N-Type Calcium Channels in the Developing Rat Hippocampus:
Subunit, Complex, and Regional Expression

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The expression of multiple classes of voltage-dependent calcium channels (VDCCs) allows neurons to tailor calcium signaling to functionally discrete cellular regions. In the developing hippocampus a central issue is whether the expression of VDCC subtypes plays a role in key phases such as migration and synaptogenesis. Using radioligand binding and immunoblotting, we show that some N-type VDCCs exist before birth, consistent with a role in migration; however, most N-VDCC subunit expression is postnatal, coinciding with synaptogenesis. Immunoprecipitation studies indicate that the increased expression of N-VDCCs in early development occurs without subunit switching because there is no change in the fraction of β3 subunits in the N-VDCC α1B−β3 heteromers. Fluorescence imaging of cell surface N-VDCCs during this period reveals that N-VDCCs are expressed on somata before dendrites and that this expression is asynchronous between different subfields of the hippocampus (CA3–CA4 before CA1–CA2 and dentate gyrus). Our data argue that N-VDCC expression is an important cue in the genesis of synaptic transmission in discrete hippocampal subfields.

Key words: rat; development; hippocampus; pyramidal neurons; voltage-dependent calcium channels; subunits; dendrites; ω-conotoxin

In neurons, voltage-dependent Ca2+ channels (VDCCs) orchestrate diverse functions, including neurotransmitter release (Wheeler et al., 1994; Dunlap et al., 1995; Scholz and Miller, 1995), excitability (Llinás and Sugimori, 1979; Llinás, 1988), and gene expression (Bading et al., 1993). Growing evidence indicates that VDCCs are also important in establishing the functional cytoarchitecture of the brain (Llinás and Sugimori, 1979; Mills and Kater, 1990; Vigers and Pfenninger, 1991; Komura and Rakic, 1992; Johnson and Deckwerth, 1993; Spitzer et al., 1994), but their precise role is uncertain. In situ hybridization studies have revealed mRNAs encoding VDCCs, which mediate high voltage-activated (HVA) Ca2+ currents in those regions of pre- and postnatal brain undergoing active proliferation and migration (Tanaka et al., 1995). In contrast, electrophysiology in vitro and in vivo suggests that neurons only express HVA currents once the cells are polarized and are no longer migrating (Peacock and Walker, 1983; Yaari et al., 1987; Reece and Schwartzkroin, 1991; Scholz and Miller, 1995). One explanation is that VDCC expression is phasic and mirrors, or even orchestrates, key developmental events (Jacobson, 1991). Unfortunately, how VDCCs might contribute to such events is complicated by their diversity.

Until recently, VDCCs were classified according to their biochemical and pharmacological characteristics into T, L, N, or P/Q subtypes. Molecular cloning, expression, and biochemical studies now show that this scheme is too simplistic (Hofmann et al., 1994; Dunlap et al., 1995). In brain, VDCCs are large (>400 kDa) heteromers composed of an α1, α2/δ, and β subunit (Wagner et al., 1988; Hell et al., 1993, 1994; Witcher et al., 1993; Hofmann et al., 1994; Leveque et al., 1994). Expression of VDCC gene products in Xenopus oocytes (Mori et al., 1991; Williams et al., 1992a) or transfected cells (Williams et al., 1992b; Fujita et al., 1993; Stea et al., 1993) shows that α1 subunits contain the ion channel pore, whereas the auxiliary α2/δ and β subunits modulate optimal cell surface expression and channel kinetics (Brust et al., 1993; Castellano et al., 1993; Isom et al., 1994; Olcese et al., 1994). In rat brain, the α1 subunits are encoded by at least five discrete classes (A–E) of cDNA. Although α1A and α1H correspond to P/Q- and N-VDCCs, respectively (Westenbroek et al., 1992, 1995; Witcher et al., 1993; Hell et al., 1994; Stea et al., 1994), the α1C and α1O classes form L-type VDCCs (Hell et al., 1993). Further diversity of VDCCs arises through multiple genes encoding the β subunits and, in many cases, alternative splicing of the α1 and β RNA transcripts (Hofmann et al., 1994; Dunlap et al., 1995). In contrast, α2/δ subunits exist as single splice variants in rat brain (Kim et al., 1992). What function does such diversity serve? Expression studies indicate that the precise composition of gene products in the α1, α2/δ, and β-VDCC heteromers defines their pharmacology and biophysical characteristics (Hofmann et al., 1994; Dunlap et al., 1995). However, specific VDCC subtypes also have unique patterns of expression in discrete brain regions and even within individual neurons (Jones et al., 1989; Robitaille et al., 1990; Westenbroek et al., 1990, 1992, 1995; Cohen et al., 1991; Hell et al., 1993; Haydon et

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al., 1994; Mills et al., 1994; Elliott et al., 1995). Thus, neurons may exploit VDCC diversity to tailor voltage-dependent Ca$^{2+}$ influx in discrete functional compartments (Elliott et al., 1995). Consequently, we hypothesize that changes in functional demand experienced by developing neurons could be reflected in the dynamics of specific VDCC complex expression.

We now provide a comprehensive analysis of the expression of the neuron-specific N-type VDCC from embryonic to adult stages in rat hippocampus. This VDCC has important roles in neurotransmitter release (Robitaille et al., 1990; Cohen et al., 1991; Haydon et al., 1994; Wheeler et al., 1994; Dunlap et al., 1995; Scholz and Miller, 1995), dendritic function (Mills et al., 1994), and neuronal migration (Komura and Rakic, 1992). Via expression (Dubel et al., 1992; Williams et al., 1992b; Brust et al., 1993; Fujita et al., 1993; Stea et al., 1993) and biochemical studies (Wagner et al., 1988; Westenbroek et al., 1992; Wither et al., 1993; Leveque et al., 1994; Scott et al., 1996), it seems that most N-VDCCs in adult brain are $\alpha_{1B}$, $\alpha_2$, and $\beta_2$ heteromers, although subpopulations containing $\beta_1$ or $\beta_2$ rather than $\beta_3$ subunits also may exist (Scott et al., 1996). Using site-directed antibodies and selective fluorophore and radioactive labels, we have found that our data support a significant role for N-VDCCs in the development of the nervous system.

MATERIALS AND METHODS

**Synthetic peptides.** Peptides corresponding to residues 582–688 of the $\alpha_{1B}$ (Dubel et al., 1992) sequence (GenBank accession number M92905) and residues 1–15 of the $\beta_1$ (Castellano et al., 1993) sequence (GenBank accession number M88751), plus a C-terminal cysteine for coupling, were synthesized by Vetrogen (London, Ontario, Canada). The identity of the peptides was confirmed by amino acid analysis and mass spectrometry ([M]+, m/z = 1017 and [M]+, m/z = 1829 for the $\alpha_{1B}$ and $\beta_1$ peptides, respectively).

**Preparation of antibodies.** Peptides were coupled to keyhole limpet hemocyanin with the heterobifunctional cross-linker $\varepsilon$-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS; Pierce, Rockford, IL), and the conjugates were dialyzed against PBS. After New Zealand White rabbits were immunized with the conjugates (Division of Comparative Medicine, University of Toronto), antisera were collected and the IgG fraction was enriched by using MAPS affinity chromatography (Bio-Rad, Mississauga, Ontario, Canada). Throughput, antisera were characterized by ELISA and immunoblotting (see below).

**Membrane preparation.** Cortical membranes were prepared (Jones and So, 1993) from Wistar rats (timed-pregnant; Harlan Sprague Dawley, Indianapolis, IN) at E16, E18, and postnatal (P) days 0 (birth), 1.5, 2.5, 3.5, 4, 6, 8, and 10. Hippocampal membranes were prepared likewise. Although subpopulations containing $\beta_1$ or $\beta_2$ rather than $\beta_3$ subunits also may exist (Scott et al., 1996). Using site-directed antibodies and selective fluorophore and radioactive labels, we have found that our data support a significant role for N-VDCCs in the development of the nervous system.

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of the maximal binding ($E_{0.5}$) seen within the first 6 weeks of birth occurred were 7.0 and 3.5 d for cortex and hippocampus, respectively. Direct assays of the $[125\text{I}]\omega$-CgTx–N-VDCC interaction (Fig. 2) showed that these binding changes reflected developmental differences in N-VDCC density rather than toxin binding affinities. Because the $\omega$-CgTx binding assay is not a true equilibrium reaction (Jones and So, 1993), the affinities were obtained by direct assay of the kinetics of toxin association with, and dissociation from, membranes prepared from P0 and P40 hippocampi. At both P0 and P40, $[125\text{I}]\omega$-CgTx binding conformed to a simple bimolecular reaction, for which the kinetics of association ($k_{\text{on}} = 3.0 \times 10^8 \text{ mol}^{-1} \cdot \text{min}^{-1}$ at P0 and P40) or dissociation [$k_{\text{off}} = 5.8 \times 10^{-4} \text{ min}^{-1}$ (P0) and $5.3 \times 10^{-4} \text{ min}^{-1}$ (P40)] were essentially identical and gave very similar $K_d$ values [1.9 pm (P0) and 1.8 pm (P40)].

### Antibody characterization

To resolve N-VDCC expression in detail, we raised specific polyclonal antibodies against the $\alpha_{1B}$ and $\beta_3$ subunits previously shown to form the major N-VDCC complex in brain (Wagner et al., 1988; Dubel et al., 1992; Westenbroek et al., 1992; Williams et al., 1992b; Brust et al., 1993; Fujita et al., 1993; Stea et al., 1993; Witcher et al., 1993; Leveque et al., 1994; Scott et al., 1996). High-titer antisera from rabbits immunized with synthetic peptides deduced from the coding sequences of $\alpha_{1B}$ and $\beta_3$ were assayed by immunoprecipitation and immunoblotting (Fig. 3). Antisera against $\alpha_{1B}$ showed dose-dependent immunoprecipitation of up to 55% of the total $[125\text{I}]\omega$-CgTx binding sites from detergent extracts of adult rat brain membranes (Fig. 3A). Control immunoprecipitations that used preimmune serum, $\alpha_{1B}$ antiserum pretreated with excess competing antigenic peptide (other peptides were ineffective; data not shown), or membranes treated with excess unlabeled $\omega$-CgTx before labeling with $[125\text{I}]\omega$-CgTx all failed to immunoprecipitate $[125\text{I}]\omega$-CgTx binding sites, as expected (Fig. 3A, inset). On immunoblots of adult rat brain, our $\alpha_{1B}$ antibodies to a peptide within the $\alpha_{1B}$ domain II–III linker recognized a band of approximate $M_r$ 220 kDa (Fig. 3B, lane 1), as reported previously (Westenbroek et al., 1992; Hell et al., 1994). This band is identical in size to that obtained with mAb CC18, an anti-N-VDCC monoclonal antibody raised against...
Figure 3. Characterization of α1B (A, B) and β3 (C, D) polyclonal antibodies. A, Immunoprecipitation of [125I]ω-CgTx-labeled N-VDCCs by anti-α1B antibodies. [125I]ω-CgTx-labeled N-VDCCs were solubilized with digitonin (see Materials and Methods), and their interaction with anti-α1B antibodies was demonstrated by the concentration dependence of immunoprecipitation. The data were fit assuming a saturation curve of the form $y = 2254 \times [1 - \exp(-x/134)]$, according to Westenbroek et al. (1992), as above. The value of 2254 dpm corresponds to 55% of the total [125I]ω-CgTx in each reaction. Inset, The specificity of the interaction between anti-α1B antibodies and solubilized [125I]ω-CgTx binding sites was determined by comparing the radioactivity in experimental immunoprecipitations (c) with that in control immunoprecipitations made with preimmune serum (a); control membranes, i.e., those pretreated with excess cold ω-CgTx before radiolabeling (b); competing antigenic peptide (25 μM) (c); and preimmune serum plus competing peptide antigen (25 μM) (d). B, Antibodies against α1B recognize a band of ~220 kDa on immunoblots (lane 7) and several bands of lower molecular weight. Staining of the 220 kDa, but not the minor bands, was eliminated if the antibody was treated first with competing competing α1B peptide antigen (40 μM) (lane 2). The band recognized by our α1B antibodies is identical in molecular weight to that recognized by monoclonal antibodies to an α1B fusion protein (lane 3) (Gift of Dr. V. Lennon, Mayo Clinic, Rochester, MN), persists on purification of digitonin-solubilized N-VDCCs with heparin–agarose (Fig. 3 inset b, c, d, e), and is intensified after an additional wheat germ affinity chromatography step (lane 5). The 220 kDa bands in lanes 4 and 5 both can be displaced by pretreatment of the α1B antibody with competing peptide (lanes 6 and 7, respectively). Blots were analyzed with MAPS-purified α1B antibody (10 μg/ml) and detected by ECL (see Materials and Methods). C, Immunoprecipitation of [125I]ω-CgTx-labeled N-VDCCs by anti-β3 antibodies. Digitonin solubilized [125I]ω-CgTx-labeled N-VDCCs immunoprecipitated as described for anti-α1B antibodies (A, above), and the data were fit to the saturation equation $y = 1717 \times [1 - \exp(-x/48)]$, as above. The value 1717 dpm corresponds to 42% of the total [125I]ω-CgTx sites in each reaction. Inset, Specificity of the β3 immunoprecipitations, determined as in A, inset. Experimental immunoprecipitations (f) were compared with the following controls: control membranes (a) (see A, inset b), competing antigenic peptide (25 μM) (b), control membranes plus competing peptide (c), immunoprecipitates with no primary antibody (d), control membranes and no primary antibody (e), and preimmune serum (g). D, Antibodies against β3 recognize a band of 55 kDa on immunoblots (lane 1), which can be displaced completely by competing peptide antigen (lane 2) (40 μM). Molecular weights were derived from prestained molecular weight standards (arrowheads at left).

a fusion protein corresponding to the entire α1B II–III linker (Scott et al., 1996) (Fig. 3B, lane 3). The specificity of the α1B antibody was confirmed by our ability to eliminate the 220 kDa band by pretreatment of the α1B antisemur with competing antigenic peptide (Fig. 3B, lane 2) and by the persistence of the 220 kDa band on affinity purification of N-VDCCs, using wheat germ agglutinin and heparin–agarose (Fig. 3B, lanes 4 and 5; peptide controls, lanes 6 and 7, respectively) (Westenbroek et al., 1992; Witcher et al., 1993). Antisera against β3 also proved effective in selectively immunoprecipitating [125I]ω-CgTx binding sites from detergent extracts of adult rat brain membranes (Fig. 3C and inset). The maximum fraction of [125I]ω-CgTx binding sites that
could be immunoprecipitated by the \( \beta_3 \) antibodies was consistently 76 \( \pm \) 4% (n = 5) of that immunoprecipitated by \( \alpha_{1B} \) antibodies. On immunoblots, our \( \beta_3 \) antibody recognized a single band of \( M_r \) 55 kDa identical to that predicted from the \( \beta_3 \) cDNA (Fig. 3D).

**Immunoblot analysis of the expression of N-VDCC subunits in hippocampal development**

The ontogeny of N-VDCC subunit proteins was determined by immunoblot analysis with anti-\( \alpha_{1B} \) and \( \beta_3 \) antibodies (Fig. 4). Although both mAb CC18 and our polyclonal \( \alpha_{1B} \) antibodies gave similar results, mAb CC18 was used because of its greater sensitivity of detection. Expression of the ubiquitous \( \alpha_\delta/\beta \) subunit was examined with a commercially available monoclonal antibody to the skeletal muscle protein that cross-reacts with that in brain (Upstate Biotechnology, Lake Placid, NY). Expression of the 220 kDa \( \alpha_{1B} \) subunit (Fig. 4A,D) was detectable but very weak at E18 (the earliest stage examined), rose markedly (48-fold increase) after birth to reach a maximum at P10, and thereafter followed a slight decline to adult levels. In contrast, a phasic profile was noted for the band corresponding to the reduced form of the \( \alpha_\delta/\beta \) subunit (\( M_r \) 150 kDa; Gurnett et al., 1996) (Fig. 4B,E). Expression of \( \alpha_\delta/\beta \) was evident as early as E18 (45% of adult levels), waned until P4, and then rose to a plateau level at P10. The expression of \( \beta_3 \) subunits also increased markedly with development but with a profile distinct from either the \( \alpha_{1B} \) or the \( \alpha_\delta/\beta \) subunits. Thus, \( \beta_3 \) subunit expression increased eightfold from birth, attained a maximum level at P25, and then declined slightly to its adult level (Fig. 4C,F).

**Immunoprecipitation analysis of the expression of \( \alpha_{1B}–\beta_3 \) N-VDCC complexes in the developing hippocampus**

Although immunoblotting delineated the ontogeny of the \( \alpha_{1B} \), \( \alpha_\delta/\beta \), or \( \beta_3 \) subunits, the degree of their coassembly was unclear. We therefore analyzed the extent of \( \alpha_{1B}–\beta_3 \) complexation during development by immunoprecipitation assays of solubilized N-VDCCs. The extent of \( \alpha_{1B}–\alpha_\delta/\beta \) complexation was not examined because of poor recognition of native \( \alpha_\delta/\beta \) antibodies in digitonin extracts by anti-skeletal muscle \( \alpha_\delta/\beta \) antibodies, as reported elsewhere (Sakamoto and Campbell, 1991), and the possibility that N-VDCCs contain \( \alpha_\delta/\beta \) isoforms that are not recognized by this antibody (Westenbroek et al., 1992). Because immunoprecipitation assays demand the use of solubilized material, we first tested for developmental differences in the ease of solubilization of \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites. Surprisingly, although the ontogeny of the \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites in each reaction (Fig. 5A, open bars) mirrored that in the membrane binding assays, as expected (see Fig. 1), the ontogeny of solubilized \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites (Fig. 5A, solid bars) showed a shallower profile, with a maximum at P4. The source of this discrepancy became apparent by examining the percentage of \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites solubilized at each developmental day. Between E18 and P10 the fraction of sites that could be solubilized declined by 40% and thereafter remained constant (30% for hippocampus). Thus, the developmental changes in the concentration of solubilized \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites shown in Figure 5A (solid bars) reflect changes in both N-VDCC expression and solubilization. At E18, \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites are poorly expressed but readily solubilized; at later stages \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites are more prevalent but less readily solubilized.

Having defined the developmental profile for the concentration of solubilized \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites, we examined that fraction that could be immunoprecipitated by \( \beta_3 \) antibodies (Fig. 6A). As shown, \( ^{[125]} \text{I} \)-\( \omega \)-CgTx radioactivity in the \( \beta_3 \) immunoprecipitates rose from low levels at E18, peaked at P10, and then declined modestly to P25. From the similar ratio in the normalized \( \beta_3 \) immunoprecipitated and solubilized \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding site profiles between E18 and P25 (Fig. 6A, inset), we infer that changes in the \( \alpha_{1B}–\beta_3 \) complexes parallel those of the entire N-VDCC population. To examine \( \alpha_{1B}–\beta_3 \) complexation further, we immunoprecipitated the \( \beta_3 \) subunits associated with \( ^{[125]} \text{I} \)-\( \omega \)-CgTx-labeled N-VDCCs, using biotinylated \( \alpha_{1B} \) antibodies bound to streptavidin–agarose. Then the \( \alpha_{1B} \) complexes were tested for associated \( \beta_3 \) subunits by immunoblotting with digoxigenylated anti-\( \beta_3 \) antibodies. The use of both biotin and digoxigenin-labeled antibodies was essential in reducing nonspecific bands from IgGs in the immunoprecipitates. As shown in Figure 6B, both the radioactivity corresponding to \( ^{[125]} \text{I} \)-\( \omega \)-CgTx binding sites and \( \beta_3 \) expression in the \( \alpha_{1B} \) immunoprecipitates have very similar developmental profiles (Fig. 6B). This was confirmed further by the similarity in the ratios of the normalized profiles for \( \beta_3 \) expression and \( ^{[125]} \text{I} \)-\( \omega \)-CgTx radioactivity between E18 and P25 (Fig. 6B, inset).

**Visualization of N-type VDCCs on hippocampal pyramidal neurons**

To examine N-VDCC expression in intact neurons, we labeled developing brain slices selectively with Fl-\( \omega \)-CgTx (Mills et al., 1994). In previous studies we have shown Fl-\( \omega \)-CgTx to be a powerful biologically active probe of N-VDCCs, which is internalized only slowly at room temperature (Mills et al., 1994). Thus, Fl-\( \omega \)-CgTx labeling highlights only those N-VDCCs at the nerve cell surface—a significant advantage compared with most immunocytochemical approaches. Hippocampal brain slices sectioned at different days were labeled with Fl-\( \omega \)-CgTx, fixed, and visualized by laser confocal microscopy (Figs. 7, 8). Elsewhere we have shown that labeling of adult hippocampal slices by Fl-\( \omega \)-CgTx can be displaced completely by pretreating the slices with native \( \omega \)-CgTx (Mills et al., 1994). To exclude any developmental artifacts in such control experiments, we compared E18 (Fig. 7A) and P40 (Fig. 7C) hippocampal slices treated with native \( \omega \)-CgTx before Fl-\( \omega \)-CgTx labeling. In both E18 (Fig. 7B) and P40 (Fig. 7D) controls, staining was completely absent. We next examined Fl-\( \omega \)-CgTx labeling in hippocampal slices from E19 to adulthood (Fig. 7E–H). At E19, Fl-\( \omega \)-CgTx labeling was barely discernible in any hippocampal subfield (Fig. 7E). However, by birth (Fig. 7F), surface expression of N-VDCCs was prevalent in the pyramidal layers of subfields CA3–CA4, the somata of the subiculum, and to a lesser extent in the external granule cell layer of the dentate gyrus and stratum radiatum of CA3–CA4. Surprisingly, staining was barely detectable in CA1–CA2 (Fig. 7F, asterisk) at this stage and only began to appear at P4. This period also demarcated the onset of expression of N-VDCCs in the internal granule cell layer of the dentate gyrus (DGii; Fig. 7G). Once initiated, N-VDCC expression increased to adult levels (Fig. 7E) throughout all hippocampal subfields. The lack of Fl-\( \omega \)-CgTx labeling in the CA1 region of rats before P4 (Fig. 7F) did not reflect simply a lack of cells in this region, because individual CA1 neurons adjacent to the subiculum (Fig. 8A, inset) could be filled with the intracellular dye Lucifer yellow at P2 (Fig. 8A) or P3 (Fig. 8B). We next exploited the late onset of Fl-\( \omega \)-CgTx labeling in CA1 neurons to examine the spatiotemporal patterns of N-VDCC expression. Hippocampal slices again were labeled with Fl-\( \omega \)-CgTx, and individual cells in stratum pyramidale were
outlined by Lucifer filling. As shown in Figure 8C, N-VDCC labeling in P7 CA1 neurons was confined mainly to regions containing the somata and very proximal dendrites (<30 μm) despite the disclosure of exuberant dendritic arbors in these neurons by Lucifer yellow (Fig. 8E). In contrast, Fl-ω-CgTx labeling of adult CA1 neurons revealed intense, often punctate, staining extending throughout the entire somatodendritic region, as detailed previously (Mills et al., 1994) (Fig. 8D).
DISCUSSION

We have defined the spatiotemporal expression of N-VDCCs in the hippocampus via selective ligand binding, immunoblotting of N-VDCC subunits, subunit coupling, and fluorescent imaging of channels expressed at the cell surface. Although some $\alpha_{1B}$, $\alpha_2/\delta$, and $\beta_3$ subunits are found at E18, most of their expression occurs...
between P0 and P16, in agreement with our \([^{125}I]\omega-CgTx\) binding data. Nevertheless, N-VDCC expression is not uniform throughout the hippocampus but occurs in subfields CA3–CA4 and the subiculum before dentate gyrus and CA1–CA2. In all regions N-VDCCs appear on somata before dendrites.

Numerous studies have shown that bona fide VDCC function depends on the coexpression of \(\alpha_1\), \(\alpha_2/\delta\), and \(\beta_3\) subunits (Brust et al., 1993; Stea et al., 1993; Isom et al., 1994; Olcese et al., 1994; Gurnett et al., 1996). The presence of \(\alpha_1\), \(\alpha_2/\delta\), and \(\beta_3\) subunits in the prenatal hippocampus argues that embryonic N-VDCCs may

Figure 7. Distribution of N-VDCCs in the developing rat hippocampus, as determined by Fl-\(\omega\)-CgTx labeling. Hippocampi were sectioned, labeled with Fl-\(\omega\)-CgTx, and imaged at low power by confocal fluorescence microscopy, as described (see Materials and Methods). A–D, Control experiments reveal lack of fluorescence (B, D) in hippocampal slices pretreated with \(\omega\)-CgTx before labeling with Fl-\(\omega\)-CgTx at both P0 (A, B) and P40 (C, D). A and C show phase micrographs corresponding to the slices in B and D. Scale bar in C, 500 \(\mu\)m. E–H, Distribution of fluorescence in hippocampal slices labeled with Fl-\(\omega\)-CgTx. E, Hippocampus at E19; note absence of marked staining. F, Hippocampus at P0; note relative absence of staining in subfields CA1–CA2 (asterisk) and the dentate gyrus, as compared with CA3–CA4 and the subiculum (Su). G, At day 4, labeling is detected in the somata and dendrites of all subfields, except the internal granule cell layer of the dentate gyrus (DGi). H, Labeling of adult hippocampus by Fl-\(\omega\)-CgTx is now evident in all fields and is consistently higher on the somata than in the dendrites. DGe, Dentate gyrus. All measurements were replicated in at least five separate experiments.
be functionally competent; nevertheless, the levels of both $\alpha_{1b}$ and $\beta_3$ subunits are very low (<5% of adult levels) at E18. In contrast, $\alpha_2 / \beta$ subunits are much more prevalent at E18 (45% of adult levels) than either $\alpha_{1b}$ or $\beta_3$ subunits, and their expression is phasic. These data presumably reflect complexation of the $\alpha_2 / \beta$ subunits with other non-N-type VDCCs (Dunlap et al., 1995; Liu et al., 1996a), the expression patterns of which are tailored to the developing prenatal hippocampus. Likewise, the more sustained expression of $\beta_3$, as compared with $\alpha_{1b}$, subunits at later stages of development is rationalized most simply via the association of the $\beta_3$ subunits with other non-$\alpha_{1b}$ subunits, notably $\alpha_1$, known to be expressed in adult brain (Liu et al., 1996a). Of greater interest is whether the degree of $\alpha_{1b}-\beta_3$ complexation changes in development. Changes in heteromer composition in development are well documented (Sheng et al., 1994; Murray et al., 1995) and would be especially significant for VDCCs because the $\alpha_{1b}-\beta_3$ subunit interaction is known to be promiscuous (De Waard et al., 1995; Liu et al., 1996a; Scott et al., 1996) and can be displaced by interaction with G-proteins (De Waard et al., 1997; Zamponi et al., 1997). Moreover, multiple $\beta$ subunits can exist in individual cell types (Liu et al., 1996b), and different $\beta$ subunits confer discrete kinetic characteristics to VDCCs (De Waard and Campbell, 1995). Nevertheless, our immunoprecipitation data clearly indicate that the ratio of $\alpha_{1b}-\beta_3$ subunits remains constant between E18 and P40 despite changes in the absolute levels of the $\alpha_{1b}-\beta_3$ complexes. The enhanced expression of N-VDCCs without accompanying changes in subunit composition that we observed in brain also has been seen during NGF-induced differentiation of PC12 cells in culture (Liu et al., 1996b). Together, these data suggest that N-VDCC subunit expression and assembly are highly coordinated.

Throughout, we failed to detect major (>5%) excursions from the anticipated sizes of the $\alpha_{1b}, \alpha_2 / \delta$, or $\beta_3$ subunits, indicating that they do not undergo extensive processing during development. Occasionally, minor bands were seen for $\alpha_{1b}$, suggesting that other variants may exist besides the 220 kDa subunits reported previously (Westenbroek et al., 1992; Hell et al., 1994) or that $\alpha_{1b}$ subunits undergo extensive differential post-translational modifications or proteolysis. The lower size of the $\alpha_{1b}$ subunits determined by SDS-PAGE, as compared with the 262 kDa predicted from the corresponding cDNA (Dubel et al., 1992), is typical of $\alpha_{1b}$ (Westenbroek et al., 1992) and other $\alpha_1$ VDCC subunits and has been attributed to anomalous migration in 5% gels (Hell et al., 1993).

Particularly intriguing is our observation that the ease of solubilization of N-VDCCs decreases between E18 and P10, presumably via an increased association of N-VDCCs with detergent-intractable components, especially those of the neuronal cytoskeleton. An interaction of N-VDCCs with the cytoskeleton is supported by the polarized distribution (Jones et al., 1989; Westenbroek et al., 1992; Mills et al., 1994; Christie et al., 1995) and the immobility of >70% (Jones et al., 1989) of N-VDCCs in mature hippocampal neurons and by the fact that the first postnatal week is a major phase for maturation of the neuronal cytoskeleton (Burgoyne, 1991).

To resolve only the surface N-VDCCs, we used high-resolution imaging of slices labeled with a selective fluorescent analog of $\omega$-CgTx (Mills et al., 1994). The validity of such CgTx-based approaches (Jones et al., 1989; Robitaille et al., 1990; Cohen et al., 1991; Komura and Rakic, 1992; Filloux et al., 1994; Haydon et al., 1994; Mills et al., 1994) is substantiated by the similar temporal expression patterns of the 220 kDa $\alpha_{1b}$ subunit, $\omega$-CgTx binding sites, and overall Fl-$\omega$-CgTx labeling. Our study also agrees with that of Filloux et al. (1994), who used $[^{125}\mathrm{I}]\omega$-CgTx autoradiography to explore N-VDCC ontogeny in the rat brain; however, we did not detect the developmental increase in $[^{125}\mathrm{I}]\omega$-CgTx affinity reported by these authors. More significantly, the ontogeny of Fl-$\omega$-CgTx labeling is very similar to that obtained via in situ hybridization (Tanaka et al., 1995) where, from E18 onward, both $\alpha_{1b}$ and $\beta_3$ mRNAs are evident in all cell body layers throughout the hippocampal formation. The only real discrepancy concerns the lower expression of Fl-$\omega$-CgTx labeling in CA1–CA2 relative to adjacent regions before P7. The simplest explanation is that neurons in discrete hippocampal subfields translate and insert N-VDCCs differentially at the nerve surface. However, disparities between Fl-$\omega$-CgTx labeling and mRNA expression also could reflect staining of N-VDCCs trafficked to presynaptic terminals impinging on the somata rather than to N-VDCCs made by translation in the postsynaptic cell. However, such an explanation would require targeting of N-VDCCs to axon terminals before their expression on somata—a result that is inconsistent with our observation that N-VDCCs are expressed initially on cell bodies.

The initial surface expression of N-VDCCs in the soma before the distal dendrites is intriguing but has been seen for other ion channels (Strichartz et al., 1984; Nicola et al., 1992; Maletic-Savatic, 1995). Previously, it has been postulated that the transport of proteins into neurites arises via their initial expression at the soma surface and subsequent diffusion in the plane of the membrane to more peripheral regions (Small et al., 1984). However, the absence of a clear somatodendritic (or dendritic) gradient of N-VDCC expression, despite the presence of dendrites in P7 or adult neurons (Westenbroek et al., 1992; Mills et al., 1994), indicates that VDCCs also are inserted (and immobilized) directly into the dendritic membrane. Thus, expression of N-VDCCs in the neurites may proceed only after these regions are mature enough to support the appropriate trafficking, insertion, and immobilization mechanisms.

The possible role of N-VDCC expression in the development of the hippocampus is especially significant. In the rat, formation of the hippocampus begins at E14 (Altman and Bayer, 1990a–c; Jacobson, 1991). Neuroblasts, which have arisen through proliferation in the neuroepithelium, migrate to specified positions, settle, and elaborate axons and dendrites. In CA1–CA4 such events are complete by birth (Altman and Bayer, 1990a,b), but in dentate gyrus they continue for several weeks postnatally (Altman and Bayer, 1990c). However, although CA3 cells are generated earlier than CA1 cells, they take longer to settle in stratum pyramidale, reflecting a subicular-to-dentate morphogenetic gradient complete by E22 (Altman and Bayer, 1990b). Thus, the

Figure 8. Comparative distributions of Fl-$\omega$-CgTx labeling and CA1 neurons identified by filling with the intracellular dye Lucifer yellow. A, Hippocampal CA1 neurons at P2 filled with Lucifer yellow show extensive arborization (arrows) but weak staining with Fl-$\omega$-CgTx (inset, asterisk). In contrast to CA1, staining with Fl-$\omega$-CgTx is much stronger in the adjacent subiculum (inset, arrow). B, Direct comparison of the distributions of Fl-$\omega$-CgTx labeling and CA1 neurons identified by Lucifer yellow filling (arrows) at P3. Note the much weaker staining of the CA1 subfield neurons, as compared with those in the cingulate cortex (top left). C, Fl-$\omega$-CgTx labeling in P7 CA1 neurons. Note the strong staining of the (Figure legend continues)
somata and the very proximal dendritic regions and the sharp decline in labeling that occur within a few soma diameters from the cell body. D, Fl-ω-CgTx labeling in adult CA1 neurons. Note that the staining, while often punctate, is sustained for distances corresponding to several soma diameters on dendrites emanating from identifiable somata (arrowheads) and pervades the dendritic arbor. E, At high magnification, Lucifer filling of P7 CA1 neurons reveals extensive dendritic arborization and stains even the most distal dendritic regions (arrows), whereas the Fl-ω-CgTx labeling is restricted to somata and very proximal dendrites (asterisk), as in C.
sequence of N-VDCC surface expression seems to parallel the postmitotic age of the neurons rather than their time of settling in stratum pyramidale. The absence of α1B subunits in neurogenic zones, but their prenatal expression elsewhere, is compatible with a role for N-VDCCs in migration, as proposed for cerebellar neurons (Komura and Rakic, 1992). However, the perinatal levels of α1B are only 2–5% of those found in adult. Thus if N-VDCCs indeed do facilitate migration, relatively few channels are needed, or few cells are migrating at any one instant. Most N-VDCCs appear within the early postnatal period, consonant with reported increases in HVA Ca\(^{2+}\) currents, such as those generated by N-VDCCs in culture (Yaari et al., 1987; Scholz and Miller, 1995).

This period is marked initially by dendritic arborization and gliogenesis, but the event that most closely defines N-VDCC expression is synaptogenesis, the bulk of which occurs in the first few weeks after birth (Jacobson, 1991). An intimate relationship between N-VDCC expression and synaptogenesis is supported by changes in α-CgTx binding in mouse brain growth cone particles (Vigers and Pfenniger, 1991). Certainly, the pattern of expression of N-VDCCs, unlike several other voltage-gated ion channels (Yaari et al., 1987; Maletic-Savatic et al., 1995; Scholz and Miller, 1995), is remarkably similar to that of other proteins implicated in synaptic function (Burgin et al., 1990; Bahn et al., 1994; Lameli et al., 1994; Melloni et al., 1994; Sheng et al., 1994). Enhanced expression of N-VDCCs during synaptogenesis agrees well with their role in neurotransmitter release (Dunlap et al., 1995; Scholz and Miller, 1995). Although the role of N-VDCCs in neurotransmission is shared with other VDCCs in adult neurons (Wheeler et al., 1994; Scholz and Miller, 1995), two lines of evidence suggest that N-VDCCs may be especially significant in the immature hippocampus. First, neurotransmission in developing cultures is dominated initially by N-VDCCs, the role of which declines as their task becomes shared by P/Q-VDCCs (Scholz and Miller, 1995). Second, the efficacy of CA1 synapses is reported to be high [release probability (P) close to unity] in very young (P4–P6) hippocampi but diminishes to a lower level (P <0.5) with maturation (Bolshakov and Siegelbaum, 1995). A purely presynaptic role for N-VDCCs is probably unlikely, however, because N-VDCCs also are found on hippocampal dendrites (Jones et al., 1989; Westenbroek et al., 1992; Mills et al., 1994; Christie et al., 1995) and their spines (Mills et al., 1994). Although the functional contribution such postsynaptic N-VDCCs make is unclear (Mills et al., 1994; Elliott et al., 1995), one plausible role is to direct afferent axons to discrete synapses. Such a role also may explain the shift in synapses from dendritic shafts to spines that is seen between P7 and P15 (Harris et al., 1992).

Several physiological correlates of hippocampal maturation emerge within the same time window as N-VDCC expression in the immature hippocampus. toward the end of the first postnatal week CA1 pyramidal cells begin to exhibit adult electrophysiological characteristics and population spiking (Bekenstein and Lothman, 1991; Bolshakov and Siegelbaum, 1995). Remarkably, this phase coincides with our initial detection of N-VDCCs in CA1 and the arrival of the commissural and perforant fibers to CA1 and dentate gyrus, respectively (Bekenstein and Lothman, 1991). The early expression of N-VDCCs in the lateral blade of the dentate gyrus merits attention because the lateral perforant pathway relays primarily olfactory inputs, whereas the medial pathway relays presubicular and nonolfactory inputs from the entorhinal cortex (Shepherd, 1990). Marked changes in activity-dependent plasticity, notably paired pulse facilitation (PPF) and long-term potentiation (LTP) and depression (LTD), also occur in the early postnatal period. LTD is greatest before P14 (Dudek and Bear, 1993) whereas LTP reaches adult levels at ~P14 (Dudek and Bear, 1993). Although the relative contribution of pre- and postsynaptic mechanisms to such events is controversial (Kullmann and Siegelbaum, 1995), the absence of PPF and LTP, but not LTD, at CA3–CA1 synapses in P4–P8 animals can be rationalized by their high-release probability (P, 0.9) (Bolshakov and Siegelbaum, 1995). Thus, N-VDCCs may be of central importance to synaptic plasticity simply by virtue of their predominant role in juvenile transmitter release (Scholz and Miller, 1995).

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