstatin A was added to a sample to 0.1 μM in the presence of constant 125I-CTX. Data presented are mean ± S.D. from three determinations done in duplicate.

Immunoprecipitation of N-type VDCC—The N-type VDCC was solubilized from P2 brain and P14 adult rat forebrain as described previously (21) with the following modifications: the N-type VDCC from P2 brain and P14 forebrain were solubilized directly from membranes using 0.75% CHAPS. Following centrifugation, the solubilized preparations were assayed for 125I-CTX binding. Approximately 9,000–12,000 cpm of specific 125I-CTX receptor activity (200 μl of the solubilized preparation) was added to individual microcentrifuge tubes that contained 20,000–40,000 cpm 125I-CTX, 0.1% BSA, plus protease inhibitors in 50 mM Hepes, pH 7.4. Identical reactions were also carried out in the presence of 50 μM unlabeled CTX to determine nonspecific binding of 125I-CTX. Following a 30-min incubation at room temperature, antibody was added in a total volume of 100–200 μl of TBS and left to incubate at room temperature for 1 h. After this time, 50 μl of protein A-Sepharose 4B (final concentration of 0.6 mg/ml) was added to each sample and rotated in the cold room overnight. 125I-CTX binding to the soluble fraction was determined by directly filtering 1.1 ml of the sample through 0.5% PEI glass fiber filters that were washed 3 × with 1 ml of 50 mM Heps/EGTA, and 50 μl of 2× sample buffer were added to the protein A beads. The samples were counted in a gamma counter for 1 min.

Quantification of VDCC Subunits by 125I-Protein A Overlay or 125I-IgG Anti-rabbit IgG—125I-Protein A and 125I-IgG anti-rabbit IgG were diluted in 50% of 3% BSA in 1× TBS. Filters previously blocked with...
5% milk in 1× TBS and probed with primary antibody were washed with 1× TBS for 15 min and then washed two additional times for 5 min. The washed filters were incubated in 50 ml of either the 125I-protein A, 3% BSA solution (approximately 500 cpm/µl) or the 125I-IgG, 3% BSA solution (approximately 30–50 cpm/µl) for 2 h at room temperature with constant shaking. Following this incubation, the 125I solution was removed, and the filters were washed 3–5 times with TBS (5 min each). The approximate wash volume was 50 ml. The filters were blotted with paper towels and exposed to film at −80 °C with the aid of intensifying screens. The position of the antigen was determined relative to the exposed film, and the corresponding band on the filter was cut and counted. Slices that corresponded to nonspecific areas of the filter were also counted and subtracted from the signal. The data were obtained from multiple determinations done in duplicate.

**General Methods and Data Analysis**—The gels were transferred to nitrocellulose at 0.45 A for 17–22 h. The filter was incubated in 5% powdered milk in TBS + 0.01% sodium azide + 0.05% Tween and blocked for either 3 h at 37 °C with constant shaking or overnight at 4 °C in the cold room. The primary antibody was diluted in 3% BSA, 1× TBS and incubated with the filter overnight at 4 °C. The filters were washed 3 times in TBS at room temperature. The secondary antibody was diluted to 1/10,000 in 3% BSA, 1× TBS, and the filter was incubated for 45 min at room temperature with constant shaking. The filters were washed as before. The antigen was visualized using ECL. Membrane protein and soluble protein were measured by the Pierce BCA assay. Bovine serum albumin was used as a standard in all cases, and all samples were normalized with respect to buffer and detergent composition. Gel electrophoresis was carried out on polyacrylamide gels according to standard procedures (45). Gel electrophoresis was carried out using a 4% stacking gel and a resolving gel of appropriate porosity (see figure legends) according to standard procedures. All samples were incubated with 5× SDS-PAGE sample buffer (325 mM Tris, pH 7.0, glycerol (25% v/v), mercaptoethanol (25% v/v), SDS (10%) without boiling. Staining of proteins in polyacrylamide gels was Coomassie Blue (0.05%), 50% methanol, 10% acetic acid. The results are expressed as mean ± S.D. Statistical analysis was evaluated by a paired t test or one-way analysis of variance with Tukey’s or Dunnett’s post hoc test. p values less than 0.05 were considered significant.

**RESULTS**

**Expression and Properties of the N-type α1B Subunit during Rat Brain Development**

**Immunological Characterization of Rat α1B—**Anti-peptide antibodies to the II–III intracellular loop of the rat α1B subunit were raised to two distinct epitopes. The first antibody, Ab CW14, was raised to an epitope present in all α1B subunits cloned to date (34). The second antibody, Ab CW8, was raised to an epitope present only in the rat α1B sequence (37). Both antibodies were analyzed in parallel to characterize the structure of the endogenous α1B as it relates to the original rat α1B cDNA clone (37).

The pan specificity of Ab CW14 was verified by its detection of 230/210-kDa proteins in HEK293 cell line G1A1 stably expressing the human α1B (46, 47) and a single 230-kDa protein in rat, rabbit, and mouse forebrain samples (Fig. 1A). Both the 230/210-kDa proteins in HEK293 cell line G1A1 were determined to bind 125I-IgG detected by 125I-IgG detected by photoaffinity labeling with derivatized 125I-IgG (21) (data not shown). The specificity of Ab CW8 for the rat epitope was evidenced by its reaction with the 230-kDa protein expressed only in rat brain. To verify the expression of the rat α1B as the isoform expressed in representative developmental samples, we quantified the amount of α1B detected by Ab CW8 and Ab CW14 in brain homogenates of E18 and adult rat. As shown in Fig. 2A, α1B detected by Ab CW14 increases from E18 to adult. The signal obtained with Ab CW8 is very similar to that obtained with Ab CW14. The signals were quantified using 125I-protein A, and the results indicate that the epitopes targeted by Ab CW8 and Ab CW14 are equivalently expressed.

**Developmental Expression of α1B Subunit in Rat Brain—**The change in expression of α1B subunit presented in Fig. 2 leads us to use Ab CW14 to evaluate the level of expression of α1B in postnatal (P0–P14) rat brain and adult forebrain homogenates. As shown in Fig. 3A, the level of expression of α1B increases during rat brain development. The results of similar Western
blots were quantified using $^{125}$I-IgG and evidenced statistically significant increase in expression of $\alpha_{1B}$ subunit throughout the period of postnatal development (Fig. 3B).

Characterization of N-type VDCC $^{125}$I-CTX Binding during Three Rat Developmental Stages—We used radioligand binding assays of the peptide neurotoxin, $^{125}$I-CTX, to determine if there were developmental differences in either the density or affinity of $^{125}$I-CTX binding to N-type VDCC. Radioligand binding assays using $^{125}$I-CTX were carried out on homogenates from embryonic day 18 brain (E18), newborn rat brain (P0), and adult rat forebrain. The results of the pseudo-Scatchard analysis are presented in Table I. A comparison of the $B_{\text{max}}$ values indicates significantly less $^{125}$I-CTX binding at E18 ($B_{\text{max}} = 0.008 \pm 0.002$ pmol/mg) versus adult rat forebrain ($B_{\text{max}} = 1.8 \pm 0.8$ pmol/mg). The high affinity $^{125}$I-CTX binding site diagnostic for the N-type VDCC is present throughout development ($K_d$ of 11.7, 21.7, and 8.3 pmol/mg for $^{125}$I-CTX binding to adult rat forebrain, P0 rat brain and E18 rat brain, respectively).

We then used the data obtained by quantitative Western blot analysis and Scatchard analysis to investigate the fraction of $\alpha_{1B}$ subunits in E18 and P0 that could support high affinity binding relative to the adult sample. This comparative analysis, which assumes that the ratio of $\alpha_{1B}$ subunits in adult is unity, identifies differences in the ratio of $\alpha_{1B}$ protein present in E18 and P0 brain as quantified by $^{125}$I-protein A versus the density of $^{125}$I-CTX binding sites (Table I). In E18 brain, ratio of $\alpha_{1B}$ binding sites is 25:1, whereas in P0 the ratio is 12.5:1. The discrepancy between the expression of $\alpha_{1B}$ protein and 125I-CTX binding sites suggests a population of $\alpha_{1B}$ present at E18 and P0 that does not support high affinity 125I-CTX binding.

Expression and Assembly of VDCC $\beta$ Subunits during Rat Brain Development

Expression of $\beta$ Subunit Isoforms during Rat Brain Development—The level of expression of calcium channel $\beta$ subunits in developing rat brain was then analyzed to evaluate possible changes in the pool of available $\beta$ isoforms. Thus, we used Ab CW24, an antibody raised to an epitope shown to be present in all $\beta$ subunits cloned to date (34), to probe a Western blot of homogenates prepared from developing rat brains. These experiments revealed two populations of $\beta$ subunits that could be easily resolved by SDS-PAGE as follows: $\beta$ subunits with apparent molecular masses of $\geq 80$ kDa comprised of $\beta_{1B}$ and $\beta_{2}$ isoforms, and smaller $\beta$ subunits ($65$ kDa) comprised of $\beta_{3}$ and $\beta_{4}$ isoforms (34). As shown in Fig. 4, direct comparison of these two populations of $\beta$ subunits indicated no statistically significant change in the level of expression of the larger $\beta$ subunits between P0 and adult, whereas the smaller $\beta$ subunits evidenced a significant 3-fold increase in expression. It is important to note that the histogram reflects the expression of the $\beta_{1B}$ isoform detected in P0 through adult with the increase commencing at the time of cerebellar maturation (P7). The $\beta_{2}$ isoform (Fig. 5B) and $\beta_{3}$ isoform (Fig. 5C) were expressed at constant levels. Interestingly, there is a 10-fold increase in the level of expression in the $\beta_{4}$ isoform in adult brain compared with P0 that also commences at the time of cerebellar maturation (P7). The $\beta_{4}$ isoform is expressed in P0 through adult (Fig. 5C). The increase in expression of the $\beta_{4}$ detected in the P7–P14 interval in these rat brain samples parallels the increased expression of the $\beta_{4}$ mRNA in cerebellum as determined by in situ hybridization (48).

It is evident from these results that the reactivity of the $\beta$-generic antibody Ab CW24 for the smaller $\beta$ subunits ($\beta_{3} + \beta_{4}$) accurately reflects the sum of their expression as verified by the isoform-specific antibodies (Fig. 5C). It is interesting that the results obtained using Ab CW24 to monitor the expression of a larger $\beta$ subunit are seemingly discrepant when compared with the results obtained using the anti-$\beta_{1B}$- and anti-$\beta_{2}$-specific antibodies. Therefore, our observations may indicate $\beta$ isoform(s) that are not detected by our anti-$\beta_{1B}$ and anti-$\beta_{2}$ antibodies at P0–P7.

Heterogeneity of $\alpha_{1B}/\beta$ Complexes during Rat Brain Development—The N-type VDCC has been previously purified from rat (21, 49, 50) and rabbit forebrain (51), where its density of expression is 2.5-fold higher than in cerebellum or other brain regions (52). Therefore, N-type VDCC were solubilized from P2 brain, P14, and adult rat forebrain and immunoprecipitated with the $\alpha_{1B}$ antibody Ab CW14, and the generic anti-$\beta$ subunit antibody Ab CW24. The N-type VDCC present at early stages of rat brain development (P2 and P14) can be quantitatively immunoprecipitated by antibodies to the $\alpha_{1B}$ (Fig. 6). However, only 60–70% of all $^{125}$I-CTX binding can be immunoprecipitated by the generic antibody reactive toward all $\beta$.
subunits (Ab CW24). These data identify a statistically significant fraction of α1B subunit protein in immature brain that are not tightly associated with a β subunit. In contrast, the N-type VDCC extracted from adult rat can be quantitatively immunoprecipitated by both anti-α1B and Ab CW24.

We then used β isoform-specific antibodies to immunoprecipitate the 125I-CTX-labeled N-type VDCC from P2, P14, and adult rat forebrain. As shown in Fig. 7, β subunit isoforms are differentially associated with the N-type VDCC during rat brain development. At P2, the antibody to the β1b subunit immunoprecipitated 37% (±5) of 125I-CTX binding, whereas the anti-β3 antibody and β2 antibodies immunoprecipitated 27% (±3) and 1% (±0.9), respectively. There was no specific immunoprecipitation of 125I-CTX by the anti-β4 antibody, which gave a signal comparable to control samples without receptor (Fig. 7). The sum of the total 125I-CTX immunoprecipitated from P2 by anti-β1b, -β2, and -β3 antibodies (65%) is in good agreement with the results obtained using Ab CW24 alone. At P14, we observed a shift in the contribution of the various β subunits to N-type VDCC channel complexes with the β3 isoform accounting for 32% (±5.8) of total N-type complexes and the β1b accounting for 13% (±2.0). The β subunit was associated with 14% (±9.0) of total N-type VDCC at P14 versus 25% (±6.3) of total N-type VDCC in adult. The sum of the total 125I-CTX immunoprecipitated by anti-β1b, -β2, -β3, and -β4 antibodies (59%) at P14 also parallels the results obtained using Ab CW24 alone.

The contribution of β subunit isoforms to the N-type VDCC solubilized from adult rat brain evidenced the following distribution: β1b (32 ± 9.0%), β2 (8 ± 2.8%), β3 (55 ± 5.6%), and β4 (25 ± 6.3%) isoforms. In comparison to the previously published findings, our results suggest a greater fractional contribution of the β1b and a lesser contribution of the β4 to the adult rat N-type VDCC (Fig. 7) and further support the observation that the adult N-type VDCC can be comprised of β1, β3, and β4 isoforms (Fig. 7) (22). The sum of the total 125I-CTX immunoprecipitated from adult brain by anti-β1b, -β2, -β3, and -β4 antibodies (60%) is similar to the amount of 125I-CTX immunoprecipitated by either Ab CW14 or Ab CW24.

**DISCUSSION**

In the past several years, investigators have struggled to make physiological sense of the vast diversity of VDCC subunit isoforms present in neural tissue. The dramatic changes that occur in calcium conductances during neuronal maturation suggest an underlying and equally dramatic change in the diversity and subtype of VDCC. Indeed, in cultured cerebellar Purkinje cells, studies have demonstrated that changes in calcium conductances were critical for neuronal maturation (53). More recently, important changes were observed in the expression and differential contribution of N-type and P/Q-type VDCC during synapse formation in cultured neurons (30–32). Similarly, changes in calcium channel currents were observed at different stages of embryogenesis (54). These studies were among the first to suggest regulation of α1 subunit expression as a possible mechanism for establishing diversity in calcium signaling during development.

In support of previous findings on the role of N-type VDCC in neuronal development, we have demonstrated an increase in the expression of α1B during rat brain development (Figs. 2 and 3) which does not correlate with the acquisition of 125I-CTX-binding sites (Table I). The parallel increase in reactivity of Ab

| Sample                  | Kd (pm) | B_max (pmol/mg) | Relative density of 125I-CTX-binding sites | Relative amount of α1B protein | Ratio α1B/binding sites |
|-------------------------|---------|----------------|-------------------------------------------|-------------------------------|-------------------------|
| Adult rat forebrain     | 11.7 ± 0.5 | 1.8 ± 0.8       | 1.0                                       | 1.0                           | 1.0                     |
| Neonatal rat brain (postnatal day 0) | 21.7 ± 2.0 | 0.05 ± 0.01     | 0.028                                     | 0.35                          | 12.5                    |
| Embryonic rat brain (E18) | 8.3 ± 2.0  | 0.008 ± 0.002   | 0.004                                     | 0.10                          | 25.0                    |

* Data represent three independent determinations carried out in duplicate.

The amount of expressed α1B protein relative to adult rat brain was determined from the quantified Western blot analysis presented in Fig. 2.

**FIG. 4.** Expression of all β subunit isoforms as detected by pan-specific antibody Ab CW24 during development. Tissue was obtained from animals at different ages, collected into sample buffer, and resolved by SDS-PAGE upon a 10% gel. The amount of protein was 150 μg/lane. The gels were transferred to nitrocellulose and probed with Ab CW24 (1/100 dilution, A) and visualized by ECL. In experiments conducted on similar samples, the transferred antigens were detected using 125I-labeled IgG, exposed to film. The bands inclusive of the larger β subunits β1 + β2 (B) and the smaller β subunits β3 + β4 (C) were excised and counted in a gamma counter. The data were obtained from four determinations done in duplicate and normalized to the signal obtained at P0 (β1b + β2 = 277 ± 88 cpm band; β3 + β4 = 210 ± 46 cpm band). #: denotes p values less than 0.05.

**TABLE I**

Comparison of the density of high affinity CTX-binding sites with the relative level of α1B subunit protein
CWS and Ab CW14 throughout development indicates that the α1B present in these samples contains two epitopes originally identified in the rat α1B cDNA (37). Functionally different isoforms of N-type VDCC have been identified in rat sympathetic ganglia (38) and embryonic (E17) tissues (55); however, these variants contain both Ab CW14 and Ab CW8 epitopes. The report of splice variants in the II–III loop of α1A subunit suggests caution in dismissing the existence of additional α1B variants. It is important to note that the region defined by Ab CWS is coincident with a region of diversity in the α1A variants (56).

In this study we have described a population of α1B detected in embryonic and P0 brain samples that does not support high affinity 125I-CTX binding (Table I). This property is reminisc-
cent of the unassembled a1B expressed in heterologous systems in the absence of α1/β and β subunits (57). Also, studies on the developmental expression of the sodium channel have noted the acquisition of high affinity [3H]saxitoxin binding occurring in parallel with channel assembly (58). Our results suggest that the acquisition of high affinity CTX binding during rat brain development (Table I) occurs by a mechanism that is similar to the sodium channel and reflects the conversion of the pool of unassembled α1B (present at E18 and P0) to mature α1B that are assembled with component α1/β and β.

This is the first report to demonstrate regulation of the β subunit component of a specific VDCC (N-type) during neurodevelopment. Previously, correlations in the localization and density of α1 and β isoform mRNA in adult, embryonic, and postnatal rat brain predicted likely α1/β complexes (48), but there were no conclusions reached in the case of the α1B/β complex. Another study concluded that there was no evidence for subunit switching in developing hippocampus as they targeted only the α1β/β3 heteromultimers for analysis (59). In this study we have identified clear developmental trends in α1B/β composition from a population predominant in α1B + β1b complexes (P2) to a population comprised of α1B + β3 and α1B + β4 complexes in mature rat brain.

The discrepancy between the fraction of 125I-CTX-labeled N-type VDCC that could be identified in P2 and P14 by anti-α1B antibodies versus those identified by anti-β antibodies (Fig. 6) suggests the presence of a β isof orm that is not identified by our pan-specific antibody nor recognized by β-specific antibodies. Alternatively, there may be some structural lability in the physical coupling of β to the α1B in early development. A conserved site on the intracellular I–II loop has been identified in all α1 subunits that bind β (60, 61). In vitro studies have determined nanomolar affinity between all β and the I–II loop interaction domain in a binding reaction that is not affected by calcium or protein kinase C phosphorylation (61). Recently, a second site that mediates α1B/β interaction has been identified at the C terminus of the α1E, α1A, and α1B (62, 63); however, the affinity for β at this site has not yet been determined. The occupancy of these two sites on α1B by β adds another dimension to α1B/β heterogeneity.

This age-dependent structural heterogeneity in N-type VDCC is anticipated to have a functional counterpart based upon recombinant studies of β isoforms co-expressed with the α1C (26, 64), α1C (65), and α1E (65). We anticipate significant cellular consequences related to differential expression of α1B/β complexes as β is required for the assembly, stabilization, and targeting of α1 (66–69) in addition to influencing the kinetic and modulatory properties of α1 (25, 26, 64, 70–72). The magnitude and duration of calcium entry via specific α1B + β subunit complexes and their localization may contribute to distinct signals that regulate neurite extension, synapse formation, and growth cone collapse (7–10).

Differential modulation of the N-type VDCC by protein kinases is another property that may result from the assembly of a specific β with the α1B. The β1b (41) and β2 (25) isoforms contain consensus sites for protein kinase A modification and no consensus site for tyrosine kinases; conversely, the β3 isoforms contain consensus sites for tyrosine kinases and no consensus site for protein kinase A (43). In the β4, both protein kinase A and tyrosine kinases consensus sites are absent (39). The functional consequences of assembling different isoforms of β subunit that act as substrates for different protein kinases into the N-type α1B/β complex suggest a possible mechanism for coupling specific intracellular signaling pathways to N-type VDCC.

The report of changes in α1 and β mRNA levels during development (48) and our results (Fig. 5) suggest differential regulation of β isoform expression. The effect of neurotrophic agents upon changes in calcium currents (73–75) and the specific expression of α1B and β isoforms have been reported (33, 34) in established neurotypic cell lines. However, these in vitro models pale in comparison to the complexity of developing rat brain. The trends identified in this study require further scrutiny at the cellular level to unravel the mechanisms that underlie both β expression and its association with α1B. We would like to address the question whether the assembly of β with an α1 subunit is reflective of specific assembly processes or simply reflects the fractional contribution of a β isoform relative to the pool of total available β. As demonstrated by our results, with the exception of the β4 subunit, there is no straightforward relationship that emerges between the relative contribution of β1b, β2, and β3 to the pool of available β subunits (Fig. 5) and the contribution of that isoform to the assembled N-type VDCC (Fig. 7).

A comparison of the heterogeneity in α1B/β complexes through development with changes in the pool of available β isoforms suggests several cellular strategies are at play which regulate α1B/β subunit assembly. The β1b subunit is detected in P2 homogenate at a fraction of its maximal adult level of expression (Fig. 5A); however, the relative amount of β1b associated with the P2 N-type VDCC is similar to the adult N-type VDCC (Fig. 7). These findings indicate a relative enrichment of the β1b in the P2 N-type VDCC complex relative to adult N-type VDCC. As previously shown, the β2 subunit is detected throughout rat brain development (Fig. 5B), yet antibodies to the β2 isoform do not immunoprecipitate 125I-CTX binding from P2 or P14 samples. In the adult samples, less than 10% of all 125I-CTX-labeled N-type VDCC were immunoprecipitated by anti-β2 antibody. These findings suggest the active exclusion of the β2 subunit from the N-type VDCC complex. Similar to β expression, the β3 subunit is also expressed at a relatively constant level in the interval between P0 and adult. However, in a manner similar to the β4, there is a statistically significant increase in its association with the α1B during development. It is very interesting to note that the onset of increased β1b and β4 expression occurs at the beginning of a well defined period of axonal outgrowth, infiltration, and synapse formation in the rat neocortex which occurs in the first 2 weeks of postnatal life (76–78).

Significantly, the 10-fold increase in the expression of the β4 isoform between P0 and adult and its parallel association with the α1B through development is in striking contrast to the other β isoforms and identifies a property unique to the β4. Interestingly, there has been a report that demonstrates the importance of the β4 isoform. Analyses of the mutation that underlies the mouse lethargic phenotype (lh/lh), a model of epilepsy which does not exhibit any neurodegeneration or other neurohistological abnormalities (79), have identified an insert in the β4 gene that leads to a truncated gene product. Specifically, the truncation of the β4 subunit protein eliminates the α1, binding domain as well as more than 60% of the protein (80). The study by Burgess et al. (80) is significant as it is the first to implicate VDCC auxiliary subunit as the basis for a neurological disease. The co-localization of the α1B/β heterogeneity and growth cone collapse (7–10).

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consequence of a defect in a single β subunit intimates a role for the β4 isofrom that cannot be complemented by the expression of the other β isofroms. Although it is clear from our study that the β4 isofrom is unique among β subunits in its magnitude of induction and temporal pattern of expression, it would be premature to suggest that it is the absence of the β4 isofrom per se that gives rise to the epileptic lh/lh phenotype. Alternatively, one might consider that alterations in the level of expression of full-length β4 in lh/lh mice may induce profound compensatory effects upon the regulation of expression of the other β isofroms.

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