EXPRESSION OF N-TYPE CALCIUM CHANNEL SUBUNITS IN THE DEVELOPING RAT HIPPOCAMPUS

by

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for the degree of Master of Science
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"... If we can conquer outer space, we should be able to conquer inner space, too. The frontier of the brain, the central nervous system, and the afflictions of the body that destroy so many lives..."

Christopher Reeve, Actor, 1996.

ABSTRACT

Voltage-dependent calcium channels (VDCCs) are diverse heteromers that mediate neuronal excitability, neurotransmitter release, and contribute to the cytoarchitecture of the brain. One major VDCC - the N-type (N-VDCC), is thought to play a role in neurodevelopment, however the precise details are unknown.

In this thesis, N-VDCC expression was investigated in the developing rat hippocampus, using antibodies to the α_{1B}, β_3 and α_2/δ subunits comprising the N-VDCC heteromer, and [^{125}I]-ω-conotoxin GVIA binding assays. Binding showed increases in N-VDCCs during development, parallel to α_{1B} subunit expression. However, the expression of β_3 and α_2 subunits were different from that of the α_{1B} subunit, suggesting that these subunits are not always coassembled. Immunoprecipitations indicated that coassembly of the β_3 subunit with α_{1B} increases throughout development, however the proportion of α_{1B}-β_3 complexes in the total α_{1B} pool varies. Thus, α_{1B} may exist alone, or coassembled with β subunits other than β_3. The prenatal expression of N-VDCCs is consistent with a proposed role in neuronal migration, while their postnatal increase suggests a role in differentiation and synaptogenesis. These studies help define N-VDCC subunit expression in development, and may provide insight into channel functionality during critical developmental periods.

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A	Absorbance
ABTS	Azidino-di-(3-ethyl-benzthiazoline-sulfonate)
Aga	Agatoxin (funnel web spider *Agelenopsis aperta* toxin)
BIS	N,N'-methylenebisacrylamide
BSA	Bovine Serum Albumin
ω-CgTx	ω-Conotoxin GVIA (marine snail *Conus geographus* toxin)
[^{125}I]-ω-CgTx	[^{125}I]-Tyr^{22}-ω-CgTx GVIA
ω-CgTx-MVIIC	ω-Conotoxin MVIIC (marine snail *Conus magus* toxin)
DHP	Dihydropyridine
DTNB	5,5'-Dithio-bis (2-Nitrobenzoic Acid)
DTT	Dithiothreitol
E	Embryonic Day
ELISA	Enzyme-Linked Immunosorption Assay
HRP-DAR	Horseradish Peroxidase Conjugated Donkey Anti-Rabbit Ig
HRP-GAM	Horseradish Peroxidase Goat Anti-Mouse Ig
HVA	High Voltage-Activated
IV	Current-Voltage
Kds	Kaleidoscope Pre-stained Molecular Mass Standards
KLH	Keyhole Limpet Hemocyanin
K^+ PB	Potassium Phosphate Buffer
LVA	Low Voltage-Activated
NFDM	Non Fat Dry Milk
N-VDCCs	N-type Voltage Dependent-Calcium Channels
P	Postnatal Day
PBS	Phosphate Buffered Saline
PMSF	Phenylmethyl-Sulfonylfluoride
Pr	Release Probability
PVDF	Polyvinyl Difluoride
SDS	Sodium Dodecyl Sulfate
SDS-PAGE	Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
sFTX	synthetic Funnel Web Spider Toxin
Sulfo-MBS	m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester
TBS	Tris Buffered Saline
VDCCs	Voltage Dependent-Calcium Channels

1. INTRODUCTION

1.1. Role of Calcium in Neurons

Calcium influx is critical for virtually every aspect of nerve function. Significant processes that depend on calcium include neuronal excitability and the generation of action potentials (Llinas and Sugimori, 1979; Llinas, 1988) and the release of neurotransmitters at synapses (Miller, 1987; Augustine et al., 1991; Wheeler et al., 1994; Dunlap et al., 1995). In recent years it has also become apparent that in neurons calcium signalling is critical for the activation of genes encoding transcription factors, such as c-fos and c-jun (Bading et al., 1993; Lerea and McNamara, 1993; Ghosh et al., 1994), and mediating communication between the synapse and the nucleus (Deisseroth et al., 1996). In addition, calcium signalling is important for the activation of diverse enzymes such as phospholipases (Farooqui and Horrocks, 1991), kinases (Favaron et al., 1990) and calcium-dependent proteases such as calpain (Siman and Noszek, 1988; Siman et al., 1989). In order to realize such signalling roles, neurons, in common with other excitable cells, generate concentration gradients across both the cell membrane and between the cytoplasm and intracellular stores. Typically, intracellular calcium is maintained at a concentration of ~100nmol/L, or 105 times lower than the extracellular calcium concentration (Mills and Kater, 1990; Hille, 1991; Tymianski and Tator, 1996). Owing to the diversity, prevalence and importance of calcium-dependent events, neurons have developed a variety of homeostatic mechanisms that maintain the intracellular calcium concentrations within a defined range (Kater and Mills, 1991). Outside of this range, calcium ions appear to be neurotoxic. High calcium influx is well correlated with neuronal cell death in prolonged seizure activity and global hypoxia/ischemia

(Heinemann and Hamon, 1986; Deshpande et al., 1987; Choi, 1988; Silver and Erecinska, 1990; Uematsu et al., 1990; Lobner and Lipton, 1993), while low calcium causes inhibition of neuronal outgrowth (Mills and Kater, 1990). Such observations have led to the postulation of a "set-point" hypothesis for neurological disorders (Ellren and Lehmann, 1989; Kater et al., 1989; Eimrel and Schramm, 1994; Kater and Lipton, 1995).

1.2. Contribution of Voltage-Gated Calcium Channels to Nerve Cell Calcium Influx

In order to permit the large changes in calcium concentration seen in calcium-based signalling with the need to keep such concentrations in a physiologically tolerable range calcium homeostatic mechanisms must be very flexibile. This is accomplished using a complex system of pumps, buffers, stores and exchange mechanisms which can respond to rapid elevations in calcium influx. In resting neurons, the extremely high concentration gradient of calcium across the cell is accomplished by extrusion of calcium across the cell membrane using ATP-dependent calcium pumps and Na^{+}/Ca^{2+} exchangers (Nachshen et al., 1986; Carafoli, 1992) in conjunction with extremely efficient buffering attained through the binding of calcium to specific proteins such as calbindin, calmodulin and parvalbumin (Celio, 1990; Baimbridge et al., 1992) and calcium sequestration within intracellular stores (Blaustein, 1988; Werth and Thayer, 1994). The signalling function of calcium ions is realized in two ways. First, through the influx of calcium through ligand or voltage-activated ion channels (Tsien, 1983; Catterall, 1993), and second, through its release from intracellular stores (McPherson et al., 1991; Llano et al., 1994; Simpson et al., 1995). At synapses of most central neurons, calcium influx arises pre- and postsynaptically. Pre-synaptic influx occurs at axon terminals and is primarily mediated by voltage dependent-calcium channels

(VDCCs) (Miller, 1987; Robitaille et al., 1990; Cohen et al., 1991; Dunlap et al., 1995). At the postsyanptic face, however, calcium influx occurs primarily through activation of NMDA-subtypes of glutamate receptors (Petrozzino et al., 1995). Contributions from certain other glutamate receptor subtypes, some nicotinic receptors and VDCCs also occurs but to a lesser extent (Kullmann et al., 1992; Sorimachi, 1993; Barrantes et al., 1995). Nevertheless, since synapses mainly occur on dendritic spines, and the axon terminals are extremely small, these regions may represent only a subfraction of the total membrane of the neuron. In the rest of the membrane calcium influx mainly occurs through VDCCs (Hell et al., 1993; Kocsis et al., 1994). Thus, VDCCs play an important role in calcium signalling in virtually every region of the neuron. Not surprisingly these channels have been linked to numerous physiological and pathological processes (Table 1).

1.3. Role of Calcium Channels In Development

In spite of the importance of VDCCs for nerve function, very little is known about their molecular, cellular or developmental biology. This latter area is particularly unexplored. In neurons development is most conveniently described in terms of a series of well defined stages (Jacobson, 1991). Initially, neuroblasts, arising from the neuroepithelium, proliferate, migrate to specified positions, settle, and elaborate axons and dendrites (polarise) (Craig and Banker, 1994) (Figure 1). Subsequent phases of neurodevelopment include synaptogenesis, apoptosis and pruning of unwanted cells and synapses (Purves et al., 1986; Johnston and Deckwerth, 1993). Whether, and how, VDCCs play a role in each of these stages is unclear. It has long been recognized that calcium influx is necessary for events such as cell division and neurite outgrowth and pruning (Cohan et al., 1987; Mattson and Kater, 1987;

Pathological and Physiological Roles of VDCCs	
Pathological and Physiological Processes Involving VDCCs	**References**
Activation of Gene Transcription	Murphy, et al., 1991.
Modulation of Ca^{2+}-dependent Enzymes	Kennedy, 1989.
Synaptic Transmission	Takahashi and Momiyama, 1993. Leveque et al., 1994.
Ca^{2+}-based Action Potentials	Elliot et al., 1995. Miyakawa et al., 1992.
Long-term Potentiation	Kullman et al., 1992.
Enhancement of Currents in Kindled Rat Hippocampus	Vreugdenhil and Wadman, 1992.
Altered Expression in Epileptic DBA/2J Mice During Development	Esplin et al., 1994.
Altered Expression in Cerebellar Mutant Mice	Maeda et al., 1989.
Mutations in VDCC α_1 Subunits Involved in Hypokalemic Periodic Paralysis	Ptacek et al., 1994.
Ischemic Cell Death	Valentino, et al., 1993.
Autoimmune Disease	Smith et al., 1994. Vincent et al., 1989.
Altered Expression During Postnatal Rat Brain Development	Filloux et al., 1994.
Synaptogenesis	Vigers and Pfenninger, 1991.
Neuronal Migration	Komuro and Rakic, 1992.
Apoptosis	Johnston and Deckwerth, 1993.
Increases in Functional Channels During Aging	Thibault and Landfield, 1996.

Table 1. Pathological and Physiological Roles of VDCCs

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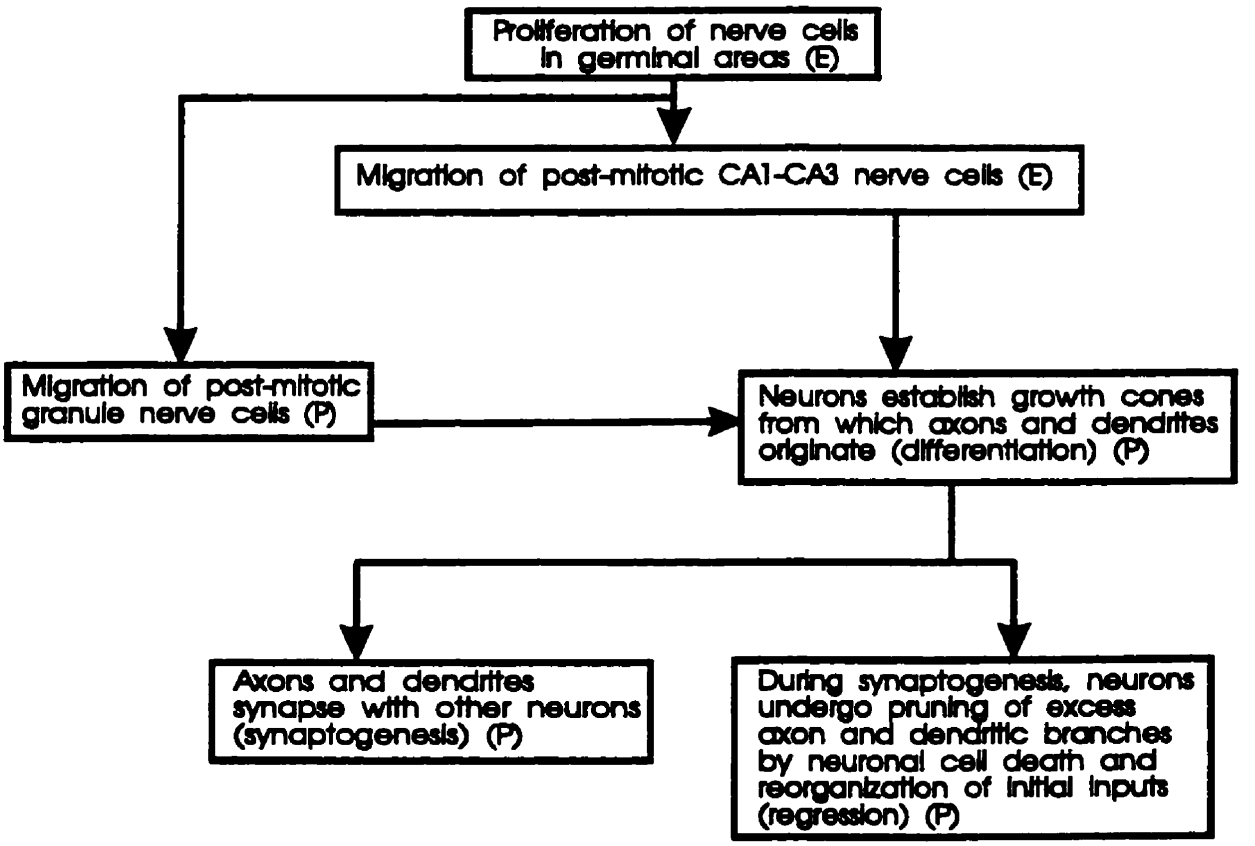

Figure 1. Stages in the Development of Hippocampal Neurons and their Connections (modified from Hopkins and Brown, 1984). Neurons undergo various processes including migration, differentiation, synaptogenesis and regression during discrete stages in embryonic (E) and post-natal (P) development.

Constantine-Paton et al., 1990; Cohan, 1992; Hepler, 1994; Liu et al., 1994; Spitzer et al., 1994), and more recently to cell polarisation (Basarshy et al., 1994; Verderio et al., 1994). How VDCCs contribute to these events is not well defined. Much recent evidence suggests that VDCCs are likely to be extremely important. For example, the expression of VDCCs appears to be necessary for migration of neuroblasts, at least in the cerebellum (Komuro and Rakic, 1992), and certainly facilitates culling of excess neurons by apoptosis (Johnson and Deckwerth, 1993). Other evidence however, is largely circumstantial. For example, *in situ* hybridization studies have revealed mRNAs encoding VDCCs in those regions of pre- and post-natal brain undergoing active proliferation and migration (Tanaka et al., 1995). Based on these studies one would predict that VDCCs are expressed very early in development. Unfortunately, this prediction is poorly supported by available electrophysiology which suggests that the bulk of VDCC expression most closely parallels synaptogenesis, consistent with biochemical studies on nerve growth cone particles (Yaari et al., 1987; Vigers and Pfenninger, 1991). Nevertheless, the presence of electrophysiologically active VDCCs at earlier stages, such as in migration, is hard to resolve because of difficulties in live cell identification. An alternate explanation is that VDCC expression is phasic and mirrors, or even orchestrates, key developmental events (Jacobson, 1991). No matter when VDCCs are expressed, the most significant factor complicating a resolution of their developmental contribution is their enormous potential for diversity.

1.4. Multiplicity of Calcium Channels in Neurons

Diverse VDCCs have been identified and classified on the basis of their biophysical (Table 2) and pharmacological (Table 3A) characteristics. In neurons, the major VDCCs

Electrophysiological Properties of Neuronal Calcium Channel Subtypes			
	Fast, inactivating		Slow, persistent
	LVA T	HVA N	HVA L
Activation Range	Positive to -70mV	Positive to -20mV	Positive to -10mV
Inactivation Range	-100 to -60mV	-120 to -30mV	-60 to -10mV
Decay Rate	Moderate (τ= 20-50ms)	Moderate (τ= 50-80ms)	Very Slow (τ<500ms)
Deactivation Rate	Rapid	Slow	Rapid
Single-channel Conductance	8pS	13pS	25pS
Single-channel Kinetics	Brief Burst, Inactivation	Long Burst	Continual Reopening
Relative Conductance	$Ba^{2+} = Ca^{2+}$	$Ba^{2+} > Ca^{2+}$	$Ba^{2+} > Ca^{2+}$

Table 2. Electrophysiological Properties of Neuronal Calcium Channel Subtypes (modified from Hille, 1991)

Calcium Channel α_1 Subunits			
Gene product		Functional Correlates	
Consensus Names	Sites of Expression	Current	Drug Sensitivity of Native Currents
α_{1S}	Skeletal muscle, BC3H1 cells	HVA L-type	Sensitive to DHPs, diltiazem and verapamil. Insensitive to sub-μM ω-CgTx-GVIA and funnel web spider venoms (ω-Aga-IVA, FTX)
α_{1A}	Brain, cerebellum, Purkinje and Granule cells, Kidney, PC12 cells, C cells	HVA Q-type?	ω-CgTx-MVIIC (>100nM); ω-Aga-IVA (>10nM) DHP insensitive
		HVA P-type?	Sensitive to ω-Aga-IVA (<10nM) and low sFTX DHP insensitive
α_{1B}	Brain, Peripheral neurons, PC12 cells, C cells	HVA N-type	Sensitive to ω-CgTx-GVIA (100-500nM) and low sFTX, DHP insensitive
α_{1C}	Heart, HIT cells, GH3 cells, Brain, Aorta, Lung, Kidney, Fibroblasts, PC12 cells, C cells	HVA L-type	DHP sensitive Insensitive to low concentrations of ω-CgTx-GVIA, ω-Aga-IVA, or sFTX
$\alpha_{1C\text{-}a}$	Heart		
$\alpha_{1C\text{-}b}$	Smooth muscle, Lung		
$\alpha_{1C\text{-}c}$	Brain		
α_{1D}	Brain, Pancreas, HIT cells, GH3 cells, PC12 cells, C cells	HVA L-type	DHP sensitive. Reversibly sensitive to ω-CgTx-GVIA, ω-Aga-IVA, or FTX
α_{1E}	Brain, Heart, C cells	HVA R-type	Sensitive to low Ni. Insensitive to DHPs or ω-CgTx-MVIIC, or to loω concentrations of ω-CgTx-GVIA, ω-Aga-IVA or sFTX

Table 3A. Calcium Channel α_1 Subunits (modified from Birnbaumer et al.,1994; Perez-Reyes and Schneider, 1995)

include the L-, N-, T-, P/Q-, and R- types. (Nowycky et al., 1985; Fox et al., 1987a,b; Bean, 1989; Llinas et al., 1989; Hess, 1990; Hillman et al., 1991; Hillyard et al., 1992; Miller et al., 1992; Mintz et al., 1992a; 1992b; Zhang et al., 1993; Randall and Tsien, 1995). However, the advent of molecular cloning and expression studies have indicated that this classification is too simplistic, as each channel type is comprised of three different subunits, including α_1, β and α_2/δ subunits in a 1:1:1 stoichiometry (Wagner et al., 1988; Hell et al., 1993; Witcher et al.,1993; Hofmann et al., 1994; Leveque et al., 1994). In neurons, each distinct VDCC α_1 subunit is transcribed from a unique gene. At present, six α_1 genes designated $\alpha_{1A\text{-}E}$ have been identified (Snutch et al., 1990; Snutch and Reiner, 1992; Soong et al., 1993; Zhang et al., 1993) (Table 3A). Like the α_1 subunits, the four known β subunits are each encoded by distinct genes (β_1-β_4) (Table 3B), although available evidence shows that these genes are much simpler in structure (Ruth et al., 1989; Pragnell et al., 1991; Hullin et al., 1992; Perez-Reyes et al., 1992; Powers et al., 1992; Williams et al., 1992b; Castellano et al., 1993a,b). To date, only a single gene has been isolated for the α_2/δ subunit (Kim et al., 1992) (Table 3C). Even further diversity arises through the existence of alternative splicing within α_1, β and α_2/δ transcripts (Hullin et al., 1992; Kim et al., 1992; Hell et al., 1993; Birnbaumer et al., 1994). Expression studies in *Xenopus* oocytes and transfected cell lines have attempted to establish the minimum requirements for calcium channel activity and pharmacology. These studies indicate that the α_1 subunit contains the ion channel pore and mechanisms responsible for voltage dependent activation (sensing), inactivation, and selectivity (Mikami et al., 1989; Perez-Reyes et al., 1989; Biel et al., 1990; Ellinor et al., 1995). This is not surprising, given that these subunits appear to share the broad structural motifs common to voltage dependent sodium channels (Schmidt and Catterall, 1986; Tanabe, et al., 1987). Thus, the α_1 subunit is comprised of twenty-four putative transmembrane

Calcium Channel β Subunits				
Gene Product	**Splice Variant**	**Proven Expression**	**Component of**	**Expression with α_1**
β_1	β_{1a}	Skeletal muscle Heart	DHP receptor	Increases current, shifts IV, alters kinetics, increases binding
	β_{1b}	Brain Heart	ω-CgTx MVIIC receptor, ω-CgTx GVIA receptor	Increases binding
	β_{1c}	Brain Heart Spleen	?	
β_2	β_{2a}	Brain Heart Lung	ω-CgTx MVIIC receptor	
	β_{2b}	Brain Heart Aorta		
	β_{2c}	Brain Heart		
	β_{2e}	Brain		
β_3	?	Brain Heart Aorta Trachea Lung Ovary Colon	ω-CgTx MVIIC receptor, ω-CgTx GVIA receptor	Increases currents, shifts IV, affects inactivation
β_4	?	Brain Kidney	ω-CgTx GVIA receptor, ω-CgTx MVIIC receptor	Increases currents, shifts IV, affects inactivation

Table 3B. Calcium Channel β Subunits
(modified from Birnbaumer et al., 1994; Perez-Reyes and Schneider, 1995; Liu et al., 1996; Scott et al., 1996)

Calcium Channel α_2/δ Subunits			
Gene Product	**Splice Variant**	**Proven Expression**	**Expression with α_1**
α_2/δ	α_{2a}	**Skeletal Muscle**	**Increases α_1 currents in oocytes, increases binding**
	α_{2b}	**Brain**	**Increases binding and currents**
	α_{2c}, α_{2d}, α_{2e}	**Aorta**	**?**

Table 3C. Calcium Channel α_2/δ Subunits
(modified from Perez-Reyes and Schneider,1995)

helices grouped into four domains (I-IV, or S1-S4), containing four negatively charged glutamate residues within the pore region for the coordination of calcium binding and calcium selectivity (Ellinor et al., 1995; Reuter, 1996). The α_1 subunit also contains a highly conserved region of positively charged amino acids in S4 that may act as the channel's voltage sensor. Depolarization moves S4 outward to generate the open channel configuration (Catterall, 1995) (Figure 2A). However, to realize these important roles it seems both the β and the α_2/δ subunits are also necessary. The β subunit binds to the I-II cytoplasmic linker of the α_1 subunit, and is itself entirely cytoplasmic (Takahashi et al., 1987; Pragnell et al., 1994). Through this interaction, the β subunit is able to modulate channel activity by increasing the transmembrane peak currents and/or altering the voltage dependence of channel activation and inactivation (Lacerda et al, 1991; Mori et al., 1991; Perez-Reyes et al., 1992; Williams et al., 1992b; Castellano et al., 1993a,b; Soong et al., 1993; Stea et al., 1993; Nishimura et al., 1993) (Figure 2B). Although the function of the α_2/δ subunits in channel modulation is not entirely clear, it appears to enhance calcium current amplitude, possibly through its glycosylated extracellular domain (Mikami et al., 1989; Mori et al., 1991; Hullin et al., 1992; Williams et al., 1992b; Brust et al., 1993; Gurnett et al., 1996), and may be responsible for the optimization of VDCC subunit interactions and surface expression (Brust et al., 1993; Gurnett et al., 1996) (Figure 2C). Exactly how α_2/δ accomplishes this role is unclear since its association with the membrane is rather bizarre. Biochemical studies indicate the α_2 gene product is clipped proteolytically to generate a large α_2 subunit and a smaller δ subunit which are linked through a disulphide bond (De Jongh et al., 1990; Jay et al., 1991). Such processing yields a subunit which exists largely extracellularly, with the exception of a single transmembrane domain and a carboxy-terminus consisting of only five amino acids (Gurnett et al., 1996). Despite much initial controversy it is now generally agreed that the formation

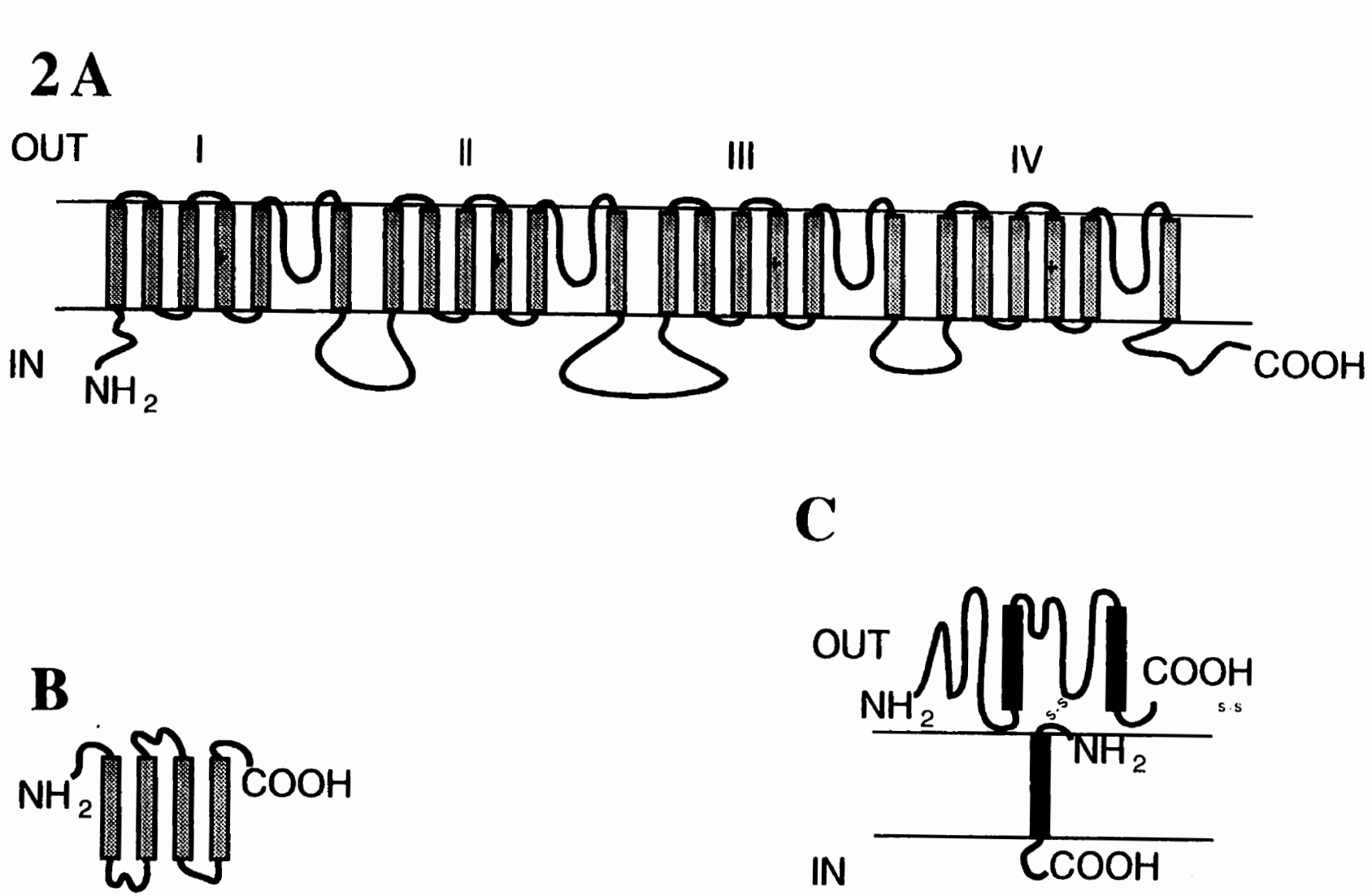

Figure 2. Diversity in Structure of the Major Components of the Voltage-dependent Calcium Channel (VDCC) Complex. The VDCC complex consists of an α_1 subunit (A), a β subunit (B) and an α_2/δ subunit (C). The positively charged residues responsible for voltage sensing by the α_1 subunit are shown (+).

of VDCCs with electrophysiological properties approximating those found *in vivo* is best achieved when both the auxilary subunits β and α_2/δ are coexpressed with the α_1 subunit (Mori et al., 1991; Williams et al., 1992a,b; Ellinor et al., 1993; Sather et al., 1993; Stea et al., 1993) (Figure 3). These results are therefore in excellent agreement with previous biochemical studies which have attempted to purify and reconstitute VDCCs from brain and other tissues. Such studies indicate that the VDCC complex consists of an α_1 subunit coassembled with a membrane-associated α_2/δ subunit and a tightly associated β subunit (Hofmann et al., 1994; Catterall, 1995). At this point it is reasonable to ask how this molecular description of VDCC structure and function fits in with their pharmacologies and with the types of channels defined by electrophysiological studies *in vivo*? Surprisingly, both reconstitution and expression systems are in remarkable agreement. While the α_{1A} gene confers properties consistent with it being the P/Q-type channel (Mori et al., 1991; Sather et al., 1993; Stea et al., 1994; Zhang et al., 1993), the α_{1B} seems to encode the N-type channel (Dubel et al., 1992; Williams et al.,1992a; Fujita et al., 1993). The dihydropyridine-sensitive L-channels are encoded by the α_{1C} and α_{1D} genes (Hui et al., 1991; Snutch, 1991; Dubel et al., 1992; Williams et al., 1992a; Hell et al., 1993). The only channel for which there is any lack of clarity is that encoded by α_{1E}, which appears to have some properties akin to the low threshold T-channel (Soong et al., 1993; Forti et al., 1994; Williams et al., 1994; Randall et al., 1995).

1.5. Role of Calcium Channel Diversity

What function does such diversity serve? Undoubtedly, diversity is exploited to some degree in providing complexes with unique biophysical characteristics (Zhang et al., 1993;

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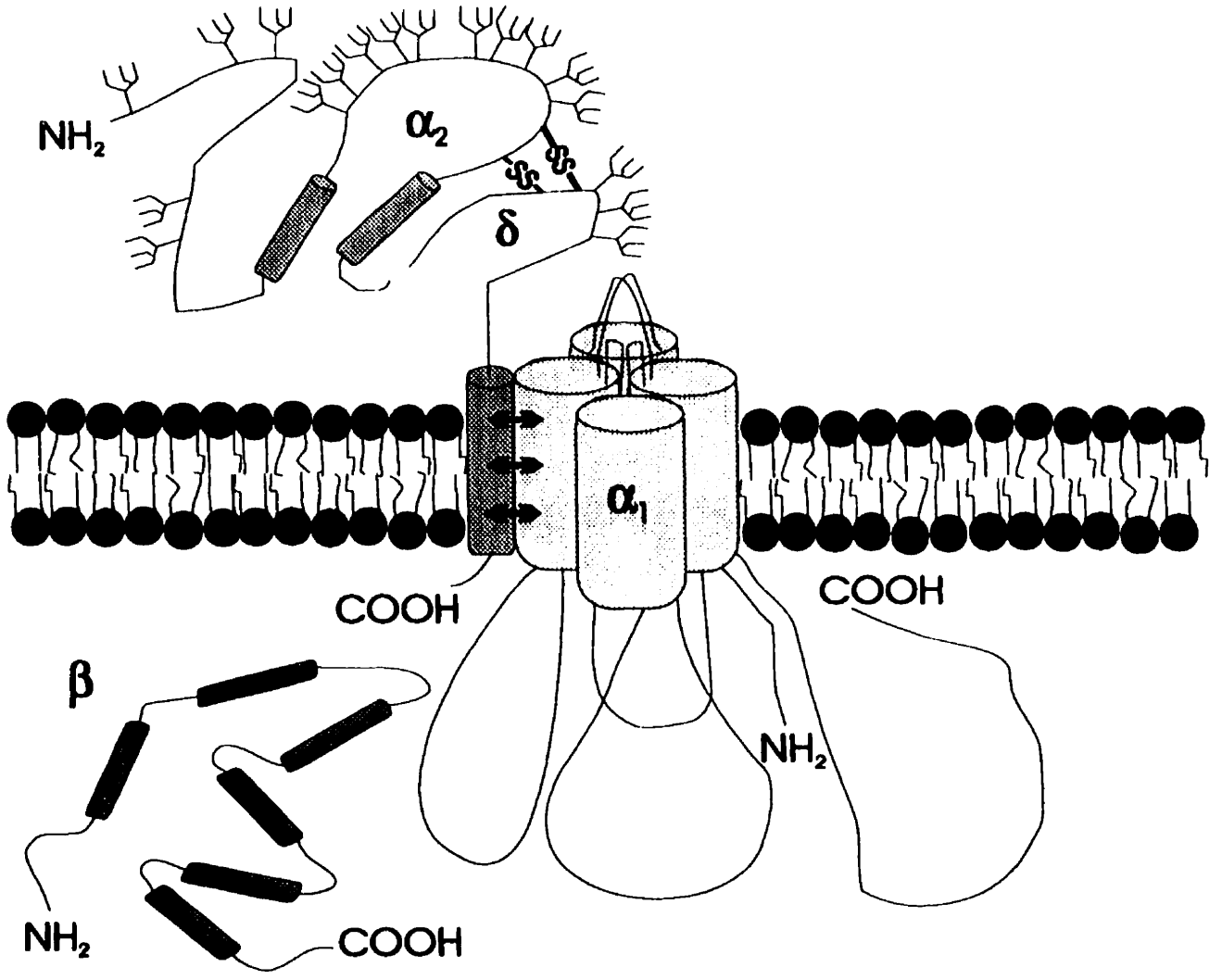


Figure 3. Three-dimensional Representation of the Assembled Voltage-dependent Calcium Channel (VDCC) Complex in the Neuronal Membrane (modified from Gurnett et al., 1996). The α_2/δ subunit consists of an extracellular glycosylated domain and a transmembrane region (in the δ subunit) that interacts with the α_1 subunit (↔). The β subunit is thought to be entirely cytoplasmic, and interacts with a cytoplasmic portion of the α_1 subunit. The α_1 subunit notably forms the channel pore.

Randall and Tsien, 1995). Nevertheless, such differences are often rather subtle. For example, the influxes of calcium through α_{1A}, or α_{1B} subunits expressed in oocytes, integrated with respect to time, are very similar (Sather et al., 1993; Stea et al., 1994). It therefore seems likely that VDCC diversity subserves other functions. One possibility is to provide a different spectrum of targets for modulation by second messengers (Hymel et al., 1988; Nunoki et al., 1989; Ahlijanian et al., 1991; Toth et al., 1996). However, recent evidence showing different VDCCs have discrete distributions over the nerve surface suggests that VDCC diversity also allows neurons to tailor specific patterns of VDCC mediated calcium influx to discrete neuronal compartments (Jones et al., 1989; Silver et al., 1990; Cohen et al., 1991; Llinas et al., 1992; Thompson and Coombs; 1992).

With the recent identification of discrete gene products corresponding to select VDCCs it has now become possible to study the developmental biology of VDCCs in a meaningful way. I now outline how I have used such information to prepare anti-peptide antibodies and thus resolve the expression of subunits corresponding to a major VDCC subtype - the N-channel - in development.

1.6. Goals of Thesis

A central hypothesis of this thesis is that VDCC expression during development is phasic. Thus, increases in VDCC expression would occur during three key phases of neuronal development: migration, differentiation and synaptogenesis. To address this hypothesis I plan to focus on one particular class of VDCCs, the N-type, as these have been unambiguously correlated with the α_{1B} gene product. Moreover, these VDCCs are especially

important for neurotransmitter release (Scholz and Miller, 1995) and have been studied intensely at all levels. Rather than examine VDCC expression throughout the brain, I have addressed the expression of N-VDCCs in the developing rat hippocampus. This region of the rat brain is an especially important locus for the plasticity thought to underlie learning and memory and for pathologies such as epilepsy and ischemic cell death (Turner et al., 1982; Lobner and Lipton, 1993; McNamara, 1994).

My specific goals were:

A) To prepare and characterize anti-peptide antibodies selective for the α_{1B} and β_3 subunits, which comprise known N-VDCC heteromers.

B) To define the expression of N-VDCC α_{1B}, β_3 and α_2/δ subunits using western immunoblots, immunoprecipitations and [^{125}I]-ω-CgTx binding assays.

The results of these studies demonstrate that the bulk of expression of N-VDCC subunits and complexes occurs within the first few weeks of birth commensurate with differentiation and synaptogenesis. However, the presence of some (5%) of N-VDCCs prior to birth indicates that N-VDCCs may also play some role in migration.

2. GENERAL MATERIALS AND METHODS

Descriptions of the materials and techniques used in thesis are provided below. Specific details and/or modifications are included in corresponding chapters.

2.1. Epitope Prediction and Peptide Synthesis

Synthetic peptides with sequences that correspond to known rat brain N-type VDCC (N-VDCC) subunit isoforms α_{1B} [Genbank accession number M92905, residues 851-867:RHHRHRDRDKTSASTPA, 2030 Da] (Dubel, et al., 1992) and β_3 [Genbank accession number M88751, residues 1-15: MYDDSYVPGFEDSEA, 1828 Da] (Castellano, et al., 1993a) were synthesized by Vetrogen Procyon (London, Ontario) using solid-phase methods (Atherton and Sheppard, 1989). The peptides were designed by Drs. Jones and Francis based on the uniqueness of their sequence at the protein level, predicted antigenicity judged visually and by algorithms (Hopp and Woods, 1983), and others' use of antibodies against similar peptide sequences (Westenbroek, et al., 1992; Witcher et al., 1993 (NH_2-terminus of β_3 subunit)). In order to facilitate subsequent conjugation to carrier proteins the peptides were synthesized with an additional cysteine residue at their carboxyl termini. The identity of the peptides was confirmed by amino acid analysis and mass spectroscopy ((M)+2; m/z = 1017 and (M) +1; m/z = 1829 for the α_{1B} and β_3 peptides, respectively).

2.2. Antibody Preparation

To enhance their antigenicity, peptides were coupled to the amino groups of a carrier protein keyhole limpet hemocyanin (KLH) (4.5 x 10^5-1.3 x 10^7 Da) (Pierce) using the water soluble heterobifunctional crosslinker m-Maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS) (416.2 Da) (Pierce) (Sheng, et al., 1992) (Figure 4). The carrier protein was activated by the addition of crosslinker, purified to remove excess sulfo MBS or its hydrolysis by-products, and then crosslinked to a sulfhydryl-containing peptide as now described. The KLH (1ml of a solution at 20mg/ml in 50mM potassium phosphate buffer pH 8.0 (K^+ PB)) was desalted by passing it through a Sephadex G-10 column (Pharmacia) equilibrated in the same buffer. Fractions were collected and elution monitored from the absorbance at 280nm (A_{280}). The peak fractions of KLH were then pooled to a concentration of 10mg/ml. To 1ml of the pooled KLH was then added a solution of 135µl of 10mg/ml sulfo-MBS (3.2µmol) in K^+PB pH 7.2 dropwise. The reaction was left for 20-35' at room temperature, with occasional mixing. To resolve free sulfo-MBS from maleimide-activated KLH, the mixture was applied to a Sephadex G-25 (medium) (Pharmacia) column (15ml bed volume), and eluted with 50mM K^+ PB pH 7.2. The progress of the elution was again determined from the A_{280} and the fractions corresponding to sulfo-MBS coupled KLH were pooled in a 3ml total volume. Coupling of the peptide to the activated KLH was achieved as follows. Lyophilized peptide was dissolved in 50mM K^+ PB pH 7.2, at 10mg/ml final concentration, 970µl of this peptide solution was then added to 3ml of the activated KLH solution and incubated for 2 hours at 4°C. Aliquots (3 x 10µl) of the free peptide solution were diluted 10-fold, and aliquots (3 x 30µl) of the peptide-KLH conjugate were diluted 30-fold in 50mM K^+ PB pH 8.0 for the thiol assay (2.10). The coupling yield was typically 80-95%. The peptide-KLH conjugate was

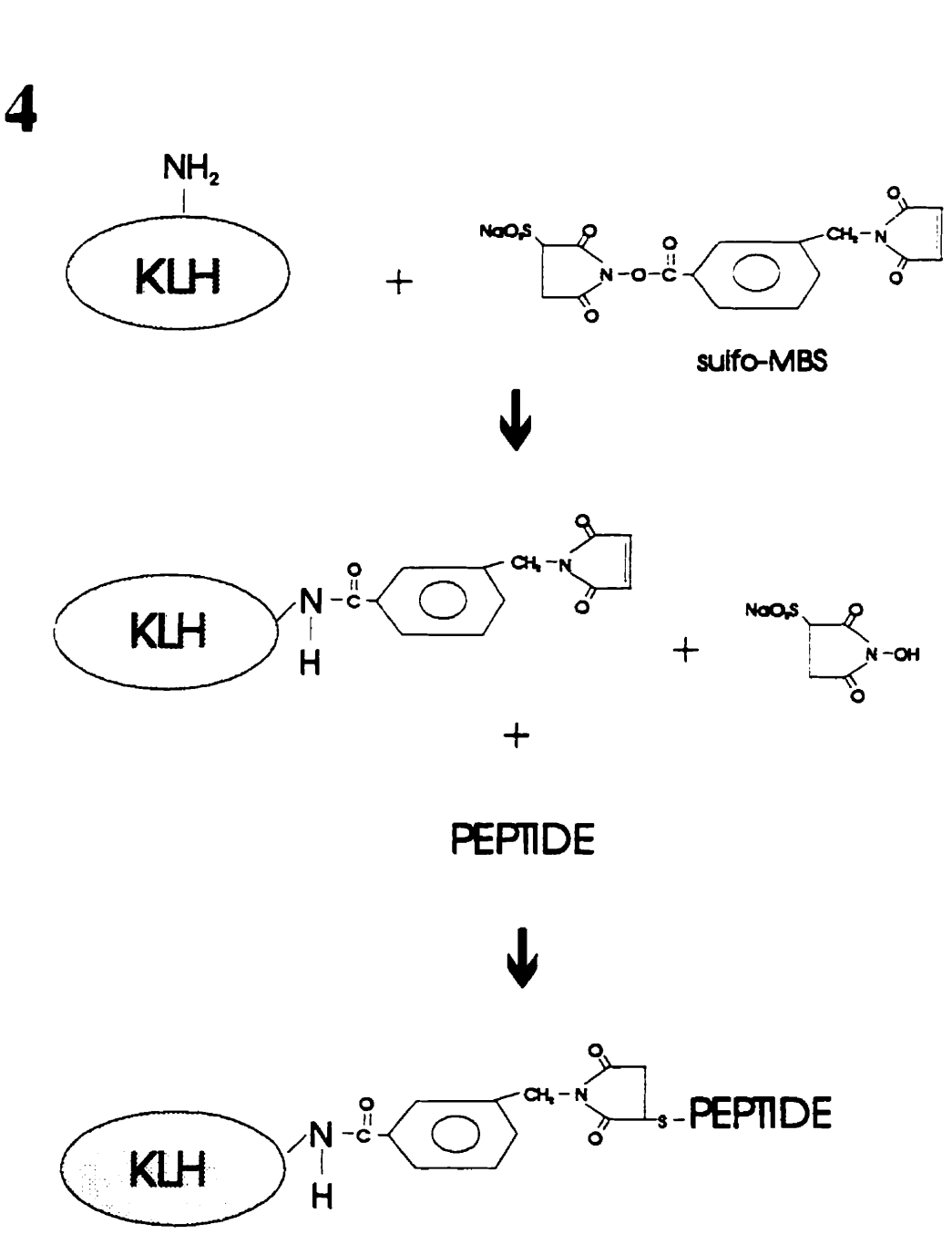


Figure 4. The Coupling of Peptides to Keyhole Limpet Hemocyanin (KLH) Using a m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBS) Crosslinker. The N-hydroxysulfosuccinimide (sulfo-NHS) ester end of sulfo-MBS reacts with primary amine groups of KLH. This reaction results in the formation of an amide bond between KLH and sulfo-MBS, releasing sulfo-NHS. The maleimide portion of sulfo-MBS is a thiol-reactive group that can be used for conjugation with a free sulfhydryl group, such as the terminal cysteines present in our α_{1B} and β_3 peptides.

dialysed for 24 hours against 2 x 2L of PBS for subsequent immunisation.

Polyclonal antibodies were raised by immunisation with the KLH-peptide complex (U. Toronto Div. Comp. Med.). Specifically, the dialysed KLH-peptide complex was emulsified in an equal volume of Freund's complete (initial injection) or incomplete adjuvant, and New Zealand White rabbits were injected in multiple subcutaneous sites. The bleeds were collected over a 3-6 month period.

2.3. Purification of α_{1B} and β_3 Subunit Antibodies

Antibodies were partially purified from antiserum by enriching the IgG fraction using MAPS affinity chromatography kits (Bio-Rad). Antiserum for MAPS purification was carefully selected by Enzyme-Linked Immunosorption Assay (ELISA) (2.5) and Western blotting (2.6) to contain a high titre of antibodies able to bind to the protein of interest in immunoblotted rat brain membrane. Briefly, a 2.5ml bed of protein A-agarose (Bio-Rad) was equilibrated in binding buffer (Bio-Rad) (31.4g binding buffer solid/100mL H_20 pH 9.0). Whole serum (approximately 2.5mg/ml IgG) was diluted 4-fold in binding buffer, and applied to the column twice over. The column was washed in 40ml binding buffer and IgG was eluted with 25 ml elution buffer (2.2g elution buffer solid/100ml H_20 pH 3.0). Fractions were collected in a predetermined volume (typically 200µl of neutralizing buffer (1M Tris-HCl pH 9.0)/660ml fraction) to bring the final pH to 7.0-7.5. Fractions containing IgG were determined from the absorbance readings at 280nm (A_{280}) and these were pooled and dialysed overnight against phospate buffered saline (PBS) (137mM NaCl/ 8.6mM K_2HPO_4/ 1.5mM KH_2PO_4 with 0.05% NaN_3 as preservative). The protein concentration was determined using

the Lowry assay (2.9). The column was regenerated and stored in regeneration buffer (Bio-Rad), at 4°C. Separate columns were used for α_{1B} and β_3 antibodies. Aliquots of 50-100µl of MAPS purified antibodies were prepared and stored frozen at -70°C.

2.4. Membrane Preparation

Rat brain membranes were purified using a modification of the protocol by Hartshorne and Catterall (1984) as follows: adult or timed-pregnant rats (Harlan) were sacrificed by CO_2 asphyxiation, decapitated and the brains microdissected to obtain the hippocampi. Peri-natal rat pups (Embryonic Day (E)18-Postnatal Day (P)1.5) were placed on ice prior to decapitation. For the developmental studies, membranes were prepared (2.1) from Wistar rats at 10 ages (E18, P0 (birth),and P1.5, P2.5, P4, P6, P10, P16, P25, and P40). The earliest developmental time point at which membrane was prepared was E18, as the hippocampus, which begins to form at E14, lacks defined tissue at earlier times (Altman and Bayer,1990 a,b; Jacobson, 1991).

The brains were homogenized by 8 strokes of a Potter-Elvejhem tissue grinder in approximately 4.5 mL total volume of buffer A (0.32M sucrose, 5mM Tris HCl, pH7.4 at 4°C). The homogenized tissue was centrifuged at $70g_{av}$ for 10' at 4°C (Sorvall combi ultracentrifuge and AH-650 swinging bucket ultraspeed centrifuge rotor (Dupont)). The supernatant was collected, and the pellet discarded. The supernatant volume was brought to 4.5mL with buffer A, and then centrifuged at $27,000g_{av}$ for 45', 4°C. The resulting pellet (P2) was collected and resuspended in 1-2 mL of buffer C (5mM HEPES-Na^+, 0.32M sucrose, pH 7.4 at 4°C), and frozen and stored in 75µl-1ml aliquots in liquid nitrogen. In order to minimize proteolysis, all buffers contained fresh protease inhibitors at the following final

concentrations: pheylmethyl-sulfonylfluoride (PMSF) (100μM), o-phenanthroline (1mM), leupeptin (1μM), aprotinin (1μg/ml), benzamidine (1mM), iodoacetamide (1mM).

Adult synaptic membranes (7.5mg/ml) for immunoblotting were prepared likewise by combining cortical and hippocampal tissues. However, the P2 pellets were resuspended by homogenising in a lysis buffer B (5mM Tris-HCl, 1mM EDTA, pH 8.2 at 4°C, + protease inhibitors). After 15' on ice, the lysate was spun at 27,000g_{av} for 45', at 4°C. The concentration of membrane proteins in these preparations was determined by Lowry assay (2.9).

2.5. Determination of Antibody Titre by Enzyme-Linked Immunosorption Assay (ELISA)

The ELISA is based on an antibody's ability to bind to a peptide antigen adsorbed onto a plastic 96-microwell ELISA plate (Corning). Microwells were coated with 50μl of 0.1mg/ml peptide in Tris buffered saline (TBS) (20mM Tris base/ 500mM NaCl pH 7.5 containing 0.05% NaN_3) and left overnight at 4°C. Each well was washed 3 times (200μl/well) in TBS and blocked with 320μl of blocking solution (3% BSA/TBS pH 7.5/0.01% thimerosal) for 2-3 hours at 20°C. Primary or pre-immune antisera in blocker were diluted in 0.5 log unit intervals (-1.0 to -4.5) added to individual wells in quadruplicate (50μl/well) and incubated 2 hours at 20°C. After 4 washes with TBS (200μl/well), the wells were treated with horseradish peroxidase conjugated donkey anti-rabbit Ig (HRP-DAR; 1:2000 dilution in blocking solution, 50μl/well), for 2 hours at 20°C. The wells were washed 3 times (200μl/well) with blocking solution, and once (200μl) with TBS. The plates were then developed by adding 100μl/well of the peroxidase substrate Azidino-di-(3-ethyl-benzthiazoline-sulfonate) (ABTS) (Boehringer), 5mg/ml in ELISA buffer (25mM borate, 25mM phosphate, 25mM citrate pH 5.0 containing

0.015% H_2O_2). The absorbances were then read at 405nm, 22°C periodically using an ELISA plate reader (UV Thermomax, Molecular Devices). The maximum absorbance used was 1.0, and any further readings were terminated to preclude any non-linearity. To determine the relative amounts of α_{1B} and β_3 antibodies, the midpoints of the antibody titration curves were compared, providing a relative measure of specific antibody concentration, as suggested by Harlow and Lane, (1988).

2.6. Gel Electrophoresis and Immunoblotting

Protein samples (2.4) were generally diluted (≥1:4 (v/v) protein:sample buffer) with an electrophoresis sample buffer A of the following composition: 0.0625 M Tris-HCl (pH 6.8); 10% (v/v) glycerol; 2% (w/v) sodium dodecyl sulfate (SDS); 5% β-mercaptoethanol; and 0.001% (w/v) bromophenol blue. However, for the high resolution blots required to determine α_{1B}, an alternate sample buffer B (Hell, et al., 1995) was used which had the following composition: 125mM Tris HCl pH 6.8; 6% (w/v) SDS; 2mM (w/v) EGTA; 10% (w/v) sucrose, 20mM (w/v) dithiothreitol (DTT); 0.001% (w/v) bromophenol blue; and fresh protease inhibitors (2μg/ml leupeptin, 4μg/ml aprotinin, 100μg/ml benzamidine). Membranes were boiled (100°C) for 5 minutes in sample buffer A, or heated at 50°C for 30-45' in sample buffer B, and resolved by SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) with the Laemmli (1970) buffer system. The samples were electrophoresed in a Bio-Rad Mini-Protean II gel apparatus using a Bio-Rad Model 1000/500 Power supply (Bio-Rad). Discontinuous polyacrylamide gels (80mm x 0.75mm or 1.5mm x 7.3mm) of the following composition were employed: stacking gel - 4%T, 2.7%C or 3%T, 2.7%C (where %T represents the total percentage [w/v] of acrylamide monomer and N,N'-methylenebisacrylamide [BIS] crosslinker,

and %C represents the amount of BIS crosslinker expressed as a percentage of the sum of acrylamide monomer and BIS), 0.125M Tris-HCl buffer (pH 6.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate, 0.05% (v/v) TEMED. Separating gel -5%T, 2.7%C or 7.5%T, 2.7%C, 0.375 M Tris-HCl buffer (pH 8.8), 0.1% (w/v) SDS, 0.05% (w/v) ammonium persulfate and 0.05% (v/v) TEMED. The electrophoresis tank running buffer consisted of 0.025M Tris-HCl buffer (pH 8.3), 0.192M glycine and 0.1% (w/v) SDS. Electrophoretic separation of the 4% stacking /7.5%separating gel occurred at 150-200V constant voltage (~ 45-60'), and for the 3% stacking/5% separating gel at 150V constant voltage (~45'). Kaleidoscope (Kds) pre-stained molecular mass standards (Bio-Rad) containing myosin (~208kDa), β-galactosidase (~144kDa), bovine serum albumin (BSA)(~87kDa), carbonic anhydrase (~44kDa), soybean trypsin inhibitor (~33kDa), lysozyme (~18kDa), and aprotinin (~7kDa), (the exact molecular weights varied between lot number), were also separated on each gel. Each standard lane contained 5µl of Kds diluted in sample buffer 6.25-fold.

The separated proteins were transferred electrophoretically to PVDF (polyvinyl difluoride) membranes (Bio-Rad) (for high resolution α_{1B} determination), or nitrocellulose sheets (for all other applications) by the method of Towbin et al. (1979). Prior to transfer, PVDF membrane was presoaked in 100% methanol for 2-3 sec., before being equilibrated in transfer buffer (0.025M Tris-HCl buffer (pH 8.3), 0.192M glycine and 20% (v/v) methanol) for 3'. Nitrocellulose sheets were equilibrated directly in transfer buffer for 20'. The gels were equilibrated in transfer buffer for 15'. Transfer occurred at 100V constant voltage, in transfer buffer, for 1 hour in a Bio-Rad Mini Trans-Blot Electrophoretic Transfer Cell using a Model 200/2.0 Power Supply (Bio-Rad).

For immunodetection of α_{1B},β_3, or α_2 subunits immobilized on PVDF or nitrocellulose membrane, non-specific sites were blocked following transfer using 5% (w/v) NFDM (Non fat

dry milk) (Bio-Rad)/ TBS/ 0.01% thimerosal (5%NFDM/TBS), overnight at 4°C. Blots that were to be probed with the α_{1B} primary antibody were washed with 3 brief (15") washes in TBS, then 3 long (5-10') washes in TBS, 20°C. All other blots were washed in TTBS (20mM Tris/500mM NaCl/0.05% (v/v) Tween-20 pH 7.5) (3 brief and 3 long washes), and then TBS (3 brief washes and 3 long washes), 20°C. Blots were then incubated for 1.5-2.5 hours at 20°C in primary antibody (rabbit anti-α_{1B} or -β_3, or mouse anti-α_2/δ (Affinity Bioreagents Inc.) diluted in blocker. Controls for specificity included probing with pre-immune sera, or blockade of antibodies by competing peptide antigens (20μM). After repeated washing (3 brief and 3 long washes in TTBS, 3 brief and 3 long washes in TBS), the blots were exposed to HRP DAR diluted in blocker from 1:2000-1:5000, or horseradish peroxidase conjugated goat-anti mouse Ig (HRP GAM; Zymed) diluted in blocker at 1:4000. The blots were then rewashed. Specificity controls included pre-immune sera and/or blockade by competing peptide antigens.

Enhanced chemiluminescence was done by mixing equal volumes of Amersham ECL detection reagents and incubating the mixture on the blots (0.125ml of mixture/cm^2 of nitrocellulose) for 1.5-2'. The sheets were drained, covered in Plastic Wrap (Saran Wrap), placed in autoradiography cassettes (Fisher Biotech; FBXC 810, 8 x 10") and exposed to Kodak scientific imaging film (X-Omat AR, 8 x 10") or Amersham Hyperfilm-ECL (high performance luminescence detection film, 8 x 10"). For high sensitivity applications, Hyperfilm-ECL was preflashed (Sensitize Pre-flash unit, Amersham). The films were developed using equipment in the Toronto Western Hospital radiology unit. For quantitative analysis, films were scanned using a Bio-Rad Model GS-670 imaging densitometer (scan mode:transmittance, pixel density:8 bit precision, max. resolution:64μm) with image analysis software (Molecular Analyst/PC Version 1.1, Bio-Rad) within the range where the

relationship between the protein loaded and densitometric response was linear (Appendix A). The Western blots were displayed pictorially using Photo-Shop 3.0 (Adobe)

2.7. Solubilization and Immunoprecipitation

Rat brain membranes (2.4), with or without preincubation with 5-10 μg/ml unlabelled ω-conotoxin GVIA (ω-CgTx), were radiolabelled with 1nM of [^{125}I]-Tyr^{22}-ω-CgTx GVIA ([^{125}I]-ω-CgTx) in buffer B (10mM HEPES-NaOH pH 7.4, 0.1M NaCl, 0.2mg/ml BSA containing fresh protease inhibitors (0.75mM benzamidine and 0.1mM PMSF)) for 15' at 20°C, by dilution of a radioactive buffer (10.5nM cold toxin, 10.6nM [^{125}I]-ω-CgTx in buffer B). The radiolabelled membranes were then diluted to 4.5ml total volume in buffer B for centrifugation at 100,000 g_{av} for 45' at 4°C (Sorvall combi ultracentrifuge/AH-650 rotor) to separate bound and free label. The pellet was solubilized (at 1mg/ml protein) by resuspension in buffer C (10mM HEPES-NaOH pH 7.4, 1.0M NaCl, 1% (w/v) digitonin, 0.2mg/ml BSA, containing fresh protease inhibitors) for 45' at 4°C. After centrifugation (100,000g_{av} 45' at 4°C) to remove insoluble material, the supernatant was diluted 10-fold in buffer A (10mM HEPES-NaOH pH 7.4, 0.2 mg/ml BSA containing fresh proteases inhibitors) for a final concentration (equivalent to buffer D) of 10mM HEPES-NaOH pH 7.4, 0.1% (w/v) digitonin, 0.1M NaCl, 0.2mg/ml BSA. Immunoprecipitations were performed using antibody-coated protein A agarose beads. Protein A agarose was washed 3 times with PBS, incubated with the appropriate MAPS purified antiserum (3.3.1) for 6 hours at 4°C and pre-equilibrated by washing 3 times with buffer D (10mM HEPES-NaOH pH 7.4, 0.1% (w/v) digitonin, 0.1M NaCl, 0.2mg/ml BSA in ice-cold water with fresh protease inhibitors). Protein A agarose-antibody complexes (100μl of 50% slurry) were mixed with a fixed volume (from

250-500µl) of labelled, solubilized membranes and gently mixed end-over-end overnight at 4°C. Immune complexes were harvested by centrifugation of protein A agarose-antibody complexed beads, washed three times with a 20 fold excess of buffer D and the pellet radioactivity determined by gamma counting. For competition analysis, free primary antisera, and protein A agarose-antibody complexes, were incubated with the appropriate peptides (0.1-10µg/ml in blocking solution) for 45'.

2.8. Radioligand Binding

N-VDCCs were determined by radioligand binding assays using [^{125}I]Tyr^{22}-ω-conotoxin GVIA [^{125}I]-ω-CgTx (Cruz and Olivera,1986) as outlined (Jones and So, 1993; Mills, et al., 1994).

2.8.1. "Equilibrium" Binding Assays

Rat brain membranes (2.4) were diluted in ice-cold reaction buffer (400mM sucrose, 5.6mM HEPES free acid, 4mg/mL BSA pH to 7.4 at 20°C with 2M Tris base) to 0.5mg/ml final protein concentration. To assay total binding, 30µl of diluted membrane were added to 40µl reaction buffer. After 30' incubation at 20°C, 30µl of [^{125}I]-ω-CgTx (81.4TBq/ mmol, Nen Dupont) labelling mixture (0.96nM cold ω-CgTx, 0.182nM [^{125}I]-ω-CgTx in reaction buffer) (0.3nM final hot + cold toxin) were added, and incubated for 45' at 20°C. Free and membrane bound [^{125}I]-ω-CgTx were then separated by vacuum filtration (Hoefer) with 3 washes of ice-cold wash buffer (160mM choline chloride, 5mM HEPES free acid, 1.2mM $CaCl_2.H_2O$, 1mg/ml BSA pH to 7.4 at 4°C with 2M Tris base) added to each sample (3 x

2.5ml). Filter bound [^{125}I]-ω-CgTx was determined by counting gamma emission. Non-specific [^{125}I]-ω-CgTx binding was determined by preincubation of membranes for 30' at 20°C with 10µl of native ω-CgTx (500nM of toxin final), and 30µl of reaction buffer. A small aliquot (30µl) of labelling mixture was sampled for standardization of the counts.

2.8.2. Association and Dissociation Assays

The rate of association of [^{125}I]-ω-CgTx to rat brain membranes was measured by scaling up the standard ω-CgTx reaction greater than 30 times (2.8.1), for an amount of material sufficient to assay 10 triplicate points. Therefore, for every sample to be assayed, 1035µl of adult rat brain membrane was diluted to 0.5mg/ml and added to 1380µl of reaction buffer. After 30' incubation at 20°C, 1035µl of [^{125}I]-ω-CgTx labelling mixture was added as above. The time at which all components were initially mixed together (including control experiments where the same reaction was pretreated with 500nM cold toxin) was defined as t_0. At subsequent times, 3 x 100µl aliquots were removed, and separated by vacuum filtration with 3 washes of ice-cold wash buffer (3 x 2.5ml) as above. The binding of [^{125}I]-ω-CgTx as a function of time was determined by counting gamma emmission.

To assay the rate of dissociation of [^{125}I]-ω-CgTx from rat brain membranes, a reaction mixture was prepared that was similar to the reaction used in the association assay. Therefore, once all the components were mixed (membrane, reaction buffer, labelling mixture in the absence and presence of 500nM cold toxin), the reaction was left to incubate for >1 hour at 20°C so that maximum binding of [^{125}I]-ω-CgTx to membrane binding sites occurred. At time t_0, cold toxin (1µM) was added in excess. At subsequent times, 3 x 100µl aliquots

were removed, and separated by vacuum filtration with 3 washes of ice-cold wash buffer (3 x 2.5ml) as above. The decline in binding of [^{125}I]-ω-CgTx as a function of time was determined by counting gamma emmission.

2.9. Protein Determination

Protein concentration was determined using the Lowry Assay (Lowry et al., 1951). Briefly, one part of solution B (2% (w/v) Na^+K^+Tartrate/ H_2O) was mixed with one part of solution C (1% (w/v) $CuSO_4$/ H_2O). This mixture was added to solution A (2% (w/v) Na_2CO_3, 0.4% (w/v) NaOH/ H_2O) to create Lowry solution 1. Protein solutions were prepared in H_2O (100µl total volume) at various dilutions, in triplicate, using BSA as standards. A 1ml volume of Lowry solution 1 was added to each sample. After 10 minutes, 100µl of Lowry solution 2 (Folin-Ciocalteu's phenol reagent (Fluka) diluted 1:1 in H_2O) were added to each sample, while mixing. Absorbances were read 45' later at 750nm, and calculations of sample concentrations were based on the BSA standard curve. The Lowry assay was adapted for the quantification of membrane protein, by the addition of 2% final SDS to each membrane sample (100µl of a 24% stock added to the membranes prior to the addition of Lowry solution 1).

2.10. Thiol Assay

Free thiol concentrations were determined using the 5,5'-Dithio-bis (2-Nitrobenzoic Acid) (DTNB) assay of Ellman (1959). Thiol-containing solutions were diluted 20-fold in 1ml total volume of 50mM K^+ PB pH 8.0 and 20µl of 10mM DTNB reagent (Pierce) was added. After

15', absorbances were read at 412nm. Concentrations were determined with a standard curve constructed using 0-80μM cysteine.

3. ANTIBODY DEVELOPMENT AND CHARACTERIZATION

3.1. Purification of α_{1B} and β_3 Subunit Antibodies

To resolve N-VDCC expression in detail, polyclonal antiserum raised against the α_{1B} and β_3 subunits were purified prior to their use in immunocytochemical experiments for removal of contaminants. The purification strategy involved MAPS purification (2.3), in which the IgG fraction of antiserum was enriched. Therefore, high titre α_{1B} and β_3 antiserum assessed by ELISAs (2.5) were MAPS purified, yielding IgG concentrations determined by Lowry assays (2.9) of 1.8-3.3mg/ml in PBS/0.02% NaN3 for β_3 antiserum and 3.3mg/ml in PBS/0.02% NaN_3 for α_{1B} antiserum.

3.2. Characterization of α_{1B} and β_3 Subunit Antibodies

The full characterization of antibodies is essential, prior to their use in more in-depth and applied studies of protein identification (Chapters 4 and 5). The characterization steps, which include immunoassays (2.5), immunoblotting (western blotting) (2.6) and immunoprecipitations (2.7) are designed to establish optimal conditions for antibody use in biochemical applications. For immunocytochemical experiments more rigorous purification through affinity based methods is invariably required.

The ELISA results indicated that antiserum collected from rabbits at different periods of time following immunization with peptide antigen varied in titre, increasing to a maximum several weeks after their first boost injection, as was expected (Not shown). After this period,

antiserum was extracted for MAPS purification (2.3). At this time the titre of the β_3 subunit antibody was 1:6300. whereas the titre of the α_{1B} subunit antibody was somewhat lower (1:731) (2.5) (Figure 5B and 5A. respectively). The ELISA was also used to test for the origin of other IgGs in addition to those specifically recognizing the peptide. Such contaminating IgGs are most likely to arise through an immune response to the carrier KLH and would go undetected in an ELISA based on coated peptide. To check for such contaminating IgGs. MAPS purified β_3 antiserum was added to ELISA plates coated with either 0.1mg/ml β_3 peptide antigen. KLH conjugated β_3 peptide (sulfo-MBS crosslinker) or KLH conjugated to an unrelated peptide - the flag epitope (IBI) (sulfo-MBS crosslinker). The plates were then developed as above, using MAPS β_3 primary antibodies. The presence of antibodies within the MAPS purified β_3 antiserum besides those recognising the β_3 peptide was evident from the high titre seen in plates coated with the KLH-β_3 peptide compared to the β_3 peptide alone. The reactivity towards the KLH-β_3 peptide appeared to result from binding of non-β_3 antibodies to the KLH immunogen, since a signal was also detected in wells coated with KLH conjugated to the flag peptide (Figure 6).

Having determined, by ELISA, that the β_3 and α_{1B} antibodies were able to recognize the corresponding peptide antigens that they were immunized against, studies were undertaken to determine the ability of the antibodies to recognize their corresponding endogenous protein antigens in immunoblotted rat brain membrane. These studies showed that the α_{1B} antibodies recognised a single broad band of molecular weight 226kDa in immunoblots of adult rat brain membranes (Figure 7A). Under some circumstances (e.g. in developmental studies), this broad band could be resolved into a doublet corresponding to polypeptides with molecular weights of and 240kDa. The 220kDa and 240kDa bands were completely abolished by

5A

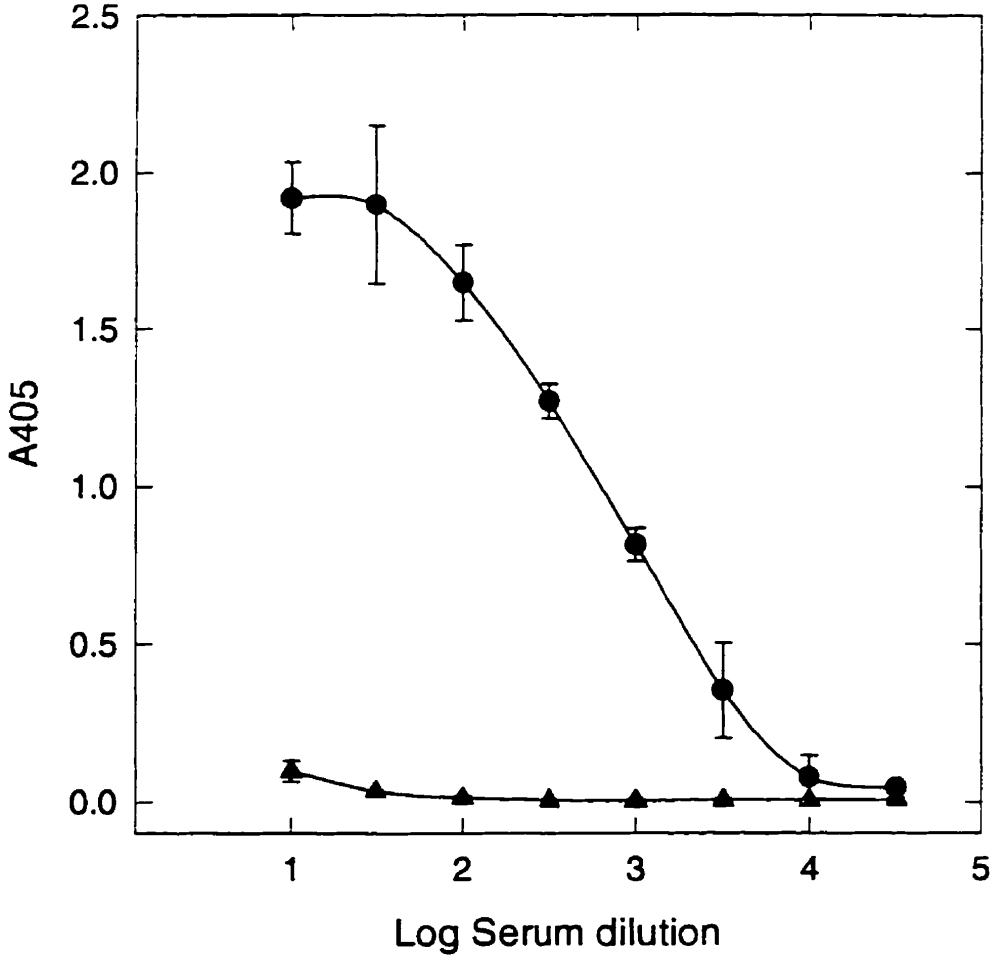


Figure 5. Enzyme-Linked Immunosorption Assay (ELISA) Indicating the Binding of α_{1B} and β_3 Immune and Preimmune Sera to their Corresponding Peptide Antigens. 5A. The binding of various dilutions of α_{1B} immune (●) and preimmue (▲) antisera to the α_{1B} peptide antigen. The bound primary antibody was complexed with HRP-conjugated goat anti-rabbit antibodies. The adsorbed peptide was assayed colorimetrically using the HRP substrate ABTS. All data is ±SEM of measurements made in at least triplicate.

5B

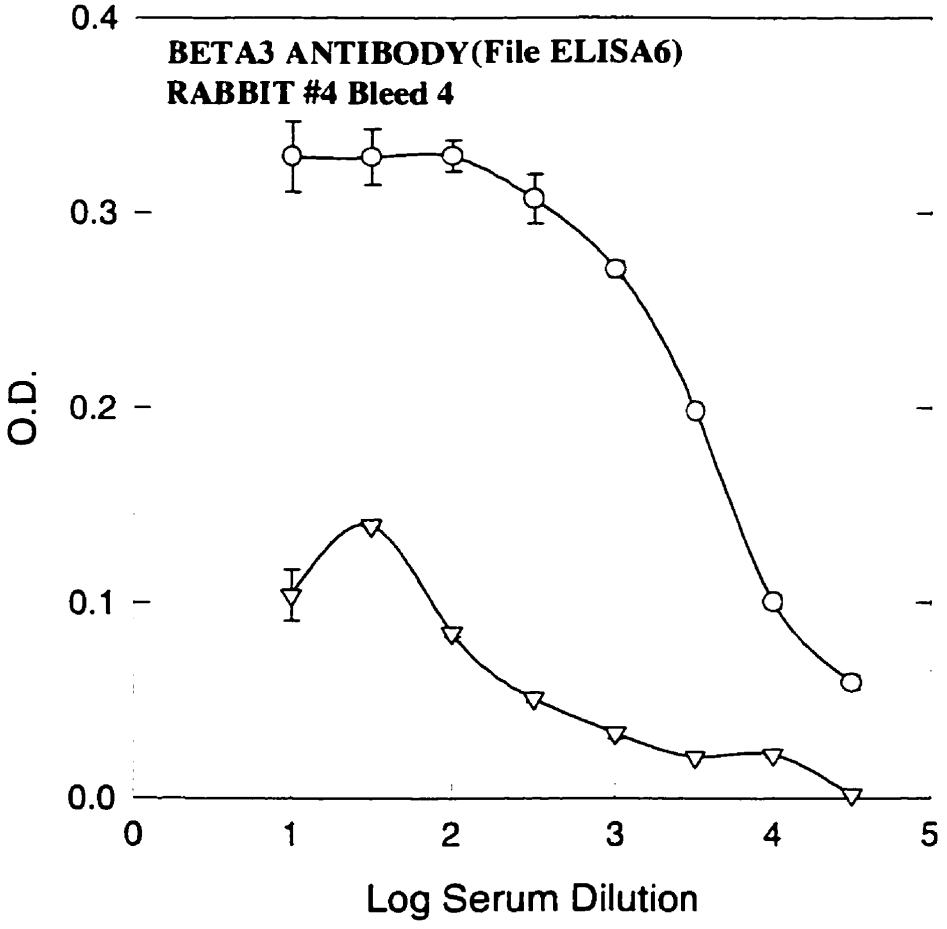


Figure 5B. The binding of various dilutions of β_3 immune (O) and preimmune (∇) antisera to the β_3 peptide antigen. The bound primary antibody was complexed with HRP-conjugated goat anti-rabbit antibodies. The adsorbed peptide was assayed colorimetrically using the HRP substrate ABTS. All data is ±SEM of measurements made in at least triplicate.

6

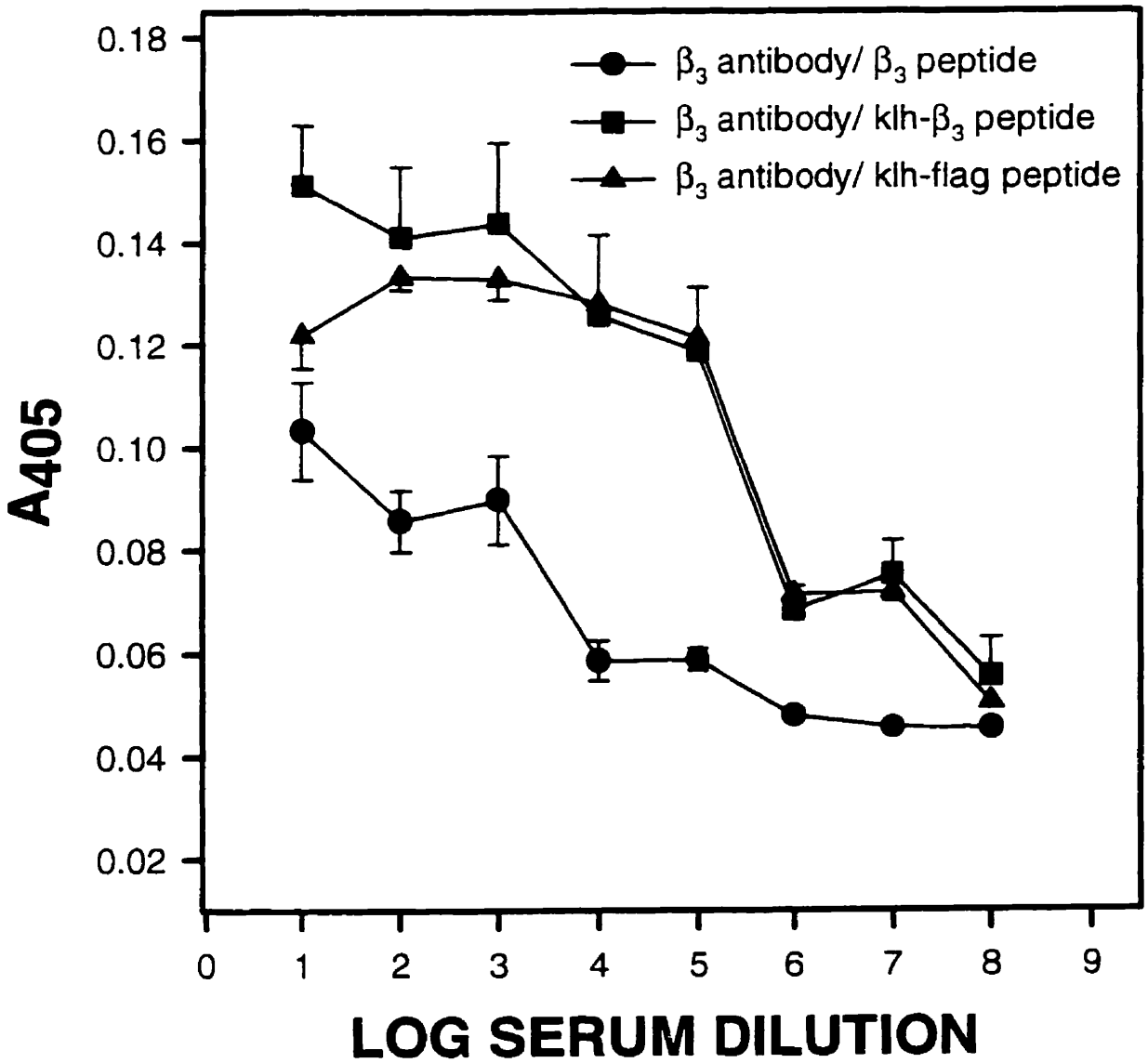


Figure 6. Enzyme-linked Immunosorption Assay (ELISA) of Specific, and Contaminating IgG Present in MAPS Purified β_3 Antiserum. MAPS purified β_3 antibody was incubated with various antigens bound to an ELISA plate, including: β_3 peptide (●); β_3 peptide conjugated to keyhole limpet hemocyanin (KLH) (■) and flag peptide conjugated to KLH (▲). All data is ±SEM of measurements made in at least triplicate.

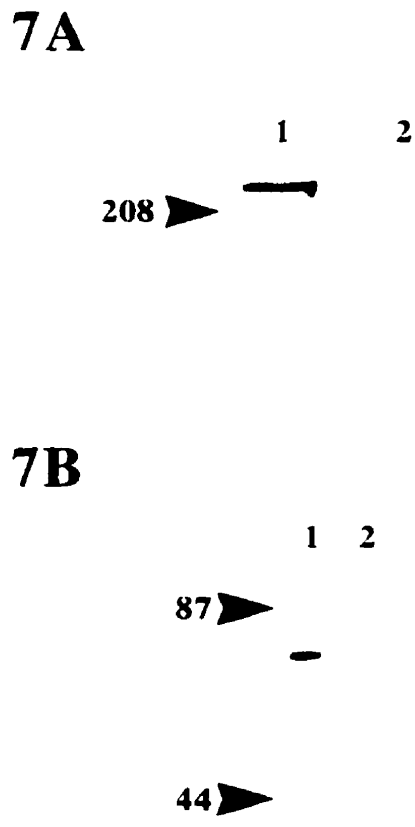
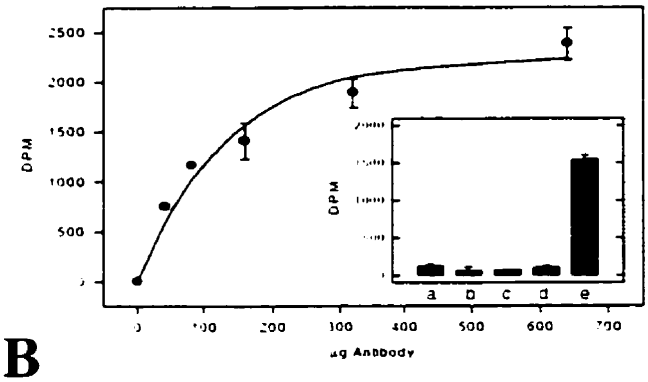


Figure 7A. Antibodies Against α_{1B} Recognize a Band of approximately 220 kDa on Immunoblots (150 µg/well of adult cortex+hippocampal membrane) (lane 1). No staining of intact (lane 2) immunoblotted membranes was observed if the antibody was first treated with competing α_{1B} peptide antigen (40µM). Blots were analyzed with MAPS purified α_{1B} antibody (10µg/ml) and detected by ECL (Methods). 7B. Antibodies Against β_3 Recognize a Band of 55 kDa on Immunoblots (5 µg/well of adult cortex+hippocampal membrane) (lane 1). The band was completely displaced by competing peptide antigen (lane 2) (40µM). Molecular weights were derived using pre-stained molecular weight standards and are shown as arrowheads at left. All data were confirmed through at least two additional experiments.

competing antigens, even following overexposure of the film. Extraneous bands that were not blocked by corresponding peptide antigens were also noted, and were thus attributed to non-specific binding. In contrast to the band(s) specifically recognized by the α_{1B} antibody, the β_3 antibody recognised a single band of molecular weight 55kDa on immunoblotted adult membrane (Figure 7B). The 55kDa band was completely abolished by the addition of competing peptide antigen, even after overexposure of the film.

In order to determine the specificity of the antibodies towards the α_{1B} and β_3 subunits and to determine the fraction of N-VDCCs containing these subunits, immunoprecipitation assays were used. The specificity of the antibodies was first defined from the extent to which the antibodies could immunoprecipitate [^{125}I]-ω-CgTx binding sites from rat brain membranes solubilized with digitonin. From the low level of counts in control immunoprecipitates, for example, those made with pre-immune serum or the absence of antibody (See Figure legend), it appeared that both α_{1B} and β_3 antibodies were highly specific. To determine the maximum amount of α_{1B} or β_3 subunit associated with [^{125}I]-ω-CgTx binding sites, immunoprecipitations were performed as above using increasing quantities of MAPS purified antibodies. Antisera generated against the α_{1B} subunit revealed a dose-dependent immunoprecipitation of up to 55% of the total [^{125}I]-ω-CgTx binding sites in solubilized adult rat brain membrane (Figure 8A). MAPS purified antisera generated against the β_3 subunit also selectively immunoprecipitated [^{125}I]-ω-CgTx binding sites from solubilized adult rat brain membranes. The maximum fraction of [^{125}I]-ω-CgTx binding sites that could be immunoprecipitated by the β_3 antibodies was 76% (n=5) of that immunoprecipitated by α_{1B} antibodies (Figure 8B).

8A



8B

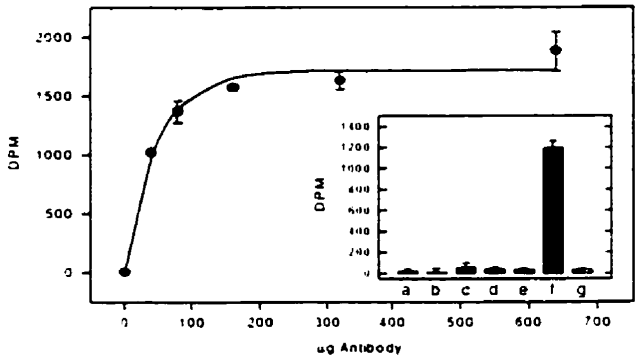


Figure 8A. Immunoprecipitation of [^{125}I]-ω-CgTx-labelled N-VDCCs by Anti-α_{1B} Antibodies. [^{125}I]-ω-CgTx labelled N-VDCCs were solubilized with digitonin (Methods) and their interaction with anti-α_{1B} antibodies was demonstrated by the concentration-dependence of immunoprecipitation. The data was fit assuming a saturation curve of the form y=2254 x [1-exp(-x/134)], according to Westenbroek et al., (1992). The value of 2254 DPM corresponds to 55% of the total [^{125}I]-ω-CgTx in each reaction. Inset: the specificity of the interaction between anti-α_{1B} antibodies and solubilized [^{125}I]-ω-CgTx binding sites was determined by comparing the radioactivity in experimental immunoprecipitations (e) with control immunoprecipitations using: pre-immune serum (a); membranes that were pre-treated with excess cold ω-CgTx prior to radiolabelling (b); competing antigenic peptide (25μM) (c); and preimmune serum plus competing peptide antigen (25μM). 8B. Immunoprecipitation of [^{125}]-ω-CgTx-labelled N-VDCCs by anti-β_3 antibodies. Digitonin solubilized [^{125}I]-ω-CgTx-labelled N-VDCCs were immunoprecipitated as described for anti-α_{1B} antibodies (8A above) and the data fit to the saturation equation y=1717 x [1-exp(-x/48)] as above. The value 1717 DPM corresponds to 42% of the total [^{125}I]-ω-CgTx sites in each reaction. Inset: experimental immunoprecipitations (f) were compared with: control membranes (see 8A) (a); competing antigenic peptide (25μM) (b); control membranes plus competing peptide (c); immunoprecipitates with no primary antibody (d); control membranes and no primary antibody (e); preimmune serum (g). All data is ±SEM of measurements made in at least triplicate.

4. EXPRESSION OF N-VDCC SUBUNITS IN DEVELOPMENT

The expression of N-VDCCs was investigated in the developing rat hippocampus using toxin binding and immunological techniques. The toxin binding assays (2.8) exploit the high affinity, irreversible binding of ω-CgTx GVIA, a peptide derived from the venom of a marine snail *Conus geographus* (Aosaki and Kasai, 1989; Plummer et al., 1989; Ellinor et al., 1994) to the N-VDCC α_{1B} subunit (Fox et al., 1987a,b; Sher and Clementi, 1991; Tsien et al., 1991). This toxin has previously been shown to be a potent inhibitor of N-VDCCs in a variety of mammalian neuronal preparations where its most notable action is to block neurotransmitter release (Kamiya et al., 1988). Throughout these studies, we used membranes from the rat hippocampus (2.4). The choice of rat tissues is based on the high cross-species conservation of N-VDCCs. The rat hippocampus was chosen for its patho-physiological significance - it is prone to epileptiform activity (Traub and Llinas, 1979), ischemic damage (Ben-Ari, 1992) and the plasticity seen in learning and memory (Bliss and Collingridge, 1993). Moreover, a considerable amount of knowledge has accumulated concerning the anatomy and electrophysiology of the hippocampus and, of particular relevance, the manner in which this structure develops is now well defined (Bayer, 1980). The range of ages selected, E18 to adult, reflects the fact that the hippocampus in rat only begins to form as a distinct structure at E16. Microdissection to remove the hippocampus from the earlier developing neocortex prior to E18 is extremely difficult, and frought with the risk of including tissues from associated cortical areas.

4.1. Ontogeny of Hippocampal N-VDCC Complexes Determined by Radioligand Binding

The initial step in defining the ontogeny of the N-VDCCs was to assess how the density of [^{125}I]-ω-CgTx binding sites changed with development. [^{125}I]-ω-CgTx binding sites were quantified using a binding assay, with rat hippocampal membranes obtained from various developmental stages. Since the binding of [^{125}I]-ω-CgTx is essentially irreversible (See below), a true equilibrium is never reached. Thus the radioligand binding assay has been modified to determine the amount of binding after a fixed time interval of 45'. Independent studies have shown that the concentration of [^{125}I]-ω-CgTx binding sites is 0.35pmol/mg in adult rat hippocampal membranes. To perform these developmental assays, the concentration of [^{125}I]-ω-CgTx used was at least 6.4-fold higher than this value. Thus, the degree of binding under these conditions equates to the number of N-VDCCs present in the tissue. Specific binding of [^{125}I]-ω-CgTx was detected as early as E18 in hippocampal membrane, which was the earliest time point examined (Figure 9). The [^{125}I]-ω-CgTx binding rapidly increased by 4-fold (0.025pmol/mg/day) between E18 and P16, and decreased modestly, prior to remaining at a constant level after P25. Thus, maximal binding was attained by P16. The half maximal binding ($E_{0.5}$) of [^{125}I]-ω-CgTx occurred at P3.5 in these assays.

In the above analysis, it was assumed that the toxin-N-VDCC interaction was essentially irreversible and thus any developmental changes in the amount of specifically bound conotoxin corresponded to changes in levels of N-VDCCs. An alternative explanation might be that the low degree of binding in early development reflects a weak interaction with the N-VDCCs rather than a paucity of sites. This was readily resolved using kinetic assays (2.8.1., 2.8.2.). In these assays, the affinity of [^{125}I]-ω-CgTx towards its membrane binding sites was

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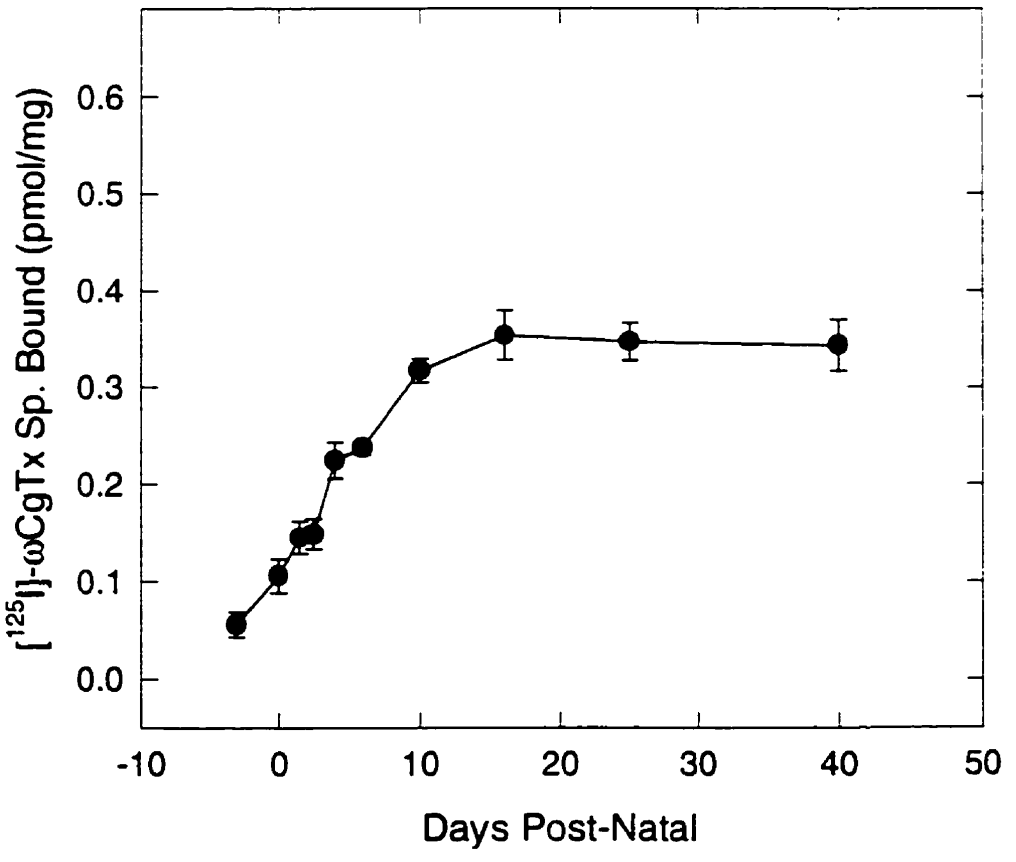


Figure 9. Expression of N-VDCCs in Development Determined by Radioligand Binding. Ontogeny of [^{125}I]-ω-CgTx binding in hippocampal rat brain synaptic membranes. Values (pmol/mg total protein) represent mean ± SEM (n=4).

determined at various stages of development by direct assay of the kinetics of [^{125}I]-ω-CgTx association with, and dissociation from, hippocampal membranes. These studies revealed that the binding of [^{125}I]-ω-CgTx to membranes prepared from P0 and P40 hippocampus conformed to a simple bimolecular reaction whose kinetics of association (k_{on} =3.0 x 10^8 lit.mol^{-1}min^{-1} at (P0) and (P40)) or dissociation (k_{off} =1.13 x 10^{-3}min^{-1} (P0) and 0.9 x 10^{-3} min-1(P40)) were essentially identical, and gave similar Kds (3.7pM (P0) and 3pM (P40)) (Figures 10A and 10B), respectively.

4.2. Immunoblot Analysis of the Expression of N-VDCC Subunits in the Developing Hippocampus

To determine how the expression of the various N-VDCC subunits changed in development, rat brain hippocampal membrane from the various developmental stages were resolved by SDS-PAGE in triplicate, with each lane of the gel representing an individual time point (2.6). The blots were electrophoretically transferred to PVDF or nitrocellulose membranes (2.3) and probed with rabbit anti-α_{1B} or anti-β_3 polyclonal antibodies. Expression of the ubiquitous α_2/δ subunit was examined using a commercially available monoclonal antibody to the skeletal muscle protein (Upstate). This antibody has been shown to cross react with α_2/δ subunits in a wide range of tissues.

Membranes probed with the α_{1B} antibody revealed that the expression of the α_{1B} subunit (average Mr 220kDa) was relatively low at E18, the earliest time point examined (Figures 11A and 11D). After P0, there was a marked increase in immunoblotted α_{1B} subunit, attaining a 48-fold increase by the time it reached a maximum value at P10. A slight decline followed, however, the α_{1B} subunit remained at a fairly constant level thereafter. Several

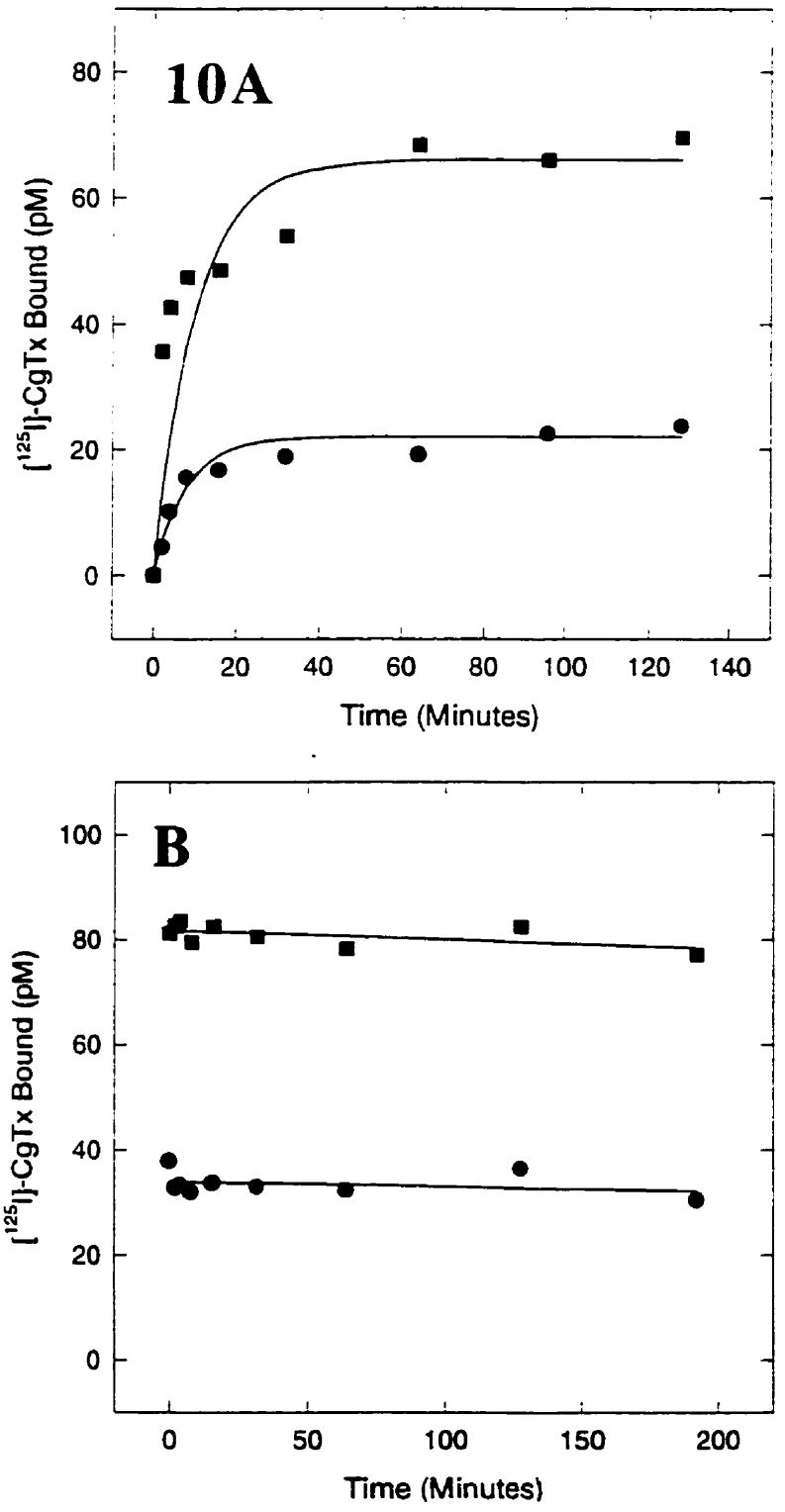


Figure 10A and 10B. Kinetics of Association and Dissociation of [^{125}I]-ω-CgTx to Hippocampal Membranes from Neonatal (P0) or Postnatal Day 40 (P40) Rats. The rates of [^{125}I]-ω-CgTx association (10A) and dissociation (10B) were determined by filtration assays (Methods) for membranes at P0 (●) and P40 (■). Curves were fit assuming bi-molecular reaction kinetics using a non-linear least squares algorithm.

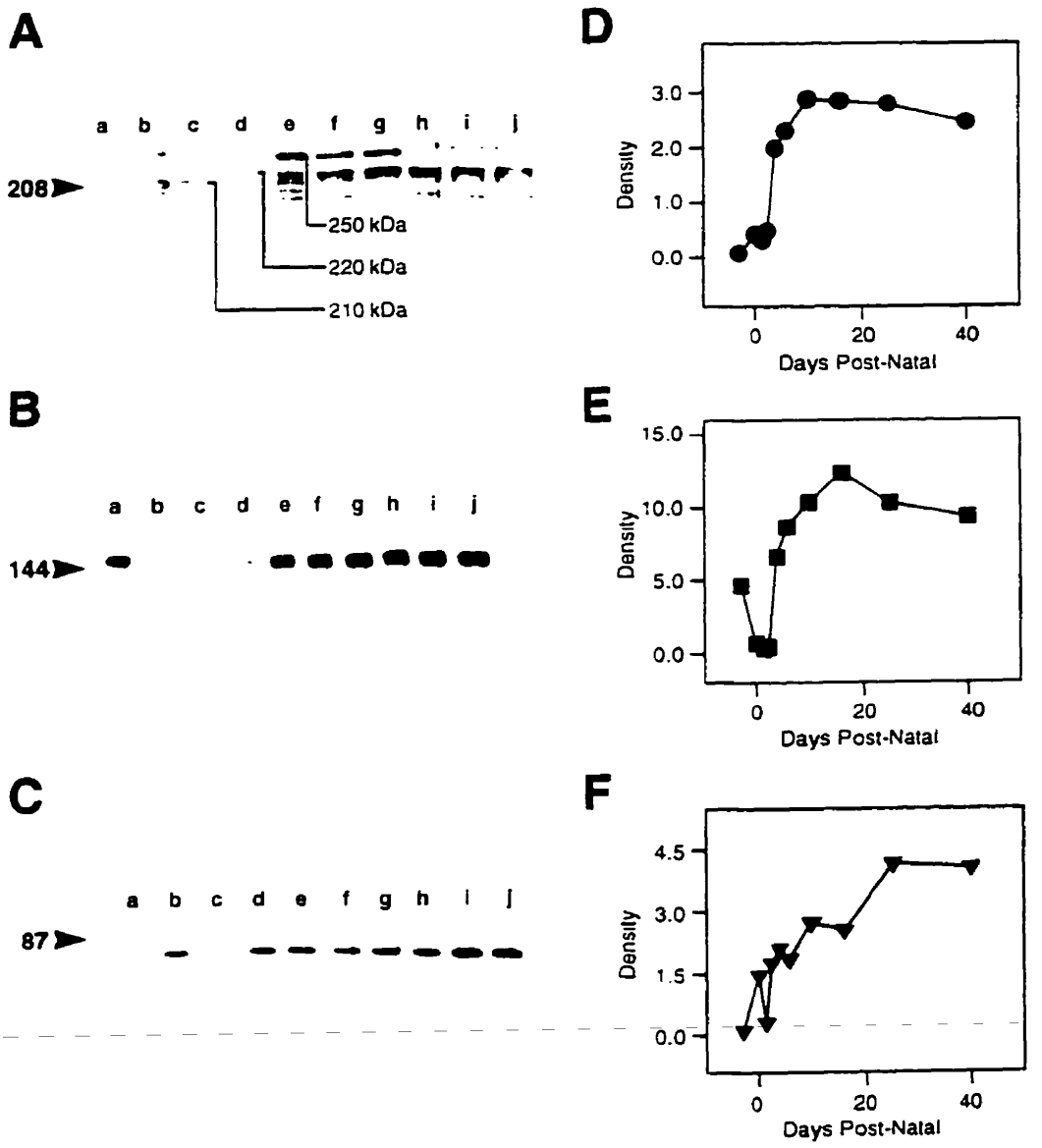


Figure 11. Ontogeny of N-VDCC Subunits in Hippocampal Membranes Determined by Immunoblotting. Immunoblots were probed with the following antibodies: α_{1B} (150 µg membrane/well) (A); α_2/δ (25 µg membrane/well) (B); and β_3 (5 µg membrane/well) (C) (Methods). Lanes a-j correspond to the following ages at which the hippocampal membranes were prepared E18, P0, 1.5, 2.5, 4, 6, 10, 16, 25 and 40. Arrowheads at left denote the positions of molecular weight markers. Densitometric scans of immunoblots corresponding to α_{1B}, α_2/δ and β_3 are shown in panels D-F, respectively (see Appendix A). All data were confirmed through at least two additional experiments.

minor bands, such as those at 210 and 250kDa whose expression varied during development, were present at molecular weights slightly above and below the molecular weight of the major α_{1B} band (Figure 11A lanes e-g). These minor bands were displaced by the addition of excess competing peptide antigen. Expression of β_3 was first evident at E18 (Figures 11C and 11F). The expression of β_3 subunits also increased markedly with development, exhibiting a 36-fold increase between E18 and P40. The increase was phasic, exhibiting peaks at P0, P4 and P10, prior to attaining a maximum level at P25 (n=3). A phasic profile was also exhibited by the band corresponding to the reduced α_2/δ subunit on immunoblots (molecular weight 150kDa; Gurnett et al., 1996) (Figures 11B and 11E). Expression of the α_2 subunit was evident at E18, and waned until P4 when it rose to a peak at P16. After P16, expression of the α_2/δ subunit declined modestly until P40, similar to that which was found with the α_{1B} subunit (Figure 11A and D).

4.3. Immunoprecipitation Analysis of the Expression of α_{1B}-β_3 N-VDCC Complexes in the Developing Hippocampus

Although the above data clearly indicated an increase in N-VDCC α_{1B} and β_3 subunits, this type of analysis by itself cannot resolve whether these subunits are actually present as separate unassembled subunits or whether they are actually together in the VDCC complex. I therefore examined the degree of co-assembly between α_{1B} and β_3 subunits during various developmental stages in solubilized hippocampal membranes by immunoprecipitations of [^{125}I]-ω-CgTx using β_3 antibodies (2.7) (Figure 12). The extent of α_{1B}-α_2/δ complexation was not examined due to the low immunoprecipitation of solubilized N-VDCCs found by others using anti-skeletal muscle α_2/δ antibodies (Sakamoto and Campbell, 1991). This may have

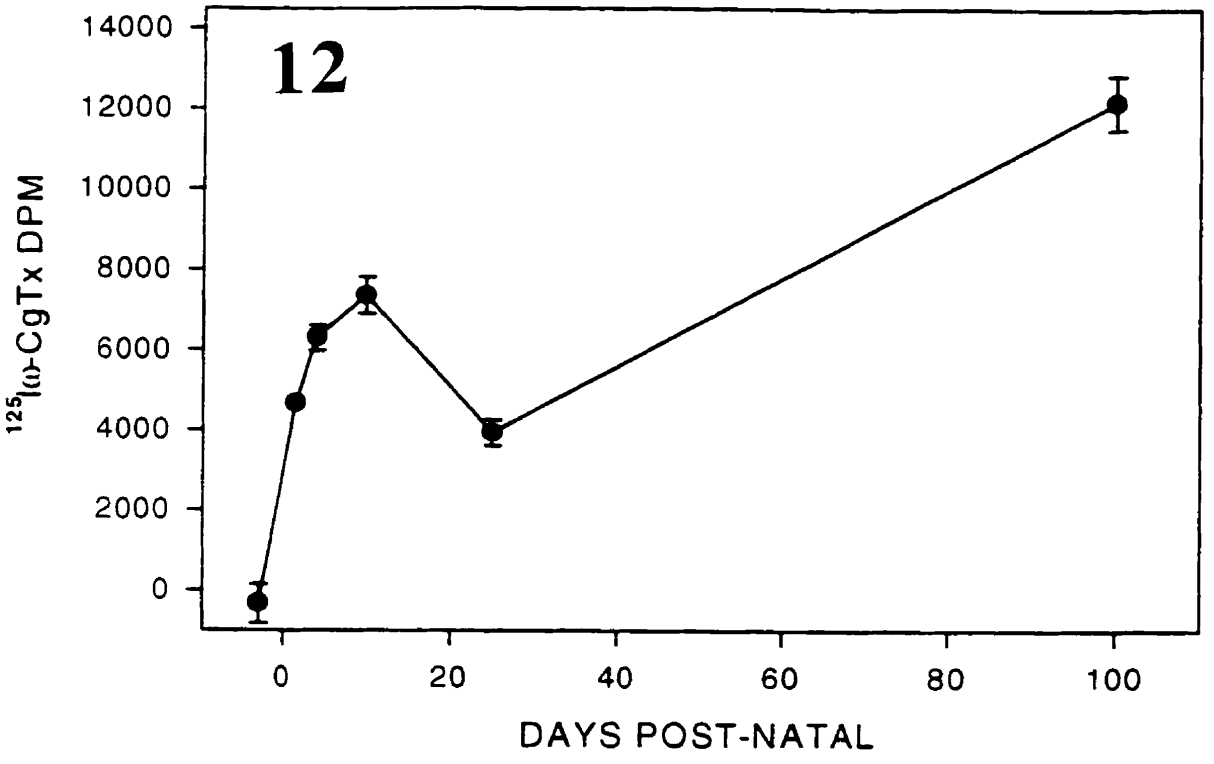


Figure 12. The expression of β_3 Subunits in N-VDCC Complexes Increases in Development. Immunoprecipitation of [^{125}I]-ω-CgTx binding sites from solubilized, P0-adult, hippocampal membranes by anti-β_3 antibodies. All data is ±SEM of measurements made in at least triplicate.

been partly due to the possibility that N-VDCCs contain α_2/δ subunits that are not recognized by this antibody (Westenbroek et al., 1992) (see Discussion). It had been previously found that, during development, the expression of solubilized [^{125}I]-ω-CgTx binding sites was similar to that of the [^{125}I]-ω-CgTx binding sites determined through binding assays (not shown). However, the fraction of solubilized [^{125}I]-ω-CgTx binding sites that could be immunoprecipitated by β_3 antibodies (Figure 12) did not have the same profile as the [^{125}I]-ω-CgTx binding assays (Figure 9). Rather, [^{125}I]-ω-CgTx in the β_3 immunoprecipitates rose from negligible levels at E18, peaked at P10, showed a modest decline to P25 and subsequently increased to adult levels. Since saturating amounts of β_3 antibodies were used in the immunoprecipitations, the changes in the [^{125}I]-ω-CgTx levels immunoprecipitated by the β_3 antibodies throughout development represented changes in the total amounts of complexed subunits. However, these experiments did not take into consideration the variations in the levels of the α_{1B} -β_3 complexes relative to the amount of total α_{1B} subunits at different periods during development. Thus, the proportion of α_{1B} subunits complexed with β_3 subunits within the total α_{1B} pool at various stages of development was determined by dividing the amount of [^{125}I]-ω-CgTx immunoprecipitated by the β_3 antibody, by the total [^{125}I]-ω-CgTx bound in binding assays (Figure 13). The ratio of α_{1B} coupled to the β_3 subunit varied throughout development, increasing from E18 to P4, maintaining a nearly steady state between P4 and P10, only to decrease after P16.

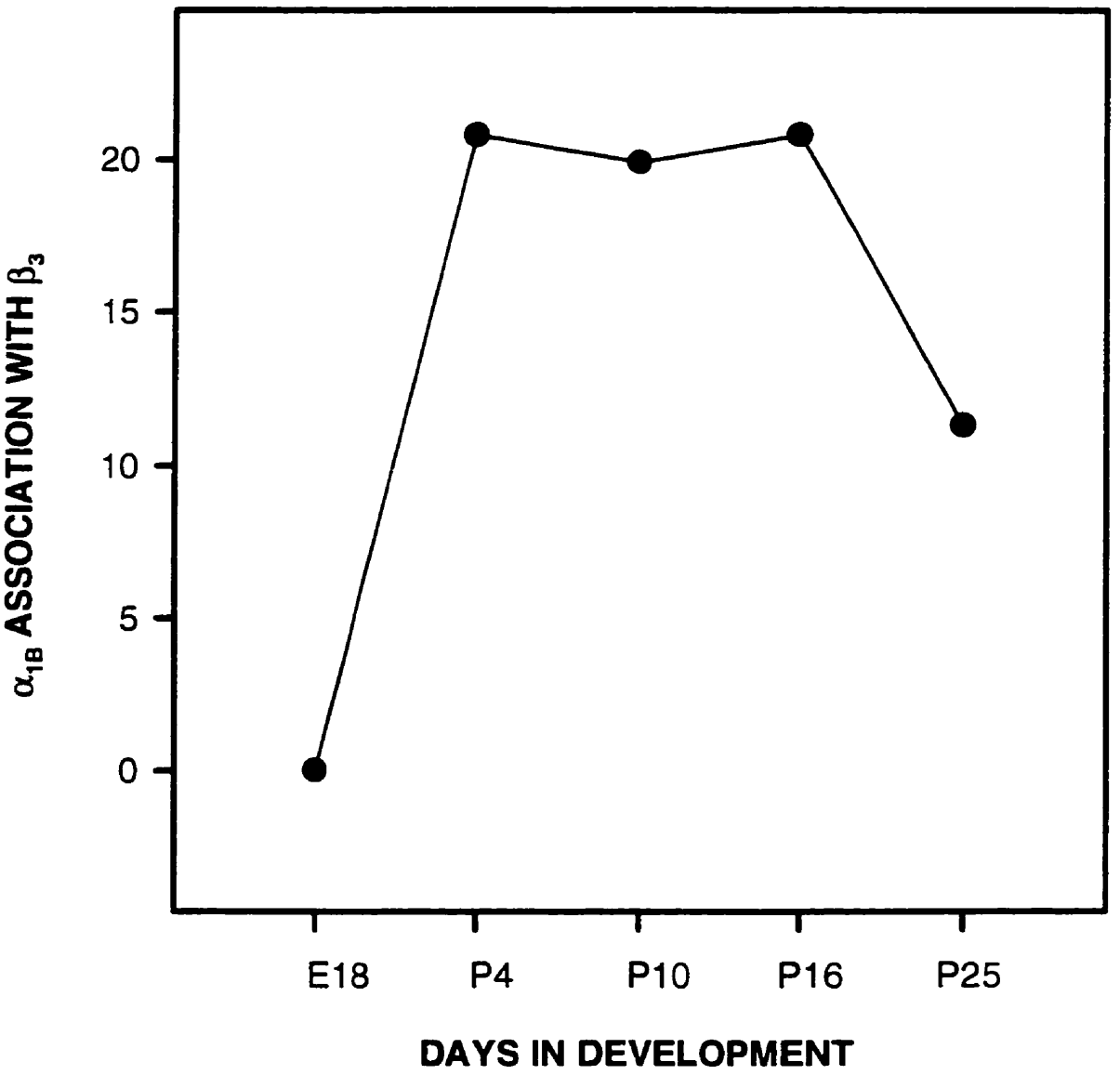


Figure 13. The Proportion of α_{1B} Subunits Complexed with β_3 Varies in Development. The proportion of total [^{125}I]-ω-CgTx binding sites that was associated with β_3 was assessed in rats ages E18-P25 by dividing the amount of [^{125}I]-ω-CgTx immunoprecipitated by the anti-β_3 antibody (12A), by the total amount of [^{125}I]-ω-CgTx bound in binding assays (9) at various stages of development.

5. DISCUSSION

5.1. Summary of Results

In this thesis I have described the successful preparation of antibodies directed against specific subunits of the N-VDCC complex. These antibodies were characterized immunologically using ELISAs, immunoblots and immunoprecipitations. The high titre anti-peptide antibodies produced by the methods in this thesis indicated that N-VDCC complexes consist of α_{1B} and β_3 subunits of molecular weights 226 and 58kDa respectively. Both the α_{1B} and β_3 subunits immunoprecipitated [^{125}I]-ω-CgTx binding sites, as was expected based on the selectivity of conotoxin binding to N-VDCCs.

5.2. Rationale for the Use of Anti-peptide Antibodies

The antibodies used in this thesis were generated against peptides that corresponded to unique regions of N-VDCC subunits. This ensured that the antibodies would specifically recognize the subunits of interest, and not other subunits Boersma et al., 1989). This is particularly important since many subunits that are similar in structure have been identified through molecular cloning, and it is conceivable that other N-VDCCs are yet to be discovered (Dubel et al., 1992; Birnbaumer et al., 1994). The ability of anti-peptide antibodies to specifically localize subunits is a tremendous advantage over traditional methods of antibody preparation. These traditional methods have usually relied on immunization with purified protein (Levey, 1993). However, the purification of many proteins, especially those like VDCCs which are found in low abundance in membranes, is not trivial (Dunlap et al.,

1995). In contrast, once sequence information comes to light through molecular cloning strategies it is relatively straightforward to design antigens based on the predicted gene products (Levey, 1993). Another problem obviated by anti-peptide antibodies is that their cross-reactivity with other known proteins can be reduced simply by choosing the appropriate target sequence. Purified proteins will often cross-react with proteins other than the protein of interest due to similarities in secondary and tertiary structures, and most importantly due to regions of the protein that contain epitopes which are shared amongst various proteins (Levey, 1993). Of course anti-peptide antibodies are not the only way to generate antibodies against gene products. A powerful alternative is to create the target protein, or large fragments of it (>25 amino acids) in a suitable cDNA expression system, typically bacteria. Similar to synthesized peptide sequences, fusion proteins can be generated in sufficient quantities for use in antibody production. Furthermore, in common with the anti-peptide strategy, fusion proteins can be used to generate antibodies against specific regions of proteins. While both approaches have been used to identify components of the VDCCs (Westenbroek et al., 1993; Scott et al., 1996), we chose to generate anti-peptide rather than anti-fusion protein antibodies for several reasons. First, since anti-peptide antibodies are made against peptides containing relatively short sequences of about 20 amino acids, these antibodies are more likely to recognize denatured proteins, such as those found in immunoblots (Boersma et al., 1988). While fusion protein antibodies may recognise proteins in their native conformation better, the recognition of native proteins can often apply to anti-peptide antibodies too (Levey et al., 1993). Indeed, there is much evidence that peptides greater than 15 amino acids in length attain a conformation similar to that of the parent protein (Scheidtmann, 1989). Second, while the longer length of fusion proteins can make them excellent representatives of native proteins through a retention of their tertiary structure (Horiuchi et al., 1987), this can be a

problem too. In particular, their size, while providing many epitopes for antibody recognition, also increases the risk of cross-reactivity with other proteins sharing those epitopes. This problem is especially acute for all subunits of the N-VDCCs, where there are very few regions greater than 20 amino acids that are absolutely unique to that subunit (Dubel et al., 1992; Castellano et al., 1993a). Therefore, the overall success of anti-peptide antibodies in the majority of immunological techniques, combined with their relative ease of production, make this a highly useful method of antibody generation (Boersma et al., 1993). As discussed below, this conclusion seemed to be valid for both our antibodies to α_{1B} and β_3.

5.3. Preparation and Characterization of Antibodies

The initial characterization of anti-peptide antibodies was achieved by using ELISA. These assays indicated that immunization with peptide coupled to KLH gave antibody titres (α_{1B} 1:731; β_3 1:6300) well above the preimmune serum values. As expected, along with the specific IgGs, the MAPS purified antiserum contained contaminating IgGs. These antibodies represent primarily IgGs present in the pre-immune serum, and more significantly those raised against the immunogenic carrier protein KLH and possibly the MBS bridge used to couple the peptide to the KLH (Hermanson, 1996).

In immunoblotted rat brain membrane, MAPS purified α_{1B} and β_3 anti-peptide IgGs were highly specific in recognizing endogenous proteins at their appropriate molecular weights. The molecular weight determined for the β_3 subunits was similar to the size predicted from its cDNA (Castellano et al., 1993a) and the molecular weight recognized by β_3 antibodies generated by others (Witcher et al., 1993; Scott et al., 1996). Although some

extraneous bands were present in the immunoblotted membrane, notably for the α_{1B} antibody, the abolition of bands at the appropriate molecular weight following preincubation of antibody with peptide antigen indicated the specificity of antibody-antigen interaction. The α_{1B} antibodies recognized a protein doublet, or possibly triplet that corresponded to the sizes found by others using similar antibodies to the same subunit (Westenbroek et al., 1992; Hell et al., 1994). However, these molecular weights are consistently lower than the 262kDa predicted from the corresponding cDNA (Dubel et al., 1992). The lower molecular weight of of α_{1B} determined by immunoblotting, in comparison to the molecular weight predicted from its cDNA sequence, is typical of α_{1B} (Westenbroek et al., 1992) and other α_1 VDCC subunits (De Jongh et. al., 1991) and has been attributed to anomalous migration in 5% gels (Hell et al., 1993).

In immunoprecipitations, both the α_{1B} and β_3 antibodies were able to immunoprecipitate [^{125}I]-ω-CgTx binding sites. This was not unexpected, as the α_{1B} subunit is thought to be the locus of ω-CgTx binding (Westenbroek, et al., 1992; Witcher, et al., 1993) and the β_3 subunit is known to form a complex with the α_{1B} subunit, at least in rabbit brain (Witcher et al., 1995). The amount of [^{125}I]-ω-CgTx immunoprecipitated by the α_{1B} subunit corresponded to that which has been found in other studies (Westenbroek et al., 1992), while the lower amount of [^{125}I]-ω-CgTx immunoprecipitated by the β_3 subunit confirmed the promiscuity of this subunit in the N-VDCC complex (Scott et al., 1996). The immunoprecipitation of the subunits with these antibodies was specific, as indicated by the low level of [^{125}I]-ω-CgTx immunoprecipitated by various controls, including preincubation of antibody with peptide antigen, immunoprecipitation using antibodies contained in the pre-immune serum, and preincubation of membrane with unlabelled ω-CgTx prior to immunoprecipitation.

A concern arises due to the necessity for solubilization of membrane proteins prior to immunoprecipitation. VDCCs immunoprecipitated under these conditions may reflect only a fraction of the total VDCCs existing in the membrane (Jones et al., 1987). Therefore, the immunoprecipitations are representative only of the solubilizable fraction of membrane proteins, and do not include the non-solubilizable proteins that exist within the membrane (e.g. those that might be anchored to the cytoskeleton). Furthermore, in addition to some proteins solubilizing less efficiently than others, proteins may have altered binding affinities, or be preferentially degraded under solubilization conditions (Levey, 1993). Thus, the results described here and elsewhere should be interpreted with some caution, as they reflect only the solubilizable N-VDCC population and their quantification may not provide a completely accurate reflection of the subunit composition of VDCCs as a whole (Levey, 1993).

5.4. Expression of N-VDCCs in Development

In the present thesis, the developmental expression of N-VDCC subunits was established through [^{125}I]-ω-CgTx binding assays, as well as through the use of antibodies to the α_{1B}, β_3 and α_2/δ subunits in Western immunoblots and immunoprecipitations. In binding assays, the [^{125}I]-ω-CgTx binding increased throughout the developmental period examined, similar to the increase observed by others postnatally in mouse brain (Litzinger et al., 1993). The increase in [^{125}I]-ω-CgTx binding in development appears to be due to an increase in the number of binding sites for ω-CgTx. Association and dissociation assays performed in the present thesis revealed that the kinetics of toxin binding were virtually identical in P0 versus late (P40) postnatal rat brain membrane. This result contradicts an earlier suggestion that the binding affinity of [^{125}I]-ω-CgTx is altered during development (Filloux et al., 1994). However, this

conclusion was based on autoradiography of thaw mounted whole brain slices. This method, which used slice preparations, is inevitably less accurate than the direct binding analysis presented here, for several reasons. First,the cell integrity in membrane preparations is superior to that of slice preparations, which contain uneven surfaces and sloughed and damaged neurons. Second, the interstitial spacing is higher in younger than older slices so controls for non-specific binding require special care. Third, toxin binding is essentially irreversible. Thus, it is impossible to derive kinetic parameters for toxin binding by incubating tissues (or indeed membranes) in different concentrations of ligand by determining Kds from the levels of bound and free ligand using analyses based on equilibrium binding such as Scatchard plots (Jones and So. 1993).

While toxin binding was used to provide an overall picture of N-VDCC expression, the detailed resolution of N-VDCC expression was made possible through the development and use of antibodies against known components of the N-VDCCs. The immunoblotted α_{1B}, β_3 and α_2 subunits exhibited distinct profiles of expression throughout the developmental period. The levels of [^{125}I]-ω-CgTx binding most closely resembled the profile of immunoblotted 220kDa α_{1B} subunit expression. This confirms other studies that found the major ω-CgTx binding site in the brain to be the α_{1B} subunit (Westenbroek et al., 1992; Witcher et al., 1993). The appearance of multiple bands in the later postnatal periods accompanying the previously reported forms (Westenbroek et al., 1992; Hell et al., 1994) suggests that variants may arise through alternative splicing, post translational modifications, or proteolysis. The possibility that these multiple bands represent novel isoforms of α_{1B} subunits is partially supported by the variable ability of saturating concentrations of α_{1B} antibodies directed against different regions of the Domain II-III linker to immunoprecipitate [^{125}I]-ω-CgTx binding sites,

as was found in other studies (Westenbroek et al., 1992).

In contrast to α_{1B}, there were no excursions in the anticipated sizes of the α_2/δ and β_3 subunits. Both these subunits had sizes exactly corresponding to those reported elsewhere. Thus it appears these subunits do not undergo marked processing in development, even though known splice variants exist. Interestingly, however, the expression of the α_2 subunit throughout development was dissimilar to that of the other subunits. This was not unexpected, as α_2/δ subunits are shared between numerous VDCCs (Kim et al., 1992). Thus, subunits recognized by our commercially prepared monoclonal antibody are likely to be associated with neuronal, as well as non-neuronal VDCCs (Hofman et al., 1994; Dunlap et al., 1995) such as those found in glia. This implies that the source of immunoblotted α_2 subunit probed by our antibodies in the developing rat brain remains unknown, and further studies of the α_2 subunits that exist in the brain are warranted prior to detailed analysis of the results of these immunoblots. Other studies have revealed that monoclonal antibodies to the α_2/δ subunit of skeletal muscle calcium channels such as the ones used by us immunoprecipitate <10% of the total [^{125}I]-ω-CgTx binding sites in solubilized rabbit brain or rat brain membrane (Ahlijanian et al., 1990; Westenbroek et al., 1990; Ahlijanian et al., 1991; Westenbroek et al., 1992). It remains unknown whether this low level of immunoprecipitation is due to the existence of alternative α_2/δ subunits that are not recognized by the available antibody, or if anti-skeletal muscle α_2/δ subunit antibodies poorly recognize solubilized α_2/δ in digitonin extracts, as has been reported (Sakamoto and Campbell, 1991).

A comparison of the expression profiles of all three N-VDCC subunits is especially

revealing. In general, all three subunits existed at a low level in embryonic tissue, and their expression subsequently increased postnatally. These increases in subunit levels do not represent enhanced total synthesis of all membrane proteins. Rather, since our experiments used concentrations of total plasma membrane proteins that were equalized between various developmental stages, our binding and immunoblotting results indicate a specific increase in N-VDCC subunit density in developing hippocampal membranes. The specific increases in the levels of subunits found in the present thesis correspond to the results of *in situ* hybridizations, which indicated low levels of α_{1B} and β_3 mRNAs in E18 hippocampus prior to increased expression in the first two postnatal weeks (Tanaka et al., 1995; Jones et al., manuscript submitted). Therefore, the onset of mRNA expression parallels that of specific protein synthesis throughout development.

An important issue is the relationship between the levels of expression of the subunits to each other - are VDCC subunits present in the membrane synchronously throughout development? In immunoblots, the levels of β_3 subunit in perinatal (E18-P2.5) tissue did not rise smoothly but showed an early peak at P0. During this same period, there was also a rise and slight dip in the level of the α_{1B} subunit. While the total α_{1B} and β_3 concentrations in the membrane seem to be phasic during this period, there are no obvious fluctuations in the [^{125}I]-ω-CgTx binding sites, which rise steadily. It is thus conceivable that α_{1B} does not account for all the binding sites in the membrane although this would be rather surprising. More likely is that any slight dip in the α_{1B} immunoblot is so small that it is not statistically significant. After the perinatal period, expression of both α_{1B} and β_3 and [^{125}I]-ω-CgTx binding sites rise more or less in parallel. Thus, the α_{1B} immunoblots and binding site data agree, and accompany an increase in the levels of the β_3 subunit at different ages. The rise (and early

dip) in β_3, and its generally phasic expression throughout development does not mirror the expression of the α_{1B} subunit, suggesting that α_{1B} and β_3 subunits may not always be coupled so that they appear in the same complexes. Strong support for this contention is provided by the immunoprecipitation data. Immunoprecipitation of solubilized [^{125}I]-ω-CgTx binding sites by β_3 antibodies rises in development, indicating that increasing amounts of β_3-α_{1B} complexes are being formed. Nevertheless, when the number of sites that can be immunoprecipitated by β_3 antibodies is expressed as a fraction of the total [^{125}I]-ω-CgTx binding sites determined by binding assays using solubilized or unsolubilized membranes it is clear that the amount of α_{1B} that is complexed to β_3 changes in development. The significance of the changes in complexation of β_3 subunits to α_{1B} subunits is unclear. Since β_3 subunits are present throughout development, one explanation is that α_{1B} subunits can exist independently, and may not be co-assembled with other subunits in early development. Pools of fully, or partially assembled N-VDCC complexes have been detected in neuroblastoma cells (Passafaro et al., 1994). On their own, these individual α_{1B} subunits are almost certainly non-functional, as N-VDCCs appear to require the α_{1B} subunit in the presence of auxiliary subunits for functional expression (Brust et al., 1993; Stea et al., 1993; Isom et al., 1994; Olcese et al., 1994; Gurnett et al., 1996). Another possible explanation for the lack of coassembly between an α_{1B} subunit and the β_3 subunit, is that the α_{1B} subunit in early development may be associated with β subunits other than β_3. Although the association of a particular β subunit with the α_{1B} subunit is a non-covalent, high affinity interaction (Scott et al., 1996), this complexation seems to be quite promiscuous (Scott et al., 1996). Interactions of β_3 and other β subunits with α_{1B} or α_{1A} have been shown to occur (DeWaard and Campbell, 1995; Scott et al., 1996). The available evidence suggests that, while promiscuous, some preferential interactions can occur, although transfection of *Xenopus* oocytes suggests that all β subunits

may deputise for each other (Hullin et al., 1992). These data would thus support the possibility of a switch in β subunits in development. Switches in the subunits used to make heteromers has been reported for several other membrane protein complexes notably the NMDA and nicotinic receptors (Ciabarra et al., 1995; Murray et al., 1995). Although it is unknown which other β subunits may complex with α_{1B} during development, the prevalence of β_4 mRNA in embryonic hippocampal tissue over adult tissue (Tanaka et al., 1995) may make this subunit a likely candidate (Hullin et al., 1992). Therefore, increases in β_3 subunit expression during development may be a means of regulating the association of β subunit to the α_{1B} subunit. The significance of such switching is highlighted by the markedly different biophysical propertes - especially inactivation and activation kinetics - seen when α_1 subunits associate with discrete β subunits. Thus any alterations in β subunit availability may indicate a requirement in the neuron for specific calcium fluxing properties.

5.5. Significance of N-VDCC Expression for Development

In the discussion that follows, N-VDCC subunit expression has been described in terms of the distinct anatomical and physiological processes which occur during the embryonic (E18- P0), early postnatal (~P0-P10) and later postnatal (~P10-P40) stages of development in rat hippocampus, that may involve N-VDCCs. This division into embryonic, early and later postnatal stages has been used by others, based on anatomical and physiological distinctiveness during these developmental periods (Murphy and Magness, 1984). The processes that occur during the embryonic period in hippocampus include neuronal migration of CA1-CA3 neurons, while migration of granule cells of the dentate gyrus, differentiation, and synaptogenesis (Grinvald and Farber, 1981; Anglister et al., 1982; Lipscombe, 1988;

Kater and Mills, 1991; Vigers and Pfenninger, 1991; Komuro and Rakic, 1992) largely occur during the postnatal periods. Therefore, in the present thesis, N-VDCC subunit expression during the early, and later postnatal periods of development are discussed individually.

5.5.1. Expression of N-VDCC Subunits During the Embryonic Period of Neurodevelopment

The results of [^{125}I]-ω-CgTx binding assays and immunoblots indicate that α_{1B}, β_3 and α_2 subunits exist in embryonic tissue around the time of migration of CA1-CA3 pyramidal neurons at E17-E22 (Altman and Bayer 1990a,b). The presence of these three subunits prenatally supports the contention that functional N-VDCCs may exist at this early stage of neurodevelopment (Stea et al., 1993; Isom et al., 1994; Olcese et al., 1994). These results correspond with other studies that suggest N-VDCCs are required for neuronal migration (Komuro and Rakic, 1992). In the present thesis, the levels of [^{125}I]-ω-CgTx binding and immunoblotted α_{1B} subunits in embryonic tissue were relatively low, suggesting that either few N-VDCCs are required for migration, or that a limited number of cells migrate at any one time. In agreement with the results of the present thesis, there is electrophysiological evidence that embryonic hippocampal neurons in culture lack high voltage-activated (HVA) calcium channels (Yaari et al., 1987), such as N-VDCCs. These channels appear later, accompanying neurite outgrowth (Yaari et al., 1987). Any economising in the expression of N-VDCCs during the migratory period may be a means of balancing a need for signalling with the need to avoid the expense of making buffering proteins, pumps, stores and channels for membrane repolarization (Spitzer et al., 1994). Furthermore, the low levels of N-VDCCs that exist during migration compared to adult levels implies that the bulk of N-VDCC

expression may occur during later stages, when N-VDCCs are required for other known aspects of brain function besides migration. Might other VDCCs be involved in migration? To address this would require antibodies against other α_1 subunits. Nevertheless, the observation that very high levels of α_2/δ occur in the early hippocampus is intriguing since these subunits are common to all known VDCC heteromers (See Future studies). Of course, even if N-VDCCs play only a minor role in migration any expression of N-VDCC α_{1B} and β_3 subunits in embryonic tissue may provide insight into neuronal migration as a whole.

The transient expression of the β_3 subunit in embryonic tissue and its decline around the time of birth gives a peak which corresponds to the final period of neuronal migration. Thus, β_3 subunit expression may demarcate the migration of neurons to their final destination. In contrast, an overall increase in α_{1B} subunit levels was shown during this time period, which corresponds to studies by Komuro and Rakic (1992), in which migrating cerebellar neurons incubated with fluorescently labelled ω-CgTx exhibited increased fluorescence as they approached their final destination (Komuro and Rakic, 1992).

5.5.2. N-VDCC Subunit Expression During the Early Postnatal Period

The enhanced expression of the various subunits during the first postnatal week in the present thesis corresponds to a period of neurodevelopment involving granule cell migration, extensive neuronal differentiation (Bayer and Altman, 1995), and synaptogenesis (Jacobson, 1991). This is not unexpected, as these events are thought to involve N-VDCCs (Anglister et al., 1982; Vigers and Pfenninger, 1991; Komuro and Rakic, 1992). The onset of adult electrophysiological characteristics and population spiking occurs in CA1 pyramidal neurons

during the first postnatal week (Bekenstein and Lothman, 1991a; Bolshakov and Siegelman, 1995), suggesting that increased levels of N-VDCC subunits may be an essential component for the development of excitatory activity in hippocampal neurons. The enhanced expression of N-VDCCs during this period may be required for their role in the release of neurotransmitter from immature excitatory synapses (Dunlap et al., 1995; Scholz and Miller, 1995). Although this role may be shared with other VDCCs (Wheeler et al., 1994; Scholz and Miller, 1995), it appears that N-VDCCs may make the highest contribution to neurotransmitter release at the immature synapse (Scholz and Miller, 1995). In agreement with these findings, other studies have found that the high efficacy of CA1 synapses in very young (P4-6) hippocampi (release probability (Pr) close to unity) diminishes to a lower level with maturation (Pr<0.5) (Bolshakov and Siegelbaum, 1995).

5.5.3. N-VDCC Subunit Expression during the Later Postnatal Period

The results of the present thesis indicate that maximal, or near maximal levels of [^{125}I]-ω-CgTx binding, immunoblotted α_{1B} subunits, and immunoprecipitations of [^{125}I]-ω-CgTx binding by β_3 antibodies are reached by P10. The high levels of N-VDCCs at this time correspond to a period of synaptogenesis, which occurs throughout the first few weeks following birth (Jacobson, 1991) and is thought to involve N-VDCC activity (Vigers and Pfenninger, 1991). Interestingly, the levels of immunoblotted β_3 subunits do not reach their maximum value at P10, but subsequently increase to reach maximal values at P40. These data are again consistent with the notion that α_{1B} subunits interact with β subunits other than β_3 in N-VDCCs, or vice versa.

After P10, there is a consistent decrease in all measures of α_{1B} subunit expression examined, at times followed by a subsequent increase to adult levels. This dip in expression has been observed with several other neuronal proteins such as the AMPA receptors (Lomeli et al., 1994). The biphasic profile of N-VDCC expression is most easily rationalized in terms of two processes. First, a continous increase in VDCC expression up to adult levels and second, a finite period during which regressive events might occur. Indeed there is good evidence that such regressive events occur in the later postnatal periods coinciding with synaptogenesis (Rihn and Claiborne, 1990). Accompanying the proliferation of dendrites during synaptogenesis, dendritic regression is thought to contribute to the stabilization of specific synapses (Litzinger et al., 1993). Such dendritic regression may result from neuronal cell death, the elimination of axon collaterals, a decrease in dendritic spine density (Ramon y Cajal., 1960; Oppenheim, 1981; Cowan et al., 1984) or dendritic elimination, which involves a decrease in the number of dendritic branches and an increase in total dendritic length (Leuba and Garey, 1984; Murphy and Magness, 1984; Rihn and Claiborne, 1990). Since there is considerable evidence that N-VDCCs are expressed on dendrites (Mills et al., 1994; Johnston et al., 1996) changes in dendritic structure might lead to a reduction in N-VDCC density.

The high levels of N-VDCC subunit expression during the later postnatal period correspond to a time of great change in neurodevelopment, when dendrites exhibit rapid growth (P 6-16), and the EEG matures to its adult form (P 10-15) (Himwich et al.,1962). Sensory systems are initiated during this period, as the rat pups' eyes open (P 11-14), and evoked potentials are produced to sound (P 10-15) (Himwich et al., 1962). These sensory systems initiate extensive synaptogenesis in various regions of the hippocampus (Rihn and

Claiborne, 1990) which may account for increased levels of N-VDCC subunit expression during this period.

The recognition of both α_{1B} and β_3 subunits by the corresponding antibodies is especially significant, and opens up many avenues for future research into the structure, function and cell biology of these important proteins (Table 4). Appropriately, the results of this thesis are compatible with several recent reports in the literature that emphasize the dynamics of subunit expression in the N-VDCC complex. These recent studies have implicated the existence of alternative subunit combinations to the previously well documented channel composition consisting of α_{1B}, β_3 and α_2/δ subunits. Thus, N-VDCCs can now be envisioned as channels that are highly variable in their subunit composition, an attribute well defined by the differential association of the N-VDCC α_{1B} subunit with various β subunits. The significance of the diversity of channel composition is probably most obvious in regards to its impact on channel functionality. Although subunit switching brings an added level of complexity to mechanisms already known to create N-VDCC channel functional diversity, it does not come entirely unexpected in light of the numerous mechanisms that exist for creating diversity of VDCCs in general. Indeed, the functional diversity of N-VDCCs may be important for mediating many events that occur throughout the neurodevelopmental period. The data in this thesis provide the first evidence that N-VDCC subunit composition may be an important factor in mediating such processes, based on temporal correlations between the variability in subunit expression and critical stages in the development of the hippocampus. The possibility that switching of these various subunits occurs in development is especially intriging, owing to the prevalence of β_4 mRNA at early times (Tanaka et al., 1995), corresponding to a period in development in which the association of the β_3 subunit with the α_{1B} subunit is low. Furthermore, the prevalence of β_3

Future Studies	
Issues Arising	**Possible Experiments**
Does subunit switching occur in N-type VDCC complexes during development?	-Immunoblot analysis of β_4 subunits, and other commercially available β subunits. -Immunoprecipitation of [^{125}I]-ω-CgTx GVIA by antibodies to the β_4 subunit. -Co-localization of N-VDCC subunits using immunocytochemical techniques.
Does subunit switching occur in other channels involved in neurotransmission during development, such as the P/Q-type VDCCs?	-Immunoprecipitation of [^{125}I]-ω-CgTx MVIIC by antibodies to the β_3 and β_4 subunits, and ligand binding assays. -Co-localization experiments (see above).
Does N-VDCC subunit expression during the period of synaptogenesis correlate with alterations in synaptic and/or apoptotic markers?	-Immunoblot analysis of synaptic vesicle protein (e.g. synaptotagmin, synapsin I and rab3a) or apoptotic markers using rat brain membrane, to be correlated with N-VDCC subunit expression.
Does the expression of N-VDCC subunits parallel the availability of corresponding mRNA during development?	-Northern blotting
Does the spatial localization of N-VDCC subunits change during development?	-Immunocytochemistry using subunit selective antibodies to label cultured hippocampal neurons.
Does the coassembly of N-VDCC subunits in pre-synaptic regions change during development?	-Use antibodies to the synaptic protein syntaxin to immunoprecipitate N-VDCC subunits at different stages of development.
Are assembled N-VDCC complexes present in the golgi apparatus during development?	-Confocal imaging using optical sections of hippocampal tissue labelled with co-localized antibodies to the various N-VDCC subunits at various stages of development (see Basarsky et al., 1994).
At which stage in channel synthesis are N-VDCC subunits complexed, and does the assembly vary during development?	-Immunoprecipitations of [^{125}I]-ω-CgTx by β subunits in the presence of Brefeldin A, which blocks the exit of newly synthesized proteins from the golgi apparatus, and nocodazole, which is a microtubule disassembling drug, (see Passafaro et al., 1994) during development.

Table 4. Future studies.

subunits at such early times suggests that the β_3 subunit may associate with α_1 subunits other than α_{1B}, as has been suggested in the literature (De Waard and Campbell, 1995; Liu et al., 1996). These data, which provide the first indication that N-VDCC composition may vary dynamically in development, suggest that alterations in N-VDCC complexes may be associated with certain pathological situations as well. For example, it is conceivable that the inappropriate association of a β subunit with an α_{1B} subunit would alter the physiological properties of VDCCs, leading to the decreased threshold of neuronal excitability found in epilepsy. The present thesis defines the variability in the expression of the major N-VDCC subunits α_{1B}, β_3 and α_2/δ in terms of well documented developmental processes that are thought to involve N-VDCCs, including migration, differentiation and synaptogenesis. The precise role of N-VDCCs in other developmental events remains undefined, and is a fertile area for future studies. One such developmental event that may involve variations in N-VDCC subunit expression is the pruning of the dendritic arbor. This thesis indicated a modest decrease in N-VDCC expression during a time when neuronal regression is thought to occur. It remains unknown whether this decrease in channel expression is due to a causal role of N-VDCCs in the regressive process, or perhaps due to a generalized loss of axons, dendrites and dendritic spines during this period (Rihn and Claiborne, 1990). Thus, it may be useful to correlate N-VDCC subunit expression with selective markers of progressive events such as synapse formation, or regressive phenomena such as neuronal cell death, the elimination of axon collaterals, decreases in spine density and dendritic elimination during the developmental period. Since the precise roles of discrete N-VDCC subunits in such developmental events remains unidentified, such data would strengthen the likelihood that subunit composition corresponds to critical stages in neuronal development.

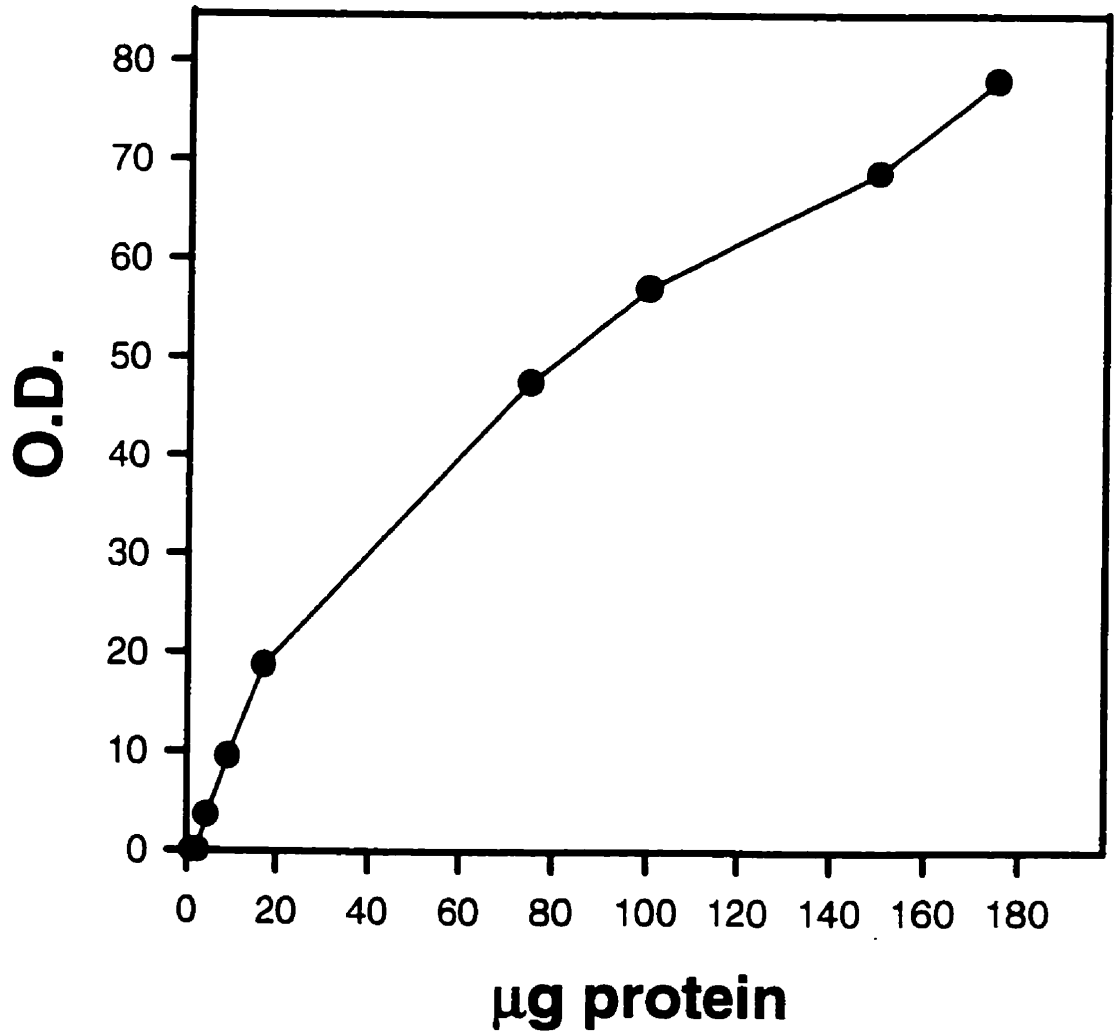
It has been postulated that the spatial localization of VDCC subtypes makes an important

contribution to the biophysical properties channels, through clustering of the channels, or the creation of channel "hot-spots" (Jones et al., 1989; Westenbroek et al., 1990). The heterogeneity of VDCC subunit complexes suggest that clustering may not be limited to VDCC subtypes, but may also be reflected at the level of the specific subunits that make up the VDCC complex. For example, VDCCs modulated by the β_3 subunit may be localized to discrete neuronal domains that differ from that of VDCCs modulated by other β subunits. In contrast, variability of VDCC subunits within clusters may act to fine-tune channel properties. Thus, although N- and L-channel clustering and immobilization has been suggested by others (Jones et al., 1989; Westenbroek et al., 1990), the specific subunits that make up the VDCC complexes in these clusters remains unknown. Thus, the colocalization of VDCC subunits within neurons, for example through an immunocytochemical approach, is especially significant, as are the mechanisms that initiate the synthesis, coassembly and maintenance of channels in distinct neuronal regions. Importantly, the spatial localization of VDCCs have been found to parallel their functional roles. For example, VDCCs in dendrites have been attributed to action potential generation (Kullman et al., 1992), whereas VDCCs in somata are thought to be important for gene transcription (Murphy et al., 1991) and VDCCs at axon terminals mediate neurotransmitter release (Dunlap et al., 1995). Interestingly, P-type VDCCs are thought to be localized to similar neuronal regions as N-VDCCs (Westenbroek et al., 1995). This is not unexpected, as P-VDCCs, similar to N-VDCCs, are thought to mediate neurotransmitter release (Dunlap et al., 1995). Thus, although a parallel expression of P- and N-VDCCs in axon terminals would be expected, there is evidence that the activity of P-channels to efficate neurotransmitter release is delayed relative to that of the N-channel during development (Scholz and Miller, 1995). Therefore, it remains unknown whether the P channel is expressed at early times in development and turned on later, or whether the density

of P channel is most marked during later periods of development. Thus, comparison of the expression of different VDCC subtypes that mediate synaptic transmission may be important for elucidation of differential functionality during development. It could be predicted that the expression of N- and P-type VDCCs would differ from that of other VDCCs, such as L-VDCCs, during development, which are involved in aspects of neuronal function other than neurotransmitter release (Murphy et al., 1991).

The possibilities for future experiments that have arisen from this thesis are numerous, and illustrate the vast amount of information about N-VDCC expression during development that remains unknown. These studies on channel subunit expression in a developmental model provide insight into the normal, and perhaps abnormal expression of subunits comprising the N-VDCC complex, as well as to help define the overall structural and functional characteristics of N-VDCCs.

APPENDIX A



Determination of the Linear Range of the Densitometer. The linear range of the densitometer was determined to asses the proportional differences between concentrations of protein within individual immunoblots. A standard curve was generated using increasing concentrations of adult rat brain membrane protein (1.1-175µg) run on SDS-PAGE. The resulting blots were probed with a primary MAPS purified β_3 antibody (1:500) and secondary goat anti-rabbit IgG (1:4000). The relative levels of α_{1B}, β_3 and α_2/δ subunits on immunoblots were determined with consideration to the maximum and minimum quantifiable signal, and the linear range of the densitometer as determined by the standard curve.

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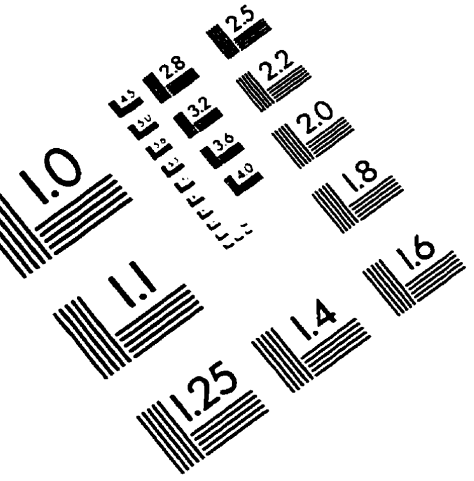
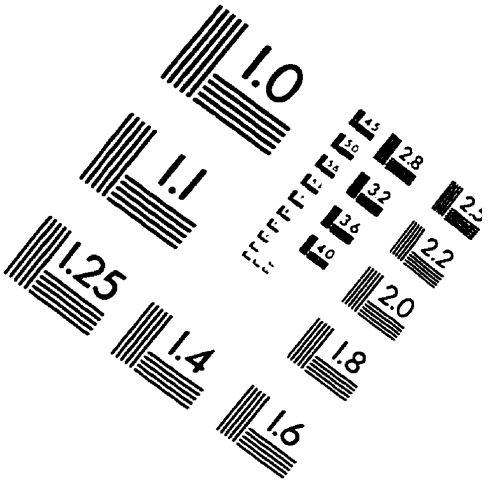
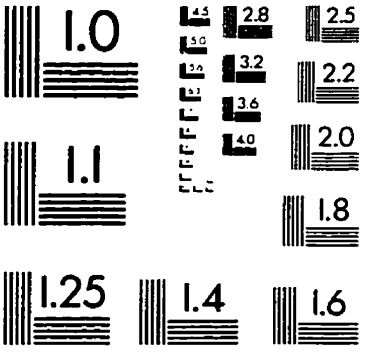
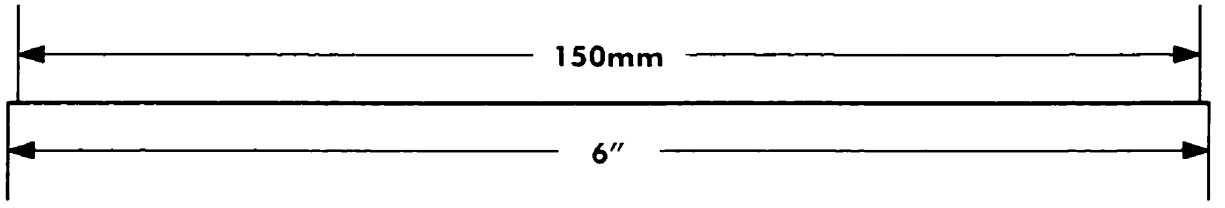
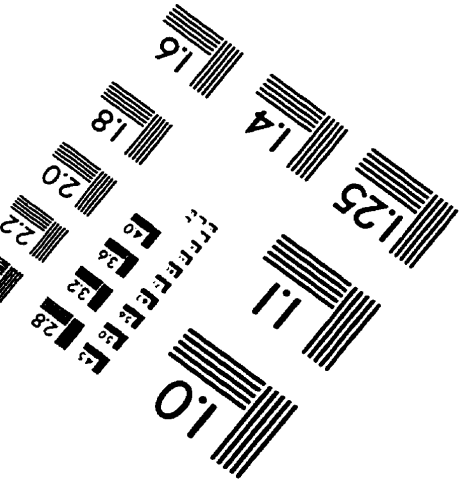
IMAGE EVALUATION TEST TARGET (QA-3)

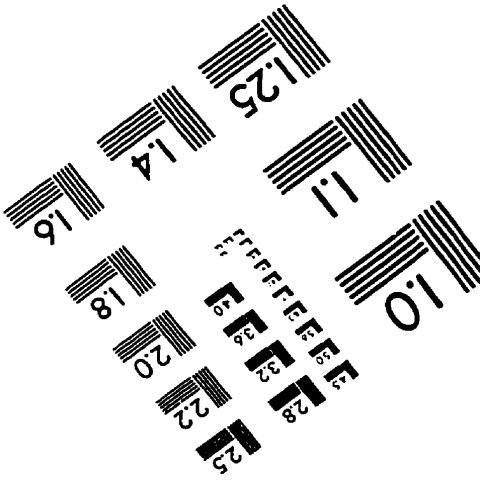












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