THE EXPRESSION OF P/Q-TYPE CALCIUM CHANNELS IN THE TOTTERING LEANER (tglo) MOUSE

by

Barbara Courssaris

A thesis submitted in conformity with the requirements for the degree of Master of Science
Graduate Department of Pharmacology
University of Toronto

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“I have no special talents. I am only passionately curious.”

Albert Einstein
ABSTRACT

The Expression of P/Q-type Calcium Channels in the Tottering Leaner (tg\textsuperscript{ia}) Mouse.

Barbara Courssaris, Master of Science, 2001. Graduate Department of Pharmacology, University of Toronto.

The tottering leaner (tg\textsuperscript{ia}) mouse has been used as a model for human absence epilepsy, ataxia, and migraine. This thesis examined the expression of P/Q-type calcium channels in wild type (wt) and tg\textsuperscript{ia} mouse cortices using \textsuperscript{[125]}\textsuperscript{I}-\textsuperscript{\omega}-conotoxin MVIIIC binding assays and western immunoblots. The function of wt and each mutant calcium channel (tg\textsuperscript{ia} long and tg\textsuperscript{ia} short) was further analyzed independently in cell lines by immunoblotting, electrophysiology, and imaging. Experiments revealed decreases in P/Q-type calcium channel binding and \(\alpha_{1A}\) subunit expression in tg\textsuperscript{ia} cortices as compared with wt. While wt, tg\textsuperscript{ia} long, and tg\textsuperscript{ia} short \(\alpha_{1A}\) subunits were expressed at approximately equal levels in HEK tsA201 cells, there was a substantial decline in calcium channel currents from both the tg\textsuperscript{ia} long and tg\textsuperscript{ia} short-transfected cells. tg\textsuperscript{ia} short channels expressed in COS-7 cells displayed perinuclear distributions. Taken together, the above data suggest a decrease in tg\textsuperscript{ia} \(\alpha_{1A}\) protein in the tg\textsuperscript{ia} mouse cortex.
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<th>Description</th>
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<tr>
<td>$\omega$-CTX GVIA</td>
<td>omega conotoxin GVIA (marine snail Conus geographus toxin)</td>
</tr>
<tr>
<td>$\omega$-CTX MVIIIC</td>
<td>omega conotoxin MVIIIC (marine snail Conus magus toxin)</td>
</tr>
<tr>
<td>CFTR</td>
<td>Cystic Fibrosis Transmembrane Regulator</td>
</tr>
<tr>
<td>COS-7</td>
<td>African Green Monkey Kidney Fibroblast cell line</td>
</tr>
<tr>
<td>CSD</td>
<td>Cortical Spreading Depression</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco's modified Eagle medium</td>
</tr>
<tr>
<td>DTT</td>
<td>Dithiothreitol</td>
</tr>
<tr>
<td>du</td>
<td>Ducky</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic Reticulum</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal Bovine Serum</td>
</tr>
<tr>
<td>GF/B</td>
<td>Glass Fibre Filter B</td>
</tr>
<tr>
<td>GFP</td>
<td>Green Fluorescent Protein</td>
</tr>
<tr>
<td>HEK</td>
<td>Human Embryonic Kidney</td>
</tr>
<tr>
<td>Ih</td>
<td>Lethargic</td>
</tr>
<tr>
<td>NFDM</td>
<td>Non-fat Dry Skim Milk</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction</td>
</tr>
<tr>
<td>P/Q-type VDCCs</td>
<td>P/Q-Type Voltage Dependent Calcium Channels</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis</td>
</tr>
<tr>
<td>stg</td>
<td>Stargazer</td>
</tr>
<tr>
<td>TBS</td>
<td>Tris Buffered Saline</td>
</tr>
<tr>
<td>TTBS</td>
<td>TBS with 0.5% Tween-20</td>
</tr>
<tr>
<td>tg</td>
<td>Tottering</td>
</tr>
<tr>
<td>$tg^{la}$</td>
<td>Tottering Leaner</td>
</tr>
<tr>
<td>$tg^{rol}$</td>
<td>Rolling Nagoya</td>
</tr>
<tr>
<td>TxB-WGA</td>
<td>Texas Red-Conjugated Wheat Germ Agglutinin</td>
</tr>
<tr>
<td>VDCCs</td>
<td>Voltage Dependent Calcium Channels</td>
</tr>
<tr>
<td>wt</td>
<td>Wild-type</td>
</tr>
</tbody>
</table>
1 INTRODUCTION

1.1 Importance of Calcium in Cells

Calcium ions are critical to cell function. In both excitable and non-excitatory cells, calcium acts as a universal second messenger (Clapham, 1995) orchestrating processes such as development, differentiation, muscle contraction, and energy metabolism. In neurons, calcium plays a vital role in the generation of action potentials, neurite outgrowth, neurotransmitter release (Llinas et al., 1981; Miller 1987; Kater and Mills, 1991; Llinas et al., 1995; Catterall, 1998) and the synaptic plasticity thought to underlie learning and memory (Kullmann et al., 1992). Calcium is not always beneficial to cell vitality however, and it is now known that, at high intracellular concentrations, it can kill cells directly or indirectly via apoptosis or other non-apoptotic processes. In neurons, abnormally high calcium influx is associated with cerebral ischaemia (Berridge et al., 1997; Berridge et al., 1998) and neurodegeneration (Kater and Lipton, 1995). Not surprisingly, calcium levels are maintained under strict yet dynamic control (Moreno, 1999). As a consequence of calcium-increasing, calcium-decreasing, and calcium-buffering mechanisms, resting levels of cytosolic calcium are typically 20–100 nM, approximately 10,000 fold less than extracellular levels (Mooren and Kinne, 1998).

1.2 Importance of Voltage-Dependent Calcium Channels in Nerve Cells

The primary means for excitable cells to convert electrical into biochemical signals is via the intense, rapid, local influx of calcium ions through voltage-dependent calcium channels (VDCCs) (Dolphin 1996; Bito et al., 1997; Blackstone and Sheng, ...
Consequently, the expression of VDCCs is a key determinant of calcium signaling in many cell types. In nerves, important examples of VDCC-dependent calcium signaling include the generation of action potentials, neurotransmitter release and the control of transcription (Morgan and Curran, 1988; Catterall, 1995; Dunlap et al., 1995; Bito et al., 1997; Catterall et al., 1998). Perhaps not surprisingly, the plethora of biological functions performed by VDCCs demands their considerable specialization. It is now recognized that such specialization is realized through the manifestation of distinct channel subtypes with discrete biophysical and pharmacological properties.

1.3 Structure and Function of VDCCs

The VDCCs are hetero-oligomeric complexes comprised of a main pore-forming α₁ subunit, and accessory β, α₂δ and γ subunits (Figures 1 and 2). The auxiliary subunits dramatically influence the properties of the channels and facilitate their surface expression (Miller, 1992; Isom et al., 1994; Gurnett and Campbell, 1996; Walker and De Waard, 1998). All VDCC subunits exhibit considerable diversity. To date, ten genes have been identified that encode variants of the mammalian α₁ subunit (α₁A-α₁I, and α₁S) (Hui et al., 1991; Snutch et al., 1991; Mori et al., 1991; Chin et al., 1991; Starr et al., 1991; Williams et al., 1992a; Williams et al., 1992b; Dubel et al., 1992; Soong et al., 1993; Zhang et al., 1993; Soong et al., 1994; Perez-Reyes et al., 1998; Cribbs et al., 1998; Lee JH et al., 1999, Mittman et al., 1999a; Mittman et al., 1999b), four that encode the beta subunits (β₁-β₄) (Pichler et al., 1997; Liu et al., 1996), three that encode the disulfide-linked α₂δ (α₂δ₁-₃) subunits (Klugbauer et al., 1999; Gao B et al., 2000), and five that encode the γ (γ₁-₅) subunits (Eberst et al., 1997; Letts et al., 1998;
Figure 1: VDCC subunit composition. Each channel contains an $\alpha_1$ subunit, a $\beta$ subunit, an $\alpha_2\delta$ subunit, and a $\gamma$ subunit. This subunit composition was originally described for skeletal muscle calcium channels but now appears to be true for neuronal calcium channels as well. The $\alpha_1$ subunit forms the pore. $\Psi$: Glycosylation. S-S: Disulfide bond. Adapted from Catterall, 1998.
Figure 2: Representation of VDCC transmembrane structure. The $\alpha_1$ subunit has four domains, each domain consisting of six transmembrane regions, the $\gamma$ subunit has four transmembrane regions, the $\beta$ subunit is a cytoplasmic subunit, and the $\alpha_2\delta$ subunit is a heavily glycosylated single transmembrane protein.
Burgess et al., 1999; Klugbauer et al., 2000). Even further diversity arises through alternative splicing of the α1, β, α2δ and γ subunit RNA transcripts in rats, mice, rabbits and humans (Hui et al., 1991; Hullin et al., 1992; Kim et al., 1992; Mori et al., 1991; Soong et al., 1994; Birnbaumer et al., 1994; Vigues et al., 1998; Ligon et al., 1998; Ertel et al., 2000).

Originally, VDCCs were classified according to their biophysical and pharmacological characteristics into L, N, P/Q, R, S and T subtypes. Just recently, however, a more systematic nomenclature has been introduced which recognizes the pre-eminent role of the α1 subunit. In this gene-based system, the VDCCs have been divided into three families: the Ca1 family representing the L-type channel subunits (α1C,D,F,S); the Ca2 family corresponding to the N, P/Q and R-type channel subunits (α1A,B,E) and the Ca3 family, representing T-type channel subunits (α1G,H,I) (Ertel et al., 2000) (Table 1).

Each α1 subunit polypeptide is composed of four contiguous domains (I-IV), with each domain containing six transmembrane spanning regions. The α1 subunit contains the channel pore and is responsible for voltage-dependent activation and inactivation. The α1 subunit is also the primary determinant of the pharmacological characteristics of the VDCC heteromer and is able to bind numerous ligands with remarkable selectivity and potency (Mintz et al., 1992a; McCleskey et al., 1987). As well, the α1 subunit can bind G-proteins (Herlitze et al., 1996; Ikeda 1996; De Waard et al., 1997; McCool et al., 1997; McCool et al., 1998; Jeong and Ikeda, 1998; Dolphin, 1998; Zamponi and Snutch, 1998; Canti et al., 1999), and is regulated by phosphorylation (Lai et al., 1990; Sakurai
<table>
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<th>Channel</th>
<th>Current Type</th>
<th>Primary Tissues</th>
<th>Name of Previous $\alpha_i$</th>
<th>Blocker</th>
<th>Functions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ca,1.1</td>
<td>L</td>
<td>Skeletal muscle</td>
<td>$\alpha_{1S}$</td>
<td>DHPs</td>
<td>Excitation-contraction coupling, calcium homeostasis, gene regulation</td>
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<tr>
<td>Ca,1.2</td>
<td>L</td>
<td>Heart, smooth muscle, Brain, pituitary, adrenal</td>
<td>$\alpha_{1C}$</td>
<td>DHPs</td>
<td>Excitation-contraction coupling, hormone secretion, gene regulation</td>
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<td>Ca,1.3</td>
<td>L</td>
<td>Brain, pancreas, kidney, ovary, cochlea,</td>
<td>$\alpha_{1D}$</td>
<td>DHPs</td>
<td>Hormone secretion, gene regulation</td>
</tr>
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<td>Ca,1.4</td>
<td>L</td>
<td>Retina</td>
<td>$\alpha_{1F}$</td>
<td></td>
<td>Tonic neurotransmitter release</td>
</tr>
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<td>Ca,2.1</td>
<td>P/Q</td>
<td>Brain, cochlea, pituitary,</td>
<td>$\alpha_{1A}$</td>
<td>$\omega$-Agatoxin, $\omega$-CTX MVIIIC</td>
<td>Neurotransmitter release, dendritic calcium transients</td>
</tr>
<tr>
<td>Ca,2.2</td>
<td>N</td>
<td>Brain, nervous system</td>
<td>$\alpha_{1B}$</td>
<td>$\omega$-CTX GVIA</td>
<td>Neurotransmitter release, dendritic calcium transients</td>
</tr>
<tr>
<td>Ca,2.3</td>
<td>R</td>
<td>Brain, cochlea, retina, heart, pituitary</td>
<td>$\alpha_{1E}$</td>
<td>None</td>
<td>Calcium-dependent action potentials, neurotransmitter release</td>
</tr>
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<td>Ca,3.1</td>
<td>T</td>
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<td>$\alpha_{1G}$</td>
<td>None</td>
<td>Repetitive firing</td>
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<tr>
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<td>$\alpha_{1H}$</td>
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<td>Repetitive firing</td>
</tr>
<tr>
<td>Ca,3.3</td>
<td>T</td>
<td>Brain</td>
<td>$\alpha_{II}$</td>
<td>None</td>
<td>Repetitive firing</td>
</tr>
</tbody>
</table>

Table 1: Nomenclature, composition and localization of $\alpha_1$ subunits. Modified from Ertel, et al., 2000; Catterall, 2000.
et al., 1995; Hell et al., 1994; Hell et al., 1995; De Jongh et al., 1996; Perets et al., 1996; Gao et al., 1997; Puri et al., 1997). In addition, the $\alpha_1$ subunit is involved in calcium/calmodulin binding (Zhou et al., 1997; Zuhlke et al., 1999; Lee et al., 1999; Lee et al., 2000) and can couple to the exocytotic machinery of the nerve terminal (Rettig et al., 1996; Kim and Catterall, 1997; Leveque et al., 1998; Sutton et al., 1999; Zhong et al., 1999). Finally, the carboxy terminus of the $\alpha_1$ subunit is thought to contain many of the features necessary for trafficking of VDCCs to appropriate subcellular domains and for binding adaptor proteins containing PDZ domains (Maximov et al., 1999; Brice and Dolphin, 1999; Proenza et al., 2000; Gao T et al., 2000).

The $\beta$ subunits are 52-78 kDa cytoplasmic proteins that can increase peak currents up to 100 fold, and can alter both the kinetics and voltage-dependence of channel activation and inactivation. As well, the expression of the $\beta$ subunit with the $\alpha_1$ subunit can result in an increase in the number of channels at the cell surface, and an increase in drug/toxin binding sites (Hullin et al., 1992; Perez-Reyes et al., 1992; Neely et al., 1993; De Waard and Campbell, 1995; Chien et al., 1995; Perez Garcia et al., 1995; Josephson and Varadi, 1996; Brice et al., 1997; Walker and De Waard, 1998; Dolphin et al., 1999; Bichet et al., 2000). Some $\beta$ subunits can also be palmitoylated, although the function of this modification remains to be clarified (Qin et al., 1998; Hurley et al., 2000). The docking of $\beta$ subunits has been proposed to occur at several distinct intracellular sites of the $\alpha_1$ subunit. The best-established interaction occurs between the $\beta$ subunit and the cytoplasmic loop between domains I and II of the $\alpha_1$ subunit (Pragnell et al., 1994; De Waard et al., 1994, Witcher et al., 1995, De Waard et al., 1996). Since the I-II loop region contains an endoplasmic reticulum (ER) retention signal, $\alpha_1,\beta$ subunit
interactions appear to regulate the expression of VDCCs at the cell surface (Bichet et al., 2000). In the absence of the β subunit, the I-Il linker signal causes the retention of the α₁ subunit in the ER. In contrast, the presence of the β subunit masks the ER retention signal, thereby allowing the VDCCs to proceed through the secretory pathway to the cell surface. Additional sites for β subunit interaction, in particular the α₁ subunit N and C-termini, have been identified through studies of the α₁A-β₁ subunit interaction (Walker et al., 1998; Walker et al., 1999).

The α₂δ subunits are 170 kDa integral glycoproteins that are the product of a single gene that is cleaved post-translationally to form an extracellular α₂ (143kDa) subunit, disulfide-linked to the transmembrane spanning δ (24-27kDa) subunit (De Jongh et al., 1990, Jay et al., 1991). The α₂δ subunit has been shown to augment current amplitudes by increasing the number of functional channels at the cell surface. This increase has been attributed to the α₂ peptide (Williams et al., 1992b; Brust et al., 1993; Wiser et al., 1996; Gurnett et al., 1996; Gurnett et al., 1997; Felix et al., 1997; Jones et al., 1998, Dolphin et al., 1999; Gao B et al., 2000). In contrast, the δ subunit seems to control the gating properties of the channel (Felix et al., 1997). The interaction between the α₂δ and α₁ subunits appears to involve extracellular protein regions, but the precise site of docking has not been determined. While such interactions can modulate drug binding to the α₁ subunit, the δ subunit is required to maintain a stable interaction between the α₂δ and α₁. (Gurnett et al., 1997). The functional importance of the α₂δ subunit is highlighted by the recent observation that these subunits serve as binding sites for the antiepileptic drug gabapentin (Gee et al., 1996).
The γ subunits, once thought to be restricted to skeletal muscle VDCCs (Eberst et al., 1997) have recently been shown to combine with neuronal VDCCs (Letts et al., 1998). Although defects in the novel γ2 subunit gene stargazer can lead to spike-wave seizures characteristic of absence epilepsy in the mouse model stargazer (Letts et al., 1998), their precise function is unknown. P/Q-type calcium channels purified to date contain α1A, β and α2δ subunits (Martin-Moutot et al., 1995; Liu et al., 1996). When co-expressed with α1, β, and α2δ subunits, the γ2 subunits modulate channel activation and inactivation by producing a small shift in the voltage-dependent activation curve to more positive values, and by shifting the inactivation curve to hyperpolarized potentials (Letts et al., 1998; Klugbauer et al., 2000). It will be interesting to see whether γ subunits are indeed associated with P/Q-type calcium channels in vivo.

1.4 P/Q-type Calcium Channels

The most abundant α1 protein in vertebrate brain is the α1A subunit (Mori et al., 1991), an integral component of the P/Q-type VDCCs (Gillard et al., 1997; Jun et al., 1999). Numerous tissue and species-specific isoforms of the α1A subunit are now known to exist, including those of rabbit (Bi-1, Bi-2; Mori et al., 1991) and rat brain, heart, pituitary (rBA-1; Starr et al., 1991) and pancreatic islets (rIA-1; Ligon et al., 1998). All these isoforms arise from a single gene and result from alternative splicing of the primary α1A RNA transcript or post-translational processing (Mori et al., 1991; Starr et al., 1991; Soong et al. 1994; Sakurai et al., 1995; Sakurai et al., 1996; Ligon et al., 1998; Scott et al., 1998). The α1A proteins are found throughout the mammalian brain and are concentrated in the dendrites, cell bodies, and presynaptic terminals of many
types of neurons. In nerve terminals, P/Q-type VDCCs mediate neurotransmitter release and are co-localized with proteins that participate in vesicle docking and exocytosis (Rettig et al., 1996; Kim and Catterall, 1997; Catterall, 1998; Leveque et al., 1998; Sutton et al., 1999; Zhong et al., 1999). Although they both contain the $\alpha_{1A}$ subunit (Jun et al., 1999), P- and Q-type channels differ in their location, pharmacology and biophysical properties. The P-type VDCCs were originally found in cerebellar Purkinje cells but are now known to have a more widespread distribution (Llinas et al., 1989; Mintz et al., 1992a; Mintz et al., 1992b). The P-type VDCCs produce slowly inactivating currents and are highly sensitive to blockade by $\omega$-agatoxin IVA and $\omega$-conotoxin MVIIIC (Mintz et al., 1992a; Mintz et al., 1992b; Hillyard et al., 1992; Olivera et al., 1994).

The Q-type VDCCs are found in hippocampal neurons, cerebellar granule cells and other neuronal tissues (Mermelstein et al., 1999) and have a greater degree of inactivation than P-type VDCCs, are partially blocked by $\omega$-agatoxin VIA and are potently blocked by $\omega$-conotoxin MVIIIC (Zhang et al., 1993; Randall and Tsien, 1995). Expression of recombinant $\alpha_{1A}$ subunits in HEK or COS-7 cells with auxiliary $\alpha_{2\delta}$ and $\beta_{1b}$ subunits produces channels that resemble P-type VDCCs in their activation, but have a somewhat decreased affinity for $\omega$-agatoxin VIA (Berrow et al., 1997; Moreno et al., 1999). In addition, recombinant channels have inactivation properties that are more similar to the Q- than P-type VDCCs (Randall and Tsien, 1995; Berrow et al., 1997; Moreno et al., 1997). The reason why expressed $\alpha_{1A}$ subunits do not faithfully generate P-type or Q-type calcium currents is thought to be due to diversity of the $\beta$ subunits (Moreno et al., 1997; Mermelstein et al., 1999) in the channels and/or alternative splicing of the $\alpha_{1A}$ mRNA (Bourinet et al., 1999).
1.5 Human Diseases Caused by Ion Channel Mutations

Given their critical role in cell function, it is not surprising that defects in voltage dependent ion channels can lead to many disease states. Pathological conditions where ion channels are directly involved are termed "channelopathies". A number of illnesses in humans are caused by mutations in voltage-dependent potassium, sodium, and calcium ion channels (Weinreich and Jentsch, 2000). Potassium channel mutations can lead to benign familial neonatal convulsions (Bievert et al., 1998; Charlier et al., 1998; Singh et al., 1998), slowly progressive dominant deafness (Kubisch et al., 1999), long QT syndrome (Wang et al., 1996), and episodic ataxia type 1 (Browne et al., 1994).

Mutations in the sodium channel can lead to paramyotonia congenita (Ptacek et al., 1992), hyperkalemic periodic paralysis (Fontaine et al., 1990; Bulman et al., 1999), and long QT syndrome (Wang et al., 1995; Bennett et al., 1995). Calcium channel mutations have been implicated in several neurological disorders. Among the best researched are those involving mutations in the $\alpha_{1A}$ gene, such as familial hemiplegic migraine (Ophoff et al., 1996), episodic ataxia type-2 (Ophoff et al., 1996) and spinocerebellar ataxia type-6 (Zhuchenko et al., 1997). Various types of mutations can be involved including mis-sense, nonsense, insertional or deletional errors as well as trinucleotide (CAG) expansions.
Figure 3: Mouse calcium channelopathies. The tottering (tg) mouse carries a mutation in the tg gene that results in a proline to leucine substitution in the $\alpha_{1A}$ subunit gene. The lethargic (lh) mouse is caused by a four base pair insertion at a splice donor consensus sequence, causing aberrant splicing and truncation of the $\beta_1$ subunit. The stargazer (stg) mouse results from an inserted Etn-class transposon into an intron in the $\gamma_2$ subunit gene. The ducky (du) phenotype leads to a decrease in $\alpha_{2}\delta$ protein. The tottering leaner (tg$^{la}$) mouse is the result of a single base pair substitution (G to A) at a splice-donor consensus sequence in the $\alpha_{1A}$ gene, leading to aberrant mRNA splicing, frameshifting, and production of two truncated $tg^{la}$ proteins. Adapted from Burgess and Noebels, 1999b.
1.6 Calcium Channelopathies in Mice

In addition to the human calcium channelopathies mentioned above, there has been much interest in mouse calcium channelopathies (Figure 3). These channelopathies can arise through mutations in any one of the VDCC subunits. For instance, the epileptic mutant mouse *ducksy* (*du*) results from a loss of $\alpha_2\delta$ subunit expression and a decrease in voltage dependent calcium channel currents in Purkinje cells (Balaguer et al., 2000), while defects in the $\gamma_2$ subunit (*stargazer*) gene cause spike-wave seizures characteristic of absence epilepsy in stargazer mice (*stg*) (Letts et al., 1998). Mutations in the $\beta_4$ subunit of the VDCCs that underlie the *lethargic* phenotype manifest as focal motor seizures, ataxia, and cortical spike-wave epilepsy (Burgess et al., 1997). Among the mouse mutations are those involving the $\alpha_{1A}$ subunit which cause diverse neurological deficits in mice and also in humans (Ophoff et al., 1998; Terwindt et al., 1998; Burgess and Noebels, 1999a; Jen, 1999; Puranam and McNamara, 1999; Fletcher and Frankel, 1999; Moreno, 1999; Steinlein and Noebels, 2000). Direct evidence that the $\alpha_{1A}$ subunit may be important in neuropathology comes from targeted ablation studies showing that $\alpha_{1A}$ knockout mice suffer from ataxia, dystonia, and absence seizures prior to undergoing premature death (Jun et al., 1999). Of the spontaneous mutations that have arisen at the *tg* locus, which encodes the mouse $\alpha_{1A}$ subunit, three have been extensively studied for their ability to disrupt P/Q-type VDCC function. These mutants include tottering (*tg*) (Fletcher et al., 1996), rolling Nagoya (*tg*<sup>dr</sup>) (Mori et al., 2000), and the tottering leaner (*tg*<sup>ln</sup>) mouse (Fletcher et al., 1996), the focus of this thesis.
1.7 What is the \( tg^{la} \) Mouse?

The \( tg^{la} \) mouse, a homozygous recessive mutant, carries an autosomal recessive mutation that is caused by a single-base pair substitution in a splice donor consensus sequence within the gene for the \( \alpha_{1A} \) subunit (Figure 4). This mutation induces a truncation in the normal reading frame beyond domain IV and leads to the expression of two major novel mRNA and C-terminal protein sequences (Figure 5) (see Appendix A for sequences) (Fletcher et al., 1996).

The \( tg^{la} \) mouse suffers from severe ataxia, absence-like seizure symptoms, and involuntary, intermittent movement disorders (Meier and MacPike, 1971; Noebels, 1984; Hess, 1996; Lau et al., 1998). Following birth, \( tg^{la} \) mice undergo progressive Purkinje and Golgi cell loss throughout their cerebella, particularly in the anterior lobe (Sidman et al., 1965, Meier and Mackpke, 1971; Heckroth and Abbott, 1994, Abbott et al., 1996). Ataxia is observed by postnatal day 10, whereas the Purkinje cell loss is not seen until around day 40 (Herrup and Wilczynski, 1982). Alterations in cell function (Abbott et al., 1996) and calcium homeostasis can be detected in the Purkinje cells of \( tg^{la} \) mice, and they may also suffer from apoptotic granule cell death (Fletcher et al., 1996; Dove et al., 2000). In addition to the above effects on the cerebellum, the tottering leaner mutation can lead to changes in other brain regions. For example, the absence-like seizures indicate potential changes in the \( tg^{la} \) cortex. Drugs to treat absence epilepsy have been tested on the tottering mouse, an allelic variant of the \( tg^{la} \) mouse. The tottering mouse is sensitive to phenobarbitol, diazepam, and ethosuximide (Hellar et al, 1983), making it a good model for absence epilepsy, and suggesting that the \( tg^{la} \) mouse could also be
Mouse $\alpha_{1A}$ gene 3’ end

A-F: Intron
41-47: Exon

Figure 4: Location of leaner mutation. The $tg^a$ mutation is located in the 3’ region of the $\alpha_{1A}$ gene. This g to a substitution (underlined a) is located at the start of intron B.
Mouse $\alpha_{1A}$ gene 3' end exons 41-47 (corresponding to the C-terminus of the $\alpha_{1A}$ protein)

**wt**

$5' \alpha_{1A} \rightarrow \begin{array}{c} 41 \ 42 \ 43 \ 44 \ 45 \ 46 \ 47 \end{array} 3' 6495$ bp

$\rightarrow COOH 2164$ aa

$\rightarrow$ Translation of Intron B sequence. Out of frame read through of subsequent exons. Premature stop codon in exon 45.

**tg$^{la}$ long**

$5' \alpha_{1A} \rightarrow \begin{array}{c} 41 \ 42 \ B \ 43 \ 44 \ 45 \end{array} 3' 6174$ bp

Translation of Intron B sequence. Out of frame read through of subsequent exons. Premature stop codon in exon 45.

**tg$^{la}$ short**

$5' \alpha_{1A} \rightarrow \begin{array}{c} 41 \ 43 \ 44 \ 45 \end{array} 3' 5937$ bp

Deletion of exon 42. Out of frame read through of subsequent exons. Premature stop codon in exon 45.

Figure 5: Sizes of wt, tg$^{la}$ long, and tg$^{la}$ short mRNA and protein sequences. The tg$^{la}$ mutation results in the production of two aberrant mRNAs and proteins with novel C-termini.
used as a model for this condition. The absence-like symptoms displayed by these mice may be a result of alterations in the thalamocortical circuit, which is thought to be involved in the generation of cortical spike wave discharges (Fletcher et al., 1996; Danober et al., 1998). Other neurological symptoms displayed by \(tg^{ja}\) mice that involve the cortex include a 10-fold resistance to cortical spreading depression (CSD), slowed propagation of CSD, and a decrease in transmitter release (Ayata et al., 2000). A decrease in cortical excitability has been linked to familial hemiplegic migraine, one of the human P/Q-type calcium channelopathies (van der Kamp et al. 1997, Ayata et al., 2000).

### 1.8 How Could the \(tg^{ja}\) Phenotype Arise?

The reason why a C-terminal mutation in a mouse \(\alpha_{1A}\) subunit should cause the devastating neurological effects of the \(tg^{ja}\) phenotype is unknown. There are four principal ways that the \(tg^{ja}\) phenotype could arise:

First, products of the \(tg^{ja}\) mutation could be directly cytotoxic. For example, the \(tg^{ja}\) protein (or mRNA) might interfere with biochemical reactions essential for cell vitality, promote undesirable intracellular reactions or disrupt organelle or membrane function. This model is not well supported by the available evidence. For example, cell death occurs several weeks after the onset of the neurological deficits.

Second, the \(tg^{ja}\) mutation could alter the biophysical properties of the P/Q-type VDCCs so that they function abnormally or not at all. Such a mechanism is not well supported, experimentally. In whole-cell recordings, Purkinje cells from \(tg^{ja}\) mice show a significant
reduction in P/Q-type currents, but alterations in their kinetic properties, such as activation and inactivation, are absent or have not been seen by all researchers (Dove et al., 1998; Wakamori et al., 1998). Single-channel recordings have also failed to reveal differences in the conductance or channel mean open time. However, a threefold reduction in the open probability and/or reduction in the number of channels at the surface has been found (Dove et al., 1998; Lorenzon et al., 1998; Wakamori et al., 1998).

Third, the $tg^{ia}$ protein(s) could be synthesized at levels similar to wild type P/Q-type VDCCs but are incorrectly processed and trafficked to (or from) the plasma membrane. Although mutations in diverse membrane proteins can cause their mis-trafficking (Sharma et al., 1999; Schwake et al., 2000), this mechanism has not been investigated for $tg^{ia}$ channels, or indeed any other VDCCs.

Fourth, the $tg^{ia}$ mutation could alter the expression levels of P/Q-type VDCCs, thereby altering their contribution to nerve functions such as synaptic transmission. In this scenario, the most likely effect of the mutation would be a reduction in channel protein arising through a decrease in protein stability, accelerated degradation, or decreased mRNA stability. Reports published on $\alpha_{1A}$ expression levels in the $tg^{ia}$ cerebellum have produced conflicting results. One study found a reduction in $tg^{ia}$ mRNA and protein levels (Doyle et al., 1997), whereas two other groups found no change in mRNA (Fletcher et al., 1996; Lau et al. 1998) or protein expression (Lau et al., 1998).
1 OBJECTIVES AND PROPOSAL

2.1 Hypothesis

Based on the preceding discussion, I hypothesized that the neurological defects seen in the \(tg^a\) mice were caused by a reduction in the cell surface levels of functional P/Q-type calcium channels arising through their mis-targeting, or through a reduction in \(tg^a\) \(\alpha_{1A}\) total protein levels.

To test the above hypothesis my specific objectives were:

1. To evaluate P/Q-type calcium channel expression in \(tg^a\) versus wt brains through immunoblotting using antibodies directed against the \(\alpha_{1A}\) subunit, and through binding assays with the P/Q-type calcium channel specific radioligand \([^{125}\text{I}]-\omega\text{-conotoxin (CTX) MVIIIC}\).

2. To examine the expression, function, and subcellular localization of \(tg^a\) calcium channels in transfected cells. Here, a two-step strategy was used. First, mammalian expression vectors were developed that contained EGFP-tagged constructs encoding \(tg^a\) long, \(tg^a\) short, and wild type \(\alpha_{1A}\) P/Q-type calcium channel subunits. Second, the corresponding EGFP-tagged channels were expressed in eukaryotic cell lines (HEK tsA201 and COS-7) by co-transfection with the necessary auxiliary \(\alpha_{2\delta}\) and \(\beta_{1b}\) subunits and characterized by electrophysiology, Western immunoblots, and imaging.
3 METHODS

3.1 Expression Levels of P/Q-Type Voltage-Dependent Calcium Channels in wt and tg\textsuperscript{la} Brains

Six 6.5 month wild type and six 7.25 month tg\textsuperscript{la} brains were obtained from Dr. Louise Abbott (Texas A&M). Cortical membranes were prepared as follows: the cortex was dissected from each animal and homogenized using a Potter-Elvejheim tissue grinder in 4 ml of ice-cold Buffer A (0.32 M sucrose, 5 mM Tris-HCl pH 7.4). The mixture was then centrifuged at 700\textsubscript{av} (2700 rpm, Sorvall AH-650 rotor) for 10 minutes at 4\textdegree{}C. The supernatant was collected and centrifuged at 27,000\textsubscript{av} (17,000 rpm, Sorvall AH-650 rotor) for 40 minutes at 4\textdegree{}C. Pellets were resuspended in 2 ml of ice cold buffer C (5 mM HEPES pH 7.4, 0.32 mM sucrose). To minimize proteolytic degradation, protease inhibitors at the following concentrations were added to the buffers immediately prior to use: PMSF (100 \mu{}M), O-phenanthroline (1 mM), leupeptin (1 \mu{}M), aprotinin (1 \mu{}g/ml), iodoacetamide (1 mM), and benzamidine (1 mM). Membranes were stored in 100 \mu{}l aliquots in liquid nitrogen. Concentrations of membrane proteins were determined with a commercial kit (D\textsubscript{C} Protein assay, BioRad). Cortical rather than cerebellar membranes were used for my experiments because the effects of the tg\textsuperscript{la} mutation on tg\textsuperscript{la} \alpha\textsubscript{1A} subunit expression in the tg\textsuperscript{la} cerebral cortex had not been previously examined.
3.1.1 Expression Levels of P/Q-Type VDCCs in tgα and wt Cortical Membranes: Western Immunoblots

To examine P/Q-type VDCC expression in tgα and wt cortical tissues, Western immunoblots were performed. Membranes were solubilized using a method introduced by Catterall's laboratory for calcium channels (Hell et al., 1995). Briefly, samples were mixed with at least 3 volumes of double strength (2X) sample buffer (12% SDS, 40 mM DTT, 250 mM Tris-HCL pH 6.8, pH 6.8, 20% sucrose, 4 mM EGTA, 4 μg/ml leupeptin, 8 μg/ml aprotinin, 200 μg/ml benzamidine) and then warmed at 55-60°C for 30 minutes. Calcium channels are often warmed (Westenbroek et al., 1992; Hell et al., 1995; Catterall et al., 1995; Sakurai et al., 1995), rather than boiled as are several other membrane proteins such as Ca²⁺/Mg²⁺-ATPase (Horgan and Kuypers, 1981), because protein aggregation can result from boiling. The samples were loaded onto 6% linear polyacrylamide gels and run at 110 V constant voltage for 100 minutes. The separated proteins were transferred electrophoretically to nitrocellulose sheets according to Towbin et al. (1992), at 100 V for 75 minutes. Following transfer, the membranes were removed and non-specific protein binding sites were blocked by incubation in 5% (w/v) non-fat dry skim milk (NFDM)/Tris-buffered saline (TBS (20 mM Tris pH 7.5, 500 mM NaCl) with thimerosol overnight at 4°C. The following day, the blots were washed using a protocol of three 10 minute washes in TTBS (TBS with 0.05% Tween-20) and three 10 minute washes in TBS at room temperature. The blots were then incubated at room temperature for 2 hours with primary antibody (α1A antibody: CNA1 (residues 865-881 in the intracellular loop between domains II and III of the rat α1A subunit, Alomone Labs) diluted 1:120 in NFDM/TBS and washed as described above. The blots were then incubated for 1 hour in secondary antibody solutions comprised of either horseradish
peroxidase-conjugated donkey-anti-rabbit antibodies diluted 1:4000 in NFDM/TBS, or horseradish peroxidase-conjugated goat-anti-rabbit antibodies diluted 1:3500 in NFDM/TBS. After washing as described above, the immunoblots were developed using Enhanced Chemiluminescence (Amersham ECL kit) and bands visualized by film exposure (Amersham Hyperfilm). To confirm the specificity of the antibody labeling, immunoblots were performed on rat brain membranes in the presence of excess antigenic peptide (Alomone Labs, 1:1 with antibody (w/w)). Statistical analysis was performed using an unpaired Student’s t test.

3.1.2 Expression Levels of P/Q-Type VDCCS in \textit{tg}/\textit{a} and wt Cortical Membranes: \textit{[\textsuperscript{125}I]}- Omega (\omega)-Conotoxin MVIIIC Binding Assay

Binding of \textit{[\textsuperscript{125}I]}-\omega-CTX MVIIIC was measured using the following protocol: 25 \mu g of membrane were incubated with increasing concentrations of \textit{[\textsuperscript{125}I]}-\omega-CTX MVIIIC (0.01-1.8 nM) at room temperature for one hour in a total volume of 200 \mu l reaction buffer (10 mM Na\textsuperscript{+}-HEPES, 100 mM NaCl, 2 mM EDTA, 0.2 mg/mL BSA, 0.75 mM benzamidine and 0.1 mM PMSF, pH 7.4). Non-specific binding was determined by incubating membrane preparations with cold \omega-CTX MVIIIC (1 \mu M) for 20 minutes prior to the addition of radioactive ligand. Membranes were collected on glass fiber filters (GF/B) soaked in 0.3% (v/v) polyethylenimine under vacuum, and washed rapidly four times with 4 ml wash buffer (10 mM Na\textsuperscript{+}-HEPES, 100 mM NaCl, 2 mM EDTA, 0.2 mg/ml BSA, pH 7.4, 0.75 mM benzamidine, and 0.1 mM PMSF). The amount of radioactivity on the filters was then counted. Specific binding was calculated by
subtracting non-specific binding from total. Statistical analysis was performed using an unpaired Student’s t test.

3.2. Characterization of EGFP-Tagged wt and tg\textsuperscript{ia} Channels in Transfected Cell Lines

3.2.1 Preparation of tg\textsuperscript{ia} and wt Constructs in pEGFP-C2

A full-length rat $\alpha_{1A}$ cDNA clone in pCDNA3 (kind gift of Dr. T. Snutch; accession number M64373) was used to create a pEGFP-C2-rat $\alpha_{1A}$ (EGFP-$\alpha_{1A}$wt) fusion construct (Figure 6). Since the clone did not contain any sites that would have allowed easy excision of the $\alpha_{1A}$ gene out of pCDNA3 and into pEGFP-C2 (Clontech), an adaptor was incorporated that contained BsiWI, SvaI, BstZ171 and BstBI sites (5’GTACGATTTAAATGTATACTT3’ top strand; 5’CGAAGTATACATTAAATC3’ bottom strand). The ligation was performed at 14°C overnight, and the adaptor was used at a 1:1000 concentration. Following the ligation of the adaptor, the $\alpha_{1A}$ gene was cut out of the modified pCDNA3 rat $\alpha_{1A}$ construct with XhoI (sticky) and BstZ171 (blunt), and ligated into pEGFP-C2 that had been pre-linearized with XhoI and SmaI (blunt). Sequencing verified the correct reading frame and junctional sequences. pEGFP-C2 encodes a red-shifted variant of wild type GFP which has been optimized for brighter fluorescence and higher expression in mammalian cells (Excitation maximum = 488 nm; emission maximum = 507 nm (Clontech pEGFP-C2 Vector Information).

To create the tg\textsuperscript{ia} constructs, the region corresponding to the C-terminus of EGFP-$\alpha_{1A}$wt was substituted with that of the tg\textsuperscript{ia} mouse cDNA (long and short forms).
1 Cut with BsiWI and BstBI and purify fragment from gel.

2 Ligate with adaptor.

3 Extract Xhol-BstZ17I fragment and insert into pEGFP-C2 digested with Xhol-Smal.

Figure 6: Schematic diagram of the synthesis of the α₁⁻ in pEGFP-C2 (EGFP-α₁⁻wt) construct. Following the ligation of an adaptor, the α₁⁻ gene was excised from pCDNA3 and inserted into the pEGFP-C2 vector.
Briefly, \(tg^\alpha\) mouse mRNA was obtained from Dr. Louise Abbott (Texas A&M). The RNA pellets were washed in 70% ethanol and resuspended in 10 \(\mu l\) DEPC water. First strand cDNA was then prepared by combining 2 \(\mu l\) of this RNA solution in a PCR tube with 1 \(\mu l\) Oligo (dT) and 9 \(\mu l\) filtered DEPC water. The mixture was then heated to 70°C for 10 minutes followed by a quick chill on ice. To this tube the following reagents were added: 4 \(\mu l\) 5X First Strand Buffer, 2 \(\mu l\) 0.1 M DTT, and 1 \(\mu l\) 10 mM dNTP mix. The contents were then mixed and incubated at 42°C for 2 minutes after which 1 \(\mu l\) of SUPERSCRIPT II was added, and the mixture was incubated for 50 minutes at 42°C. The reaction was subsequently inactivated by heating to 70°C for 15 minutes.

Amplification of both \(tg^\alpha\) long and short form cDNAs was achieved by PCR using 2 \(\mu l\) of the first strand mixture and the sequence-specific forward (\(tg^\alpha\) FOR: 5' GCCTGGGCCGCCATTCACTAT 3'; corresponding to the nucleotides 5236-5256 in the \(tg^\alpha\) long and \(tg^\alpha\) short mRNA), and reverse (LEANREV: 5' AAAATTTAAATCATGGGGCTGGTATCAGAGA 3'; corresponding to the nucleotides 6154-6174 in the \(tg^\alpha\) long, and 5917-5937 in the \(tg^\alpha\) short mRNA, and also introducing a 3' Swal site for cloning) primers. The blunt-ended long and short forms were ligated into the MCS of the pCR Blunt vector (Invitrogen) at 14°C overnight. Following transformation, the DNA was analyzed for the presence of inserts via restriction digestion. Substitution of the cDNA region encoding the carboxy-terminus of the EGFP-\(\alpha_{1A}\)wt with that encoding either the short or long form was achieved by first predigesting EGFP-\(\alpha_{1A}\)wt with NotI (5'GCGGCCGC3') and Swal (5'ATTTAAAT3'). This digestion effected the excision of DNA encoding the \(\alpha_{1A}\) C-terminus from amino acid residue 1795 through to the stop codon, together with additional downstream non-coding sequences up to the Swal site. Next, regions encoding the carboxy termini of \(tg^\alpha\) long, and \(tg^\alpha\)
short, obtained by PCR (above), were excised with NotI (5') and SvaI (3') from pCR blunt and ligated in place of the excised NotI/SvaI region of the EGFP-α1Awt vector at 14°C overnight. Faithful creation of the EGFP-α1AtgJa long, and EGFP-α1AtgJa short fusion constructs was confirmed by DNA sequencing. Since the mouse and rat α1A proteins show 98.7% amino acid identity in their C-termini, no wild type mouse substitution was made (see appendix B).

3.2.2 Expression of EGFP-tagged α1Awt, α1AtgJa long, and α1AtgJa short Channels in HEK tsA201 Cells

HEK tsA201 cells (human embryonic kidney cells stably expressing simian virus 40 large T antigen) were obtained from Dr. Peter Backx, University of Toronto, and grown on tissue culture 35 mm wells in Dulbecco’s Minimum Essential Medium (DMEM, Gibco), containing 10% fetal bovine serum (FBS) (Gibco), 10 μg/ml penicillin, and 10 U/μl streptomycin. Cells were grown in 5% CO2-balanced air at 37°C.

HEK tsA201 cells at 60-80% confluency were transfected with either EGFP-α1Awt, EGFP-α1AtgJa long, or EGFP-α1AtgJa short in combination with the rat cDNA for the auxiliary β1b (Accession number X11394) and the α2δb (Accession number M86621) subunits using LipofectAMINE reagent (Life Technologies) with a total of 2 μg DNA (0.67 μg of each subunit). Briefly, DNA diluted in 100 μl DMEM was combined with LipofectAMINE reagent diluted in 100 μl DMEM and incubated for 30 minutes at room temperature. Following incubation, 800 μl of DMEM was added to the DNA/LipofectAMINE mixture and applied to HEK tsA201 cells that had been prewashed with DMEM to remove any residual FBS. Cells were then incubated with this mixture for
5 hours at 37°C. After 5 hours, the above mixture was removed and replaced with 2 ml of DMEM containing 10% FBS. The medium was replaced again at 24 hours following the start of transfection, and then incubated for another 24 hours at 37°C. Cells were not exposed to antibiotics at any point during the transfection experiments. The transfection efficiency was generally around 47%, based on the expression of EGFP fluorescence.

After 48 hours incubation, the cells were washed twice with 1X PBS, and the cells were scraped off the dishes into 400 µl of 2X Catterall's buffer (without dithiothreitol (DTT) and bromophenol blue, which interfere with protein determination) per 35 mm well. Cell suspensions were sheared with an 18-gauge needle 15-20X, and a small aliquot was removed for protein assay (DC Protein Assay, BioRad). DTT (2.33 mg) and bromophenol blue (15 µl of 0.5% solution) were then added to the mixture and the samples re-sheared 5X with an 18-gauge needle and incubated at 55-60°C for 35 minutes to extract the proteins. Samples were then run immediately on a 6% polyacrylamide gel or stored at -70°C for later use. Blots were transferred and processed as described above (Section 3.1.1). The channels were detected with the GFP 1° antibody (Molecular Probes, 1:1000).

3.2.3 Fluorescence Imaging of EGFP-tagged wt and tgα Channels in COS-7 Cells.

Cells from the African Green Monkey kidney fibroblast COS-7 line (ATCC CRL-1651) were transfected either with EGFP-α1αwt, EGFP-α1αtgα long, or EGFP-α1αtgα short constructs, in combination with the auxiliary β15 and α2δ subunit vectors using the LipofectAMINE PLUS reagent (Life Technologies). COS-7 cells were used for the
imaging studies because they were found to be more adherent to tissue culture wells than HEK tsA201 cells and because of their extensive use in GFP trafficking studies (Presley, et al., 1997; Hirschberg et al., 1998). Briefly, cells were grown to 60% confluency and 2 μg total DNA (0.67 μg of each subunit) in 100 μl serum-free DMEM was pre-complexed with 5 μl PLUS reagent in serum-free DMEM and incubated at room temperature for 15 minutes. Pre-complexed DNA was then combined with LipofectAMINE reagent diluted in 100 μl DMEM and incubated at room temperature for 15 minutes. This mixture was added to each 35 mm well already containing 800 μl fresh DMEM on the COS-7 cells. Cells were then incubated for 3 hours at 37°C at 5% CO₂.

Following incubation, the transfection medium was replaced with fresh DMEM, containing 10% FBS and 10 nM α-CTX MVIIC, to block calcium entry into cells and improve the health of the cells for imaging purposes, and incubated once more at 37°C, 5% CO₂. Transfection efficiencies for the EGFP-α1Ashort, EGFP-α1Atgα long, or EGFP-α1Awt vectors were 24-33%. At 48 hours post-transfection, the medium was removed and the cells were washed once with osmotically-balanced 1X PBS, fixed in 4% paraformaldehyde and mounted in Mowiol (Calbiochem, CA). In cases where it was important to resolve the cell surface, the cells were labelled with Texas Red-conjugated wheat germ agglutinin (TxA-WGA) (Molecular Probes, OR) prior to fixation. Briefly, cells were removed from the incubator, washed in osmotically balanced 1X PBS, and chilled on ice to suppress internalization of surface proteins. A solution of TxA-WGA in osmotically-balanced PBS (500 μl of 20 μg/mL TxA-WGA) was then added and the cells were incubated on ice for 1 hour. Following incubation, the cells were washed twice with ice-cold 1X PBS to remove excess label, fixed with 4% paraformaldehyde and mounted in Mowiol.
Cells were imaged with a low magnification objective (20-40X) and images were collected using a Nikon Eclipse E800 microscope equipped with a cooled 3CCD camera and the fluorescence of the EGFP and Texas Red-WGA labels viewed using fluorescein and Texas Red filter blocks respectively. Images were collected with Image ProPlus (Media Cybernetics) software and analyzed using Photoshop 5.0 (Adobe). This microscope is located within the research space of Dr. Fehling’s laboratory and undergoes routine maintenance by trained laboratory technicians and Nikon specialists. One would not anticipate that any technical difficulties such as improper calibration of the collection system would occur with such a microscope (Dr. Fehling’s laboratory, personal communication). Higher objective (60X) images and images for z-series were collected with an inverted scanning confocal microscope (BioRad MRC-600) equipped with an argon-ion laser (ILT), using dual excitation and fluorescein and rhodamine filter sets and either fluor (20X and 40X) or planapo (60X) objectives as detailed earlier (Mills et al., 1994). This microscope is located within Dr. Mills’ laboratory and also undergoes routine checks to ensure that it is properly calibrated. Controls were used to test for correct alignment prior to the utilization of filter blocks, and images were checked following collection for proper alignment using fiduciary markers (Dr. Mills, personal communication). During collection, images were managed with COMOS (BioRad) software. Split images (red and green channels) were colorized and merged using Confocal Assistant. To facilitate subsequent z-series processing, either 16 or 32 optical sections were taken. Digitized images were adjusted in Photoshop 5.0 (Adobe) for variations in background intensities and displayed without any further manipulation, using identical color, brightness and contrast settings.
As controls, subcellular localization vectors for the nucleus, endoplasmic reticulum (ER) and trans-medial region of the Golgi apparatus (Clontech) were also transfected into COS-7, labeled with Tx-WGA as described above, fixed, and viewed using fluorescence microscopy.
4 RESULTS

4.1 Expression Levels of P/Q-Type VDCCs in $tg^{la}$ and wt Cortical Membranes

The expression of P/Q-type calcium channels was investigated in the $tg^{la}$ and wt mice using toxin binding assays and immunochernical techniques.

4.1.1 Expression of P/Q-Type VDCCs as Determined by Western Immunoblotting

To examine the expression levels of the $\alpha_{1A}$ subunit of P/Q-type VDCCs in wt and $tg^{la}$ mice, cortical membranes were analyzed by immunoblotting with CNA1, a rabbit anti-$\alpha_{1A}$ subunit polyclonal antibody. As immunoblotting of VDCCs with commercially available $\alpha_{1A}$ antibodies has been problematic, and not described for mouse tissues, the specificity of the $\alpha_{1A}$ antibody was first tested using rat cortical membranes. As shown in Figure 7, immunoblots revealed a major band of 190 kDa and several minor bands as described elsewhere (Sakurai et al., 1996). The major and minor bands were displaced by the addition of excess competing peptide antigen illustrating the specificity of the antibody (Figure 7). The band sizes for the $\alpha_{1A}$ subunits in wt and $tg^{la}$ mouse cortical membranes were also approximately 180-190 kDa, with the $tg^{la}$ membranes producing a doublet corresponding to $tg^{la}$ long and $tg^{la}$ short. The immunoblot revealed major differences in the intensities of the bands from the two $tg^{la} \alpha_{1A}$ subunits compared to the wt $\alpha_{1A}$ protein. Specifically, the $tg^{la}$ membranes showed consistently lower band intensities over a similar range of protein concentrations for both the $tg^{la}$ long and short forms of the protein (Figure 8A, 8B) versus wt. Moreover, these reductions were not
Figure 7. The $\alpha_{1A}$ CNA1 antibody is specific. Following preparation of rat cortical membranes, the membranes were run on SDS-PAGE and probed with an antibody against $\alpha_{1A}$ (CNA1 antibody). The major band recognized is approximately 190 kDa with numerous minor bands (A). No staining of immunoblotted membranes was observed when the antibody was pre-treated with competing $\alpha_{1A}$ peptide (B). Blots were detected by ECL (Methods).
Figure 8A. $\alpha_{1A}$ subunits are expressed at lower levels in $tg^{la}$ cortical membranes. The expression levels of $\alpha_{1A}$ in $tg^{la}$ and wt mouse cortical membranes was examined over a range of concentrations using the CNA1 $\alpha_{1A}$ antibody. Note the lower levels of expression of the $tg^{la}$ mutant $\alpha_{1A}$ subunits versus wt mouse brain. Both wt and $tg^{la}$ membranes were run on the same immunoblot. Blot was detected by ECL (methods).
Figure 8B. Densitometric analysis reveals lower expression of $\alpha_{1A}$ subunits in $tg^{a}$ than wt brains.

The Figure 2A blot was scanned by laser densitometry and the density of the wt $\alpha_{1A}$ 190kDa band corresponding to P/Q-type calcium channels was plotted as a function of the amount of protein loaded in the SDS-polyacrylamide gel and was compared to the $tg^{a}$ long and $tg^{a}$ short bands.
Figure 9. Expression of $\alpha_{1A}$ in $tg^{la}$ and wt mouse cortical membranes (n=6).

Immunoblot was prepared by loading identical amounts (60 $\mu$g) of cortical membrane protein from six $tg^{la}$ and six wt mice. The blot was probed with antibody directed against the $\alpha_{1A}$ subunit (CNA1). Both wt and $tg^{la}$ cortical membranes were run on the same immunoblot. Blot was detected by ECL (methods). Identical exposures were used for Figure 8A and Figure 9 blots.
restricted to a subset of brains but were seen consistently in all $tg^{la}$ cortices compared to wt (P<0.0001) (Figure 9). Based on densitometry, the level of expression of the $tg^{la} \alpha_{1A}$ long band plus the $tg^{la} \alpha_{1A}$ short band was approximately 30% of that seen in their wt counterparts at 60 $\mu$g of protein loaded. It should be noted that although the amount of protein loaded in each lane was always calculated, and wt and $tg^{la}$ proteins were run on the same blot and exposed for the same amount of time, no independent markers were used. Such markers could be included in the future and would ensure equal protein loading.

4.1.2 Expression of P/Q-Type Calcium Channels as determined by $[^{125}I]-\omega$-CTX MVIIC binding

Toxins from naturally occurring marine and spider venoms represent some of the most powerful tools for quantifying ion channels and defining their contributions to specific physiological processes (Bowersox et al., 1995). One such toxin, $\omega$-CTX MVIIC, a peptide derived from the venom of the marine mollusc Conus magus (Hillyard et al. 1992), has been found to be a potent blocker of P- and Q- type VDCCs in a variety of mammalian neuronal preparations (Turner and Dunlap, 1995; Bowersox et al., 1995; Liu et al., 1996). Confirmation of the selectivity and potency of $\omega$-CTX MVIIC comes from membrane binding studies which indicate that, at low concentrations, it binds exclusively to P/Q-type VDCCs, with a Kd of 0.4-1.8 nM (Liu, et al., 1996; Martin-Martout et al., 1995). Such studies also indicate that MVIIC binding assays are a facile means to quantify P/Q-type VDCCs and could therefore be used to resolve differences in P/Q-type VDCC expression in $tg^{la}$ and wt brains. To test this possibility it was first
Figure 10: Binding of $[^{125}\text{I}]-\omega$-conotoxin MVIIIC to rat brain membranes. The membranes were incubated with increasing concentrations of $[^{125}\text{I}]-\omega$-conotoxin MVIIIC in the presence (non-specific binding) or in the absence (total binding) of 1 μM unlabelled $\omega$-conotoxin MVIIIC. Specific binding was determined by subtracting total binding from non-specific binding.
Figure 11: Binding of $^{[125]}\omega$-conotoxin MVIIIC to $tg^{ia}$ and wt mouse cortical membranes. The membranes were incubated with increasing concentrations of $^{[125]}\omega$-conotoxin MVIIIC in the presence or absence of 1µM unlabelled $\omega$-conotoxin MVIIIC. Specific binding was determined by subtracting total binding from non-specific binding. The results were obtained by pooling data from a number of animals ($tg^{ia}$ n=6; wt n=5).
considered prudent to confirm the binding characteristics of MVIIC using whole rat brain membranes. As shown in Figure 10, the total binding of $[^{125}\text{I}]$-\(\omega\)-CTX MVIIC increased in a concentration-dependent, non-linear fashion. In contrast, non-specific binding, determined as that seen in the presence of excess unlabelled MVIIC, rose linearly with increasing $[^{125}\text{I}]$-\(\omega\)-CTXMVIIC concentration, and was lower than total binding over the entire range examined. Subtraction of non-specific from total binding data at equivalent concentrations yielded a curve for specific binding that showed clear evidence of saturation (Figure 10). Analysis of the binding data using a non-linear least squares approach afforded an excellent fit assuming binding to a single class of sites with a \(B_{\text{max}}\) of 1.74 pmol/mg and a \(K_d\) of 0.395 nM. These studies were then extended to include a comparison of MVIIC binding in the wt and \(tg^{a}\) mouse cortical membranes. Analysis of the specific binding curves revealed that there was a 3-fold lower \(B_{\text{max}}\) for the binding of MVIIC to \(tg^{a}\) membranes (0.391 pmol/mg) as compared to the wt membranes (1.18 pmol/mg). In contrast, the affinities of MVIIC binding to the \(tg^{a}\) (\(K_d\) 0.409 nM) and wt (\(K_d\) 0.415 nM) membranes were essentially identical (Figure 11). Together, these data argue that there is a significant decrease in the concentration of $[^{125}\text{I}]$-\(\omega\)-CTX MVIIC binding sites in the \(tg^{a}\) cortical membranes as compared to wt membranes (\(P<0.0003\)).

4.2 Analysis of the \(tg^{a}\) Mutation in Transfected Cell Lines

While the immunoblotting studies and binding assays point to significant differences between \(P/Q\)-type VDCC expression in \(tg^{a}\) and wt mice, these approaches suffer from several major drawbacks. The first problem concerns the potential expression of multiple species of aberrant proteins. The \(tg^{a}\) mutation arises through a single base-pair substitution at a splice donor consensus sequence in the mouse \(\alpha_{1A}\)
gene that leads to a mis-splicing event and the production of two primary aberrant mRNAs. Both the $tg^l$ and $tg^s$ transcripts are predicted to produce aberrant $\alpha_{1A}$ proteins with altered C-termini (Fletcher et al., 1996). Thus, the $tg^l$ phenotype could result from either one or both of these mutant proteins. A second issue concerns the difficulty of identifying the mechanisms underlying the $tg^l$ phenotype at a molecular or cellular level. The paucity of material available from mouse brain precludes many of the biochemical studies required to study aberrant $\alpha_{1A}$ protein expression, especially in cultures. In addition, the absence of numerous labeling reagents, such as fluorescent toxins, limits channel detection, precluding trafficking studies. More important, most ligand-based methods are incapable of resolving events involving discrete molecular species. The purpose of this section of my thesis research, therefore, was to develop an alternative strategy that could be used to investigate the effects of the two principle $tg^l$ mutant proteins, $tg^l$ long, and $tg^l$ short, independently in a heterologous expression system.

The strategy that was employed involved the design and construction of independent plasmids encoding the wt, $tg^l$ long, and $tg^l$ short $\alpha_{1A}$ channels each bearing an in-frame EGFP gene tag fused to the 5' end of the $\alpha_{1A}$ coding sequence. The EGFP tag is a version of the 27 kDa green fluorescent protein (GFP) of the bioluminescent jellyfish *Aequorea victoria* that has been genetically modified to optimize its fluorescence intensity and spectral characteristics and expression in mammalian cells. Owing to its ability to fold spontaneously and form a highly fluorescent chromophore, GFP is now finding widespread application in all areas of modern biology, in particular those studies where it is advantageous to monitor protein distributions in live cells (Pines, 1995; Cubitt et al., 1995; Wacker et al., 1997; Presley et al., 1997). In
addition, as EGFP is highly antigenic, this tag allows the ready immunodetection of proteins such as the α1A subunit which are difficult or expensive to detect using available anti-protein antibodies.

Each mutant construct was prepared using a two-step approach. First, DNA encoding the wt rat α1A was cloned into the eukaryotic expression vector pEGFP-C2. Second, cDNAs corresponding to the tgα long, and tgα short α1A channel C-termini were obtained via RT-PCR of mRNA from tgα mice and inserted in place of the C-terminus of the EGFP-α1Awt.

4.2.1 Preparation of EGFP-α1Awt

The EGFP attached α1Awt construct was prepared using the protocol described in the methods. The rat α1A gene in pCDNA3 was modified to include new restriction sites, by incorporating an adaptor at the 3' end of the α1A sequence. Restriction digests (Figure 12) verified the correct insertion of the adaptor. Cloning of an Xhol-BstZ171 fragment (~7 kb) of the modified α1A in pCDNA3, into pEGFP-C2, yielded a full length α1A gene in pEGFP-C2 as confirmed by restriction digestion analysis (Figure 13) and sequencing.

4.2.2 Preparation of EGFP-α1Atgα long and EGFP-α1Atgα short Constructs

First strand tgα long and tgα short cDNA was prepared from tgα mouse mRNA by PCR using primers designed to select for the 3' end corresponding to the C-terminus of the α1A protein in tgα mice. Agarose gel electrophoresis identified two PCR products,
Figure 12: Restriction enzyme digestion to confirm the correct insertion of the adaptor into the $\alpha_{1A}$ in pCDNA3 (modified $\alpha_{1A}$ in pCDNA3) vector. DNA bands were separated by gel electrophoresis in a 0.7% (w/v) agarose gel. Lane 1 (in bp), 1 kb DNA ladder (Gibco BRL); lane 2, modified $\alpha_{1A}$ in pCDNA3 uncut; lane 3, $\alpha_{1A}$ in pCDNA3 digestion with Swal/NotI to produce fragments of approximately 6.2 kb, 5.4 kb, and 1.3 kb; lane 4, modified $\alpha_{1A}$ in pCDNA3 digested with Swal/NotI to produce bands of 5.4 kb, 3.6 kb, 1.3 kb, and 0.3 kb; lane 5, modified $\alpha_{1A}$ in pCDNA3 digested with Swal/Xhol to produce bands of 7.2 kb and 3.4 kb; lane 6 modified $\alpha_{1A}$ in pCDNA3 digested with Xhol to produce a single band of 10.6 kb; lane 7, modified $\alpha_{1A}$ in pCDNA3 digested with BstZ171I to produce bands of 10.3 kb, and 0.3 kb; lane 8 modified $\alpha_{1A}$ in pCDNA3 digested with Xhol/BstZ171I to produce fragments of 7.1 kb, 3.2 kb, and 0.3 kb.
Figure 13: Restriction enzyme digestion to analyze for the correct insertion of $\alpha_{1A}$ in pEGFP-C2 (EGFP-$\alpha_{1A}$wt). Lane 1, 1 kb DNA ladder (Gibco BRL); lanes 2-16 were digestions with BamHI. Positive clones produced bands of 2.5 kb, 1.9 kb, and 7.8 kb. All lanes except lane 7 showed positive clones.
Figure 14: Polymerase chain reaction amplification of \(tg^{ia}\) 3' end corresponding to the C-terminus of the \(tg^{ia}\) \(\alpha_{1A}\) protein, from \(tg^{ia}\) mouse first strand cDNA. The primers \(tg^{ia}\) FOR and LEANREV were used in the amplification. As seen in lanes 1 and 2, two bands were detected following gel electrophoresis. The first band was 942 bp in length and corresponds to the \(tg^{ia}\) long cDNA, and the second band at 709 bp corresponds to the \(tg^{ia}\) short cDNA. Lane 3, 1kb DNA ladder (Gibco BRL).
Figure 15: Restriction enzyme analysis of \(tg'^a\) long and \(tg'^a\) short in pCR-Blunt. Lanes 1-8 correspond to \(tg'^a\) long in pCR-Blunt and lanes 10-14 correspond to \(tg'^a\) short in pCR-Blunt. Correct clones cut with NotI/Swal revealed bands of 0.9 kb and 3.5 kb for \(tg'^a\) long and 0.7 kb and 3.5 kb for \(tg'^a\) short. The 3.5 kb band is the released pCR-Blunt vector. Lane 9: 1kb ladder (Gibco BRL).
Figure 16: Restriction enzyme analysis of EGFP-α1A*tg* long and EGFP-α1A*tg* short. Miniprep DNA was digested with NotI/Swal and separated by gel electrophoresis on a 1% agarose gel. Lanes 1-6, EGFP-α1A*tg* long, correct sizes, 0.9 kb and 10 kb; lane 7, 1kb DNA ladder (Gibco BRL); lanes 8-12 EGFP-α1A*tg* short, correct sizes 0.7 kb and 10 kb. Positive EGFP-α1A*tg* long clones include lanes 1-4 and positive EGFP-α1A*tg* short clones include lane 6 and lanes 9-10.
one for $tg^a$ long at 942 bp and one for $tg^a$ short at 709 bp (Figure 14). The $tg^a$ long and $tg^a$ short C-terminal cDNAs were then ligated into the multiple cloning site of the pCR Blunt vector (Figure 15). The EGFP-$\alpha_{1A}$-wt was then pre-digested with NotI and Swal to remove the $\alpha_{1A}$ C-terminal coding region and re-ligated with the $tg^a$ long or $tg^a$ short C-terminal cDNAs to yield the EGFP-$\alpha_{1A}tg^a$ long and EGFP-$\alpha_{1A}tg^a$ short constructs. Restriction digests (Figure 16) and sequencing of the regions of ligation confirmed the fidelity of the constructs.

4.2.3 Immunoblot Analysis of EGFP-tagged $\alpha_{1A}$-wt, $\alpha_{1A}$-$tg^a$ long and $\alpha_{1A}$-$tg^a$ short VDCCs Expressed by Transient Transfection of HEK tsA201 Cells

To determine if the EGFP-$\alpha_{1A}$ fusion constructs expressed proteins of the correct size, Western immunoblot analysis was performed on HEK tsA201 cells transfected with constructs encoding each EGFP-tagged $\alpha_{1A}$wt, $\alpha_{1A}tg^a$ long, or $\alpha_{1A}tg^a$ short subunit. To preclude any mis-trafficking arising through the absence of auxiliary subunits in the VDCC complex, the cells were co-transfected with constructs encoding $\alpha_{2\delta}$ and $\beta_{1b}$ proteins. At 48 hours post-transfection, cell lysate proteins were separated on SDS-polyacrylamide gels, and immunoblated using anti-GFP antibody. Analysis of cells transfected with EGFP-$\alpha_{1A}$wt revealed a band of 217 kDa. In contrast, immunoblots of cells transfected with the EGFP-$\alpha_{1A}tg^a$ long and EGFP-$\alpha_{1A}tg^a$ short constructs yielded bands that were slightly smaller (Figure 17). A non-specific band of 125 kDa was seen in all samples, including the mock transfected cells. In no case was a band corresponding to EGFP alone (27 kDa) observed. There were no major differences in the levels of protein between the mutant and wt $\alpha_{1A}$ subunits. If anything, the mutant proteins were expressed better than the wt.
Figure 17: Total protein expression from EGFP-α_{1A}wt, EGFP-α_{1A}tg^{a} long, and EGFP-α_{1A}tg^{a} short constructs co-transfected with the α_{2δ} and β_{1b} DNA in HEK tsA201 cells. Cell lysates were run on SDS-PAGE and probed with an antibody against GFP. The α_{1A}wt protein is approximately 217 kDa (190 kd plus 27 kDa for EGFP) and the α_{1A}tg^{a} long, and α_{1A}tg^{a} short proteins are slightly smaller (A). A non-specific band seen in all lanes of approximately 125 kDa was also present in the mock (B). There is no difference in the total amount of α_{1A}wt, α_{1A}tg^{a} long, and α_{1A}tg^{a} short proteins. If anything there is less α_{1A}wt. 70 and 105 µg protein was loaded for each transfection condition. Blots were detected by ECL (Methods).
4.2.4 Current Analysis of EGFP-Tagged $\alpha_{1A}^{\text{wt}}$, $\alpha_{1A}^{tg^{\text{a}}}$ long and $\alpha_{1A}^{tg^{\text{a}}}$ short Calcium Channels.

Whole cell patch clamp recordings were obtained by Dr. Xiaolei Zhang (Dr. Peter Carlen's laboratory, TWH) from HEK tsA201 cells transfected using LipofectAMINE reagent (section 3.2.2), with either EGFP-$\alpha_{1A}^{\text{wt}}$, EGFP-$\alpha_{1A}^{tg^{\text{a}}}$ long, or EGFP-$\alpha_{1A}^{tg^{\text{a}}}$ short, in combination with the auxiliary $\beta_{1b}$ and $\alpha_{2\delta}$ subunit vectors. At +20 mV, the EGFP-$\alpha_{1A}^{\text{wt}}$ channels produced currents of approximately -202 pA, the EGFP-$\alpha_{1A}^{tg^{\text{a}}}$ long channel currents were observed at -63 pA, and the EGFP-$\alpha_{1A}^{tg^{\text{a}}}$ short channel currents were at 14 pA. At +20 mV, the currents were approximately maximal. These results indicate a reduction in calcium currents in the $tg^{\text{a}}$ long and $tg^{\text{a}}$ short expressing cells as compared to the wt.

4.2.5. Subcellular Distribution of EGFP-Tagged $tg^{\text{a}}$ and wt Calcium Channels

The large difference in currents seen between cells transfected with $tg^{\text{a}}$ and wt channels is not matched by differences in total protein levels. In immunoblots of total cell lysate proteins, mutant and wild type channels are found at similar levels (see Section 4.2.3). One of the most plausible explanations for this observation is a reduction in the surface expression of $tg^{\text{a}}$ mutant channels due to mis-trafficking of the mutants. A comparison was therefore made of the subcellular distribution of $tg^{\text{a}}$ mutant and wt channels, exploiting the intrinsic fluorescence of the EGFP tag and high resolution imaging techniques. To facilitate the imaging, the EGFP-$\alpha_{1A}^{tg^{\text{a}}}$ long, EGFP-
α1Aδ short or EGFP-α1Awt constructs were transfected, in combination with the auxiliary subunits into COS-7 cells.

**4.2.5.1 Subcellular Distribution of Channels Bearing wild-type EGFP-α1A Subunits**

The pattern of fluorescence seen with the EGFP-α1Awt: α2δ: β1b transfectants was quite distinct from that obtained from cells transfected with ECFP-tagged subcellular localization vectors for the nucleus, ER or Golgi apparatus (Figure 18A). COS-7 cells transfected with the nuclear marker showed staining of the nucleus, the ER marker showed punctate fluorescence throughout the cytoplasm, and the trans-medial Golgi marker was more localized to the peri-nuclear region in a ring. In the EGFP-α1Awt: α2δ: β1b transfected cells, the fluorescence distribution was non-homogeneous and displayed a range of patterns and intensities. This was seen using both fluorescence imaging (Figure 18B (green)) and laser scanning confocal microscopy in conjunction with a 60X objective (Figures 19A, 19B, and 19C (green). Notable patterns included strongly fluorescent ribbons and puncta extending from the perinuclear region (2/15 cells-13%) and through the Golgi apparatus (5/15 of cells-33%). As well, most cells displayed EGFP fluorescence in both their cytoplasm (although the fluorescence did not normally fill the entire cytoplasm) and in a less intense fluorescence pattern towards the cell periphery (8/15 cells-53%). Some, but not all, of the EGFP-α1Awt: α2δ: β1b transfected cells exhibited a fluorescent halo around their margins. These data are consistent with a distribution of the wt channel complexes throughout the membranous structures of the secretory pathway including the plasma membrane.

To test this notion further, the distribution of the EGFP-α1Awt fluorescence was compared with that of the plasma membrane by labeling the cells with Texas Red-
Figure 18: Examples of Fluorescence Imaging: Nucleus, ER, Golgi, and EGFP-α_{1A}wt. (A) ECFP-tagged nuclear, ER and Golgi subcellular markers. B) EGFP-tagged α_{1A}wt constructs co-transfected with α_2δ and β_{1b} vectors. Texas Red wheat germ agglutinin was used to label the COS-7 cell surfaces and cells were then fixed and viewed using fluorescence microscopy. N: Nucleus. ER: Endoplasmic Reticulum.
Figure 19A. Example of Confocal imaging (EGFP-α_{1A}wt). EGFP-tagged α_{1A}wt constructs were co-transfected with α_{2δ} and β_{1b} vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Single slice from middle of cell.
Figure 19B. Example of Confocal imaging (EGFP-α1Awt). Another example of EGFP-tagged α1Awt subunits in COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Slice from middle of cell.
Figure 19C. Example of Confocal imaging (EGFP-α_{1A}wt). Another example of EGFP-tagged α_{1A}wt subunits in COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Slice from middle of cell.
conjugated wheat germ agglutinin (TxR-WGA). Since TxR-WGA is membrane impermeant, this fluorescent lectin should only interact with glycoproteins at the cell surface (under conditions that preclude internalization such as incubation on ice). Following treatment of the EGFP-α1Awt transfected cultures with TxR-WGA, fluorescence was observed over the entire cell surface (Figures 18B, 19A, 19B, 19C (red)). The highest levels of TxR-WGA fluorescence were seen at the cell periphery and occurred both as a ruffled pattern extending around its perimeter and on the processes. Away from the cell margins, the TxR-WGA fluorescence was less intense but still present, indicating that at least some TxR-WGA was internalized by the cells. The cell periphery though, was always clearly defined. From the above, it appeared that the pattern of fluorescence corresponding to the EGFP-α1Awt channels was similar in some parts, but not identical, to that seen with TxR-WGA. To explore this point further, the extent of any fluorescence overlap was ascertained by merging the two image sets (Figures 18B, 19A, 19B, 19C (merge)). Based on this comparison, the fluorescence that was seen near the cell surface emanating from cells transfected with the EGFP-α1Awt channels resembled the distribution of the TxR-WGA plasma membrane marker, but there was only limited overlap. The regions of high, punctate or ribbon-like EGFP fluorescence corresponded poorly with any surface labeling and were clearly intracellular. Fluorescence was absent in images of EGFP-α1Awt transfectants captured using conditions for excitation of TxR-WGA (and vice-versa), thus, excluding the possibility that the EGFPα1A-wt channel fluorescence arose through “bleed-through”. This point was further underscored by the absence of EGFP fluorescence in many of those regions where the TxR-WGA fluorescence was high (or vice-versa).
Figure 19D. Confocal Slices of cell from Figure 19C. EGFP-tagged $\alpha_{1A}^{wt}$ constructs were co-transfected with $\alpha_\delta$ and $\beta_{1b}$ vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. 16 slices were taken for 3D projections. Z-series images were taken starting at the bottom of the cell and moving to the top of the cell. 0.5μM increments were used in the z-series collections.
Further confirmation of the membrane disposition of the EGFP-α₁Awt channels was obtained by using laser scanning confocal microscopy to collect images (XY optical sections) at different depths (Z-axis positions) through the cell. The resulting image stack could then be displayed as a consecutive series of images or used as the basis for more sophisticated processing, notably, image enhancement through deconvolution, surface rendering, and the generation of 3D images. Consecutive images from a stack obtained from transfected COS-7 cells expressing EGFP-α₁Awt channels are shown in Figure 19D. Here, images were collected in tandem, with the left-hand image corresponding to the EGFP signal and the right-hand image corresponding to the cell membrane outlined with TxR-WGA. Images corresponding to the top of the cell showed limited EGFP fluorescence emanating from the cell surface. However, z-series images taken at various depths within the cell displayed EGFP fluorescence with a pronounced halo pattern. Images corresponding to the bottom of the cell were similar to those from the top surface and did not exhibit a halo pattern.

Together, the above imaging data indicate that cells transfected with constructs encoding EGFP-α₁Awt, α₂δ and β₁b express EGFP-α₁Awt channels that are located in vesicular structures within the cell and approaching the cell surface.

4.2.5.2 Subcellular Distribution of Channels Bearing tg/α mutant EGFP-α₁A

Subunits

In cells co-transfected with EGFP-α₁A tg/α long, α₂δ and β₁b constructs, the fluorescence appeared to be heterogeneous and mainly localized to the Golgi (7/15
Figure 20: Three Examples of Fluorescence Imaging of EGFP-α1Agtα long. EGFP-tagged α1Agtα long constructs were co-transfected with α2δ and β1b vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surfaces. Cells were then fixed, and viewed under 40X using fluorescence microscopy.
Figure 21A. Example of Confocal imaging (EGFP-α₁A,tg²a long). EGFP-tagged α₁A,tg²a long constructs were co-transfected with α₂δ and β₁b vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Slice from middle of cell.
Figure 21B. Example of Confocal imaging (EGFP-α_{1A}tg/a long).
Another example of EGFP-tagged α_{1A}tg/a long subunits in COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil.
I: 3D projection. II: Slice from middle of cell.
cells-47%), and peri-nuclear (6/15 cells-40%), regions. Most of the EGFP fluorescence appeared in punctate patches or ribbons and as above, was deemed to be intracellular, based on images obtained by labeling the plasma membrane with TxR-WGA (Figures 20, 21A, 21B). These double-labeling studies also indicated some overlap between the less intense EGFP and TxR-WGA fluorescence, suggesting that at least some channels might be present at the cell surface (2/15 cells-13%). Nevertheless, the halo-like fluorescence distribution, found in some of the cells transfected with EGFP-α₁₅wt channels, was not observed. To explore this point further, the fluorescence distribution was examined by collecting cell slices at higher magnification by confocal imaging. Images were collected in tandem, with the left-hand image corresponding to the EGFP signal and the right-hand image corresponding to the cell membrane outlined with TxR-WGA. Throughout the series, the EGFP fluorescence was most pronounced in peri-nuclear regions (Figure 21C). Nevertheless, there were several sections where an overlap between plasma membrane and EGFP fluorescence was indicated although a halo-like pattern of fluorescence at the cell margins could not be discerned. Together, these data argue that cells transfected with constructs encoding EGFP-α₁₅tg long, α₂δ and β₁₅ express channels that have similar, but not identical dispositions compared to wild type channel complexes. Specifically, Tg long channels are largely, but not entirely, located intracellularly.

The most profound differences were found with cells co-transfected with EGFP-α₁₅tg short, α₂δ and β₁₅ constructs. Their fluorescence distribution diverged considerably from that seen with cells expressing wt or Tg long channels. In every case, the bulk of the fluorescence was found in ribbons (6/17 cells-35%) or patches of high intensity (11/17 cells-65%) in close proximity to the nucleus (Figure 22, 23A, 23B).
Figure 21C. Confocal slices of cell from Figure 21B.
EGFP-tagged \( \alpha_{1A}^\text{tg} \) long constructs were co-transfected with \( \alpha_2\delta \) and \( \beta_{1b} \) vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. 16 slices were taken for 3D projections. Z-series images were taken starting at the bottom of the cell and moving to the top of the cell. 0.5\( \mu \text{M} \) increments were used in the z-series collections.
Figure 22: Three Examples of Fluorescence Imaging of EGFP-α1A\textsuperscript{tg} short. EGFP-tagged α1A\textsuperscript{tg} short constructs were co-transfected with α2δ and β\textsubscript{1δ} vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surfaces. Cells were then fixed, and viewed under 40X using fluorescence microscopy.
Figure 23A. Example of Confocal imaging (EGFP-\(\alpha_{1A}tg^{\alpha}\) short). EGFP-tagged \(\alpha_{1A}tg^{\alpha}\) short constructs were co-transfected with \(\alpha_{2\delta}\) and \(\beta_{1b}\) vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Slice from middle of cell.
Figure 23B. Example of Confocal imaging (EGFP-α_{1A}tg^{a} short). Another example of EGFP-tagged α_{1A}tg^{a} short subunits in COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surface and cells were fixed and viewed under 60X oil. I: 3D projection. II: Slice from middle of cell.
Figure 23C. Confocal Slices of cell from Figure 23B. EGFP-tagged $\alpha_{1A^{\prime} C/1C}$ short constructs were co-transfected with $\alpha_2\delta$ and $\beta_1\nu$ vectors into COS-7 cells. Texas Red wheat germ agglutinin was used to label the cell surfaces and cells were fixed and viewed under 60X oil. 16 slices were taken for 3D projections. Z-series images were taken starting at the bottom of the cell and moving to the top of the cell. 0.5$\mu$M increments were used in the z-series collections.
A halo of fluorescence at the cell periphery was not observed. Double labeling with TxF-WGA indicated that most fluorescence was intracellular. There was no real overlap between the EGFP and the TxF-WGA fluorescence at the cell surface, suggesting that these channels did not make it to the cell surface. A similar picture emerged in confocal slices obtained using the 60X objective. Throughout each confocal series, the EGFP fluorescence was most pronounced in perinuclear regions (Figure 23C). Moreover any fluorescence emanating from the peripheral cytosol appeared to be largely located in vesicular structures. There was no evidence for channels at the plasma membrane. Together, these data argue that cells transfected with constructs encoding EGFP-\(\alpha_{1A}tG^{\alpha}\) short, \(\alpha_{2}\delta\) and \(\beta_{1b}\) constructs express channels that are restricted to intracellular structures.
5 Discussion

5.1 Summary of Results

In this thesis, numerous complimentary approaches have been used to examine the hypothesis that the $t_g^{ia}$ mutation in the $\alpha_{1A}$ subunit of P/Q-type calcium channels causes changes in the expression or function of P/Q-type VDCCs. Analysis of $[^{125}]\omega$-CTX MVIIIC toxin binding to native P/Q-type channels in mouse cortical membranes revealed a three-fold reduction in the number of binding sites in $t_g^{ia}$ versus wt with no change in binding affinity. These data are compatible with quantitative immunoblotting data that also showed a three-fold reduction in the levels of $\alpha_{1A}$ subunits in $t_g^{ia}$ versus wt cortical membranes. To further probe the basis for such changes, EGFP-tagged DNA constructs corresponding to the $t_g^{ia}$ long and $t_g^{ia}$ short mutations were prepared and expressed in a heterologous cell culture system. Although no major difference in the expression levels of $t_g^{ia}$ wt, $t_g^{ia}$ long, or $t_g^{ia}$ short $\alpha_{1A}$ subunits was found in immunoblots of whole cell lysates, marked differences were seen in currents detected by whole cell patch-clamp electrophysiology. While P/Q-type currents were readily detected in cells expressing wt channels, those expressing channels containing $t_g^{ia}$ long or $t_g^{ia}$ short $\alpha_{1A}$ subunits were reduced 3-4 fold or were absent, respectively. Analysis of images obtained through fluorescence or confocal microscopy showed differences in the subcellular locations of the mutant channels versus wt in transfected cells, with $t_g^{ia}$ short P/Q-type channels showing an almost entirely perinuclear distribution.
5.2 Alterations in \( tg^{\alpha} \) and wt \( \alpha_{1A} \) Subunit Expression in Cortical Membranes

In this study, immunoblots of cortical membranes revealed a 3-fold decrease in the expression of \( tg^{\alpha} \alpha_{1A} \) compared to wt protein. Although the main wt and two \( tg^{\alpha} \alpha_{1A} \) proteins had molecular weights somewhat lower than those predicted from their corresponding cDNA sequences (Starr et al., 1991; Fletcher et al., 1996), this finding is consistent with results from our laboratory and others and has been attributed to the anomalous migration of \( \alpha_{1} \) subunits in polyacrylamide gels (De Jongh et al., 1991; Sakurai et al., 1995). In spite of these migration artifacts, the molecular weights of the two \( tg^{\alpha} \) and major wt \( \alpha_{1A} \) proteins exhibited the predicted size differences. A second observation is the existence of multiple minor bands of lower molecular weight in the rat and wt mouse membranes. While such bands could have resulted from proteolysis, every attempt was made to keep the samples at 4°C, and a cocktail of protease inhibitors was included during every membrane preparation and solubilization step. Consequently, it seems more likely that, as reported elsewhere, these bands can be attributed to the existence of numerous \( \alpha_{1A} \) mRNA splice variants differing in their II-III loops and cytoplasmic regions (Mori et al., 1991; Sakurai et al., 1995; Sakurai et al., 1996; Scott et al., 1998; Bourinet et al., 1999) and isoforms arising through post-translational processing (Scott et al., 1998). Since alternatively spliced isoforms of \( \alpha_{1A} \) vary in their inactivation kinetics (Soong et al., 1994), and sensitivities to phosphorylation (Sakurai et al., 1995), it is plausible that each isoform performs a specific functional role. The differential expression and regulation of multiple isoforms of other \( \alpha_{1} \) subunits suggests that this may be a general phenomenon (Lai et al., 1990; De Jongh et al., 1991; Snutch et al., 1991; Westenbroek et al., 1992; Hell et al., 1994; Hell
et al., 1995). Interestingly, considerably fainter, minor bands were also present in the $tg^{ia}$ membranes and could also represent additional mutant splice variants. One alternative explanation for the multiple bands detected on the Western immunoblots is that they are due to cross-reactivity caused by non-selective binding of the $\alpha_{1A}$ antibody to other unrelated proteins that contain sequences similar to those found in $\alpha_{1A}$.

Independent confirmation of a reduction in $\alpha_{1A}$ expression in $tg^{ia}$ versus wt cortical mouse membranes comes from the [$^{125}$I]-$\omega$-CTX MVIIC binding assays. Such assays were chosen for their selectivity and their convenience, especially compared to electrophysiological methods (that would have required live brain slices and thus breeding of $tg^{ia}$ mice). Moreover, since MVIIC-P/Q-type channel interactions are conformation-dependent and are affected by the level of glycosylation (Martin-Moutot et al., 1995), binding assays are a better index of native P/Q-type channel expression than immunoblotting. While MVIIC binding assays have been used to characterize P/Q-type channels at a biochemical level (Liu et al., 1996), they have not been used to examine P/Q-type calcium channel expression in $tg^{ia}$ mice.

Assuming a single class of binding site, the values obtained for the affinity of [$^{125}$I]-$\omega$-CTX MVIIC binding to rat brain membranes and the number of available sites ($B_{max}$) are similar to those reported elsewhere (Liu et al., 1996). Unfortunately, the assumption of a single class of sites requires some justification. Studies in other laboratories have provided evidence for a second class of binding sites in nerve membranes thought to arise through the interaction of MVIIC with N-type calcium channels (Liu et al., 1996). Although the reported binding affinity of MVIIC for such sites is over eleven-fold lower (Kd approximately 4 nM) than that found for its binding to the P/Q-type channels (Liu et al., 1996), if sufficiently dense, they could contribute to the
binding data, and complicate its interpretation. Unfortunately, determining the density of low affinity sites in the presence of high affinity sites is difficult as binding occurs preferentially to the latter (Feldman et al., 1972). A direct analysis of binding in rabbit brains suggests P/Q-type calcium channels represent over 90% of the high affinity MVIIC binding sites (Liu et al., 1996). Consequently, these values provide a range over which it is possible to predict the contribution that binding to N-type calcium channels would have on the MVIIC binding curves and more specifically on the Kd and B_max of the P/Q-type calcium channel binding sites. Using this approach, it is evident that any low affinity N-type calcium channel binding sites should have a minimal effect on either the Kd or B_max for MVIIC binding to the P/Q-type channels over the concentration range employed. But despite this up to 11-fold difference in affinity between ω-CTX MVIIC binding to P/Q-type as compared to N-type calcium channels, because N-type calcium channels are found in the cerebral cortex (Westenbroek et al., 1992), to be certain that ω-CTX MVIIC binding as presented here reflects just P/Q-type calcium channel binding, one could include ω-CTX GVIA, an N-type VDCC specific pore blocker (Ellinor et al., 1995) into the binding reaction mixture. Since ω-CTX MVIIC also acts as a pore blocker (S.I. McDonough, Soc. Neuroscience Abstract 21, 140.9, 1995), ω-CTX MVIIC binding to N-type calcium channels should be occluded in the presence of ω-CTX GVIA, and any N-type calcium channels would not contribute to the MVIIC binding results. A final point regarding the validity of the single site model is that it not known for certain if N-type calcium channel expression is equal in wt and tg^a mice. For example, cells in the tg^a mouse may compensate for the decrease in P/Q-type VDCC expression with an increase in N-type calcium channels that have a low affinity for ω-CTX MVIIC. Again, by
including the N-type calcium channel blocker ω-CTX GVIA the binding of N-type calcium channels to ω-CTX MVIIC would be eliminated.

If a single site model is valid, it is clear that the tg\(\text{ia}\) cortical membranes show a three-fold reduction in MVIIC binding when compared to their wt counterparts. This reduction in binding appears to be due to a decrease in the number of available binding sites (\(B_{\text{max}}\)) rather than any change in toxin binding affinity (Kd). Thus, the MVIIC binding data closely mirror the Western immunoblot data, with both methods demonstrating a decrease in \(\alpha_{1A}\) subunits and P/Q-type calcium channel levels in the leaner versus wt cortex.

5.3 Using a Model System to Resolve the Mechanisms Underlying Expression of the tg\(\text{ia}\) Phenotype

There are several major problems in resolving the mechanisms underlying the tg\(\text{ia}\) phenotype. As shown above, the P/Q-type calcium channels are complex proteins. Even if one were to ignore the diversity arising through heteromerization with different auxiliary subunits, the native \(\alpha_{1A}\) subunit exists in multiple forms as a result of alternative splicing and post-translational modification. This situation is even more serious with the tg\(\text{ia}\) P/Q-type channels where aberrant splicing generates two major RNA species tg\(\text{ia}\) long and tg\(\text{ia}\) short, and possibly several additional minor forms as well.

To preclude the interpretational difficulties intrinsic to a comparison of membranes from wt and tg\(\text{ia}\) mouse brains, the approach adopted involved analysis of EGFP-tagged tg\(\text{ia}\) proteins in a model heterologous cell expression system. Such a
reductionist approach has numerous merits - in particular, its potential applicability to subcellular distribution studies. Traditionally, such experiments have been based on 'snapshots' of protein distributions determined through immunocytochemistry in fixed cells. In contrast, EGFP technology introduces the possibility of continuously imaging the distribution of proteins in live cells (Presley et al., 1997).

5.3.1. Expression of $tg^{la}$ and wt $\alpha_{1A}$ Subunits in Heterologous Cells

Immunoblots of HEK tsA201 cell lysates transfected with EGFP-tagged wt or $tg^{la}$ channels yield a single major band for wt, $tg^{la}$ long, or $tg^{la}$ short $\alpha_{1A}$ and are therefore considerably less complex than wt or $tg^{la}$ channels present in brain cortical membranes. Since the expressed $\alpha_{1A}$ subunits are encoded by single cDNAs (EGFP-tagged rbA-1 isoform or the engineered $tg^{la}$ derivatives), these data support strongly the earlier contention that the multiple bands seen in immunoblots of brain membranes arise through the translation of diverse transcripts generated by alternative splicing of brain RNA, rather than protein degradation. Moreover, as the molecular weights of the major bands seen with HEK tsA201 cells mirror those seen with the brain membranes, it seems likely both wt and $tg^{la}$ $\alpha_{1A}$ subunits experience similar processing events. In contrast to brain membranes, the mutant $\alpha_{1A}$ subunits are expressed as well as or slightly better than the wt subunits in transfected HEK tsA201 cell lysates. Possible reasons for the difference between cortical membranes and transfection experiments will be discussed below.

Support for a reduction in leaner $\alpha_{1A}$ subunits compared to wt comes from whole cell recordings of $tg^{la}$ and wt $\alpha_{1A}$ channels expressed in the HEK cells. The experiments
conducted by Dr. Xiaolei Zhang showed that whole cell currents obtained by expression of $tg^{ia}$ long channels are substantially reduced, while those from $tg^{ia}$ short channels are absent. These data are clearly consistent with previous examinations of whole Purkinje cell currents which showed major (>65%) reductions in $\alpha$-agatoxin-IVA currents in acutely dissociated Purkinje cells of $tg^{ia}$ versus wt mice (Dove et al., 1998; Wakamori et al., 1998; Lorenzon et al., 1998). While there are several explanations for the reduction in current densities in the $tg^{ia}$ Purkinje cells, they do not seem to involve large changes in the macroscopic properties of the currents such as the kinetics and voltage-dependence of current activation or inactivation (Dove et al., 1998; Lorenzon et al., 1998). To provide further insights, several researchers have therefore examined the properties of the individual channels underlying the macroscopic currents. The whole-cell current $I$ is the product of the single-channel current $i$, the number of functional channels $N$, and the open probability of these channels, $P_o$. Thus, changes in any of these parameters could contribute to the reduced currents seen in the $tg^{ia}$ cells. In single channel recordings three (slope) conductance levels were detected of 9, 13, and 19 pS in wt, and 9, 13 and 18 pS in $tg^{ia}$ Purkinje neurons (Dove et al., 1998). These numbers are in agreement with the 9, 14, and 19 pS conductances described previously for native P-type calcium currents in Purkinje cells (Usowicz et al., 1992). Similarly, no change was found in the mean open time of the channels. However, a three-fold reduction in the patch open-probability ($NP_o$) was found between the $tg^{ia}$ versus wt Purkinje cells. Such differences were seen even before the onset of major neurodegeneration, which begins to occur at day 40 (Dove et al., 1998) and can be attributed to a lower frequency of channel openings or a decrease in the numbers of functional channels (Dove et al., 1998). While a lower frequency of channel opening is possible, the higher frequency of inactive patches from $tg^{ia}/tg^{ia}$ Purkinje cells strongly
supports the possibility of reduced functional channels on the membrane. Precisely which of the $tg^{ia}$ products, short or long, underlies the reduction in current density cannot readily be resolved in recordings from the Purkinje neurons but can be discerned using the heterologous expression system described here. As seen, both the long and short forms of the $tg^{ia}$ P/Q-type calcium channels display reduced currents compared to wt channels. However, this loss of current is much more pronounced for the $tg^{ia}$ short versus long form. Therefore, the long form may account for most of the calcium current observed in leaner Purkinje cells. These results can be contrasted with those reported by Wakamori et al. (1998) while this work was in progress. In their study, a hybrid construct comprised of the rabbit $\alpha_{1A}$ and $tg^{ia}$ genomic carboxy terminal DNA was expressed in BHK cells, allowing the BHK cells to perform the splicing events and therefore generate each mutant. In this instance, the $tg^{ia}$ long channels had a current density comparable to the normal control, although there was a shift in the voltage-dependence of activation and inactivation. The $tg^{ia}$ short channel, in contrast, showed a significant reduction in current density. Why these workers found little reduction in current with $tg^{ia}$ long channels is unclear but could reflect differences in the host cells, the sequences of the rabbit and rat $\alpha_{1A}$ subunits (which are dissimilar in 8% of their overall amino acid sequence) (Sakurai et al., 1995) or BHK cell splicing events (Berrow et al 1997). Irrespective of the magnitude of the current reduction seen with the $tg^{ia}$ long form, it is clear that $tg^{ia}$ short channel current is severely impaired, compared to wt. Since all the recordings for this thesis were made using cells which showed EGFP fluorescence, these differences cannot be simply attributed to an absence of expression of the short (or long) forms. Based on the electrophysiology experiments conducted by Dr. Xiaolei Zhang for this thesis, it is not yet clear if the reductions in currents from the $tg^{ia}$ long and $tg^{ia}$ short transfected cells are the result of decreases in the number of
mutant channels at the cell surface. It is possible that the number of \( tg^{a} \) long and \( tg^{a} \) short channels at the plasma membrane is approximately equal to \( wt \), but that the mutant channels do not function properly. Experiments examining whole cell channel kinetics and single channel properties of the \( wt \) and mutant channels would help determine if the observed decreases in currents in the mutant-transfected cells are caused by reductions in the cell surface expression of mutant channels rather than changes in channel properties.

Semi-quantitative image analysis indicates that the levels of \( \alpha_{1A} \) subunits at the plasma membrane are \( tg^{a} \) short \(<< \) \( tg^{a} \) long \(< \) \( wt \). Determining the magnitude of these differences requires a detailed quantitative analysis that is beyond the scope of this M.Sc. thesis, although my collection of 3D data sets should greatly facilitate such an approach. Interestingly, even the \( wt \) channels showed little cell surface expression as determined by EGFP and TxA-WGA overlap. Based on the electrophysiology, which demonstrated robust currents in \( wt \)-transfected cells, one would expect to see \( wt \) channels at the cell surface. It should be noted that the electrophysiology was performed in HEK tsA201 cells whereas the imaging was done in COS-7 cells. Researchers have examined the expression and function of wild type P/Q calcium channels (\( \alpha_{1A}, \alpha_{2}\delta \) and \( \beta_{1b} \)) in COS-7 cells (Berrow et al., 1997; Brice et al., 1997) using both electrophysiology and imaging, and found that \( wt \) channels are expressed at the cell surface (Berrow et al., 1997; Brice et al., 1997).

As the overall expression of the \( tg^{a} \) short and \( tg^{a} \) long subunits is not reduced, it is pertinent to ask where these channels are since they are not at the cell surface. Any re-distribution of the \( tg^{a} \) short and \( tg^{a} \) long subunits should be resolvable in the EGFP images. In practice, this can be quite an involved process, requiring consideration of
several factors. Perhaps the most common observation with EGFP-tagged plasma membrane proteins is the existence of a halo of fluorescence around the cell margins. However, the lack of such a pattern does not necessarily indicate the absence of the protein at the plasma membrane. Interestingly, both the wt and mutant channels exhibited high levels of intracellular EGFP fluorescence. One possible explanation is that the majority of channels are contained in large intracellular pools and therefore only a proportion is found at the plasma membrane. This has been demonstrated for both P/Q-, L-, and N-type calcium channels (Passafaro et al., 1996; Brice and Dolphin, 1999). Second, the distribution of membrane protein in a cell is thought to be highly dynamic. The clearest picture of the distribution of membrane proteins in the secretory pathway comes from studies by Lippincott-Schwartz and colleagues using a GFP-tagged chimera of the vesicular stomatitis virus glycoprotein (GFP-VSVG). These studies show that trafficking in the secretory pathway is highly active, involving the movement of vesicular intermediates to and from the cell surface (Presley et al., 1997; Hirschberg et al., 1998). Differences in protein distribution can therefore be rationalized in terms of their residence times in each locale. Such studies also show that the flux of proteins in the secretory pathway is rapid compared to the lifetime of the protein, so live fluorescent images can be regarded as reflecting a “steady state” distribution, even when collected a few hours after transfection. Consequently, it is not strictly accurate to describe membrane proteins as being residents of a specific compartment, such as the plasma membrane. Rather, the protein is likely to be distributed in multiple compartments. Focusing on the \( \text{tg}^{\alpha} \) short channel, where the changes are most marked compared to the wt, it seems likely that the \( \text{tg}^{\alpha} \alpha_{\text{1A}} \) short protein is localized to a perinuclear region that is distinct from the ER or Golgi.
5.4 Possible Mechanisms Underlying the $t^A$ Phenotype

Taken together, while the cortical membrane studies demonstrated a three-fold reduction in both $\alpha_{1A}$ expression and MVIIC P/Q-type calcium channel binding sites, the transfected cell experiments indicate no real reduction in $t^A \alpha_{1A}$ subunit expression in transfected cells while confirming the reduction in currents seen in the Purkinje cells of $t^A$ mice. In addition, cells expressing wt and $t^A$ EGFP-$\alpha_{1A}$ proteins show differences in their channel distributions that cannot be resolved in immunoblots of whole cell lysates. How then can the data be rationalized? The experiments using cortical membranes clearly indicate a reduction in the number of P/Q-type calcium channels due to a decrease in the amount of $\alpha_{1A}$ protein in the $t^A$ cortex. Such a decrease in expression could result from reduced protein stability, increased protein degradation, decreased mRNA stability, or a combination of these (Haardt et al., 1999). Some of the most detailed mechanistic studies on the effects of gene mutations have been made on the cystic fibrosis transmembrane regulator (CFTR). For example, mutations that result in the truncation and premature termination of the C-terminus of the CFTR protein produce a mature protein that is less stable and is quickly targeted for degradation. These experiments emphasize that mutations can decrease the expression of proteins at the plasma membrane not only by impairing their biogenesis, but also by accelerating their degradation (Haardt, et al., 1999). Although the underlying mechanisms are far from clear, the role of the C-terminus in different aspects of proper folding and trafficking of proteins appears to manifest at several levels, including the presence of targeting signals for degradation. Studies suggest that the $\beta$ subunits of P/Q-type VDCCs interact with the $\alpha_{1A}$ C-terminus and that such interactions are likely to be severely disrupted in
the $tg^a$ mutant channels with consequent exposure of undesirable residues (e.g. degradation signals) (Walker et al., 1998). Interestingly, a failure of VDCC $\alpha_1$ subunits to assemble with $\beta$ subunits at the I-II linker region is now known to expose an ER retention signal that may also trigger $\alpha_1$ subunit degradation through a “quality control” mechanism by the ER (Bichet et al., 2000). The immunoblotting and binding data obtained from the $tg^a$ mice in this thesis, as well as the electrophysiological data described elsewhere, suggest that the $tg^a$ mutation leads to a reduction in $\alpha_{1A}$ expression. How then can these findings be reconciled with the experiments performed in cell lines that showed a decrease in calcium currents but no change in the amount of $\alpha_{1A}$ protein? One possible explanation may be related to how nonendogenous, overexpressed protein can be processed by the degradative machinery in a transfected cell (Kopito, 2000). In murine neurons, the mutant $tg^a$ $\alpha_{1A}$ subunits may be rapidly and effectively degraded because of their native expression at relatively low levels. While the mutation in the $tg^a$ $\alpha_{1A}$ subunits may target them for degradation in a heterologous system, they may not be degraded as efficiently in this context. Studies have demonstrated that the degradation machinery in heterologous systems can become saturated by the expression of incorrectly folded proteins caused by mutations, or unstable proteins that have been targeted for degradation, or simple protein overexpression (Johnston et al., 1998). This could explain why there was no decrease in the amount of $tg^a$ $\alpha_{1A}$ long and short proteins relative to the wt form in transfected cells.

A well-studied example of inefficient in vitro protein degradation is the $\Delta 508F$ CFTR mutant (Johnston et al., 1998). Most proteins in a cell are degraded through the
ubiquitin-proteasomal pathway, in which proteins are first marked for destruction through the covalent attachment of multiple ubiquitin molecules and are subsequently degraded by a multi-protein complex known as the proteasome (Ciechanover et al., 2000). Under basal conditions in cell lines such as HEK and COS-7, proteasomes, ubiquitin and certain cell stress chaperones concentrate at the centrosome (also referred to as the microtubule organization centre), which is located perinuclearly, surrounded by ER and adjacent to the Golgi apparatus (Wigley et al., 1999). In cells expressing the Δ508F CFTR folding mutant, which is largely retained in the ER and rapidly degraded by proteasomes, this structure recruits additional proteasomes, ubiquitin and cell stress chaperones from cytoplasmic pools, becomes ensheathed in vimentin and expands four times in size (Johnston et al., 1998; Wigley et al., 1999). This enlarged structure was called an "aggresome" because it contained large aggregates of undegraded, misfolded Δ508F CFTR protein (Johnston et al., 1998). Aggresomes are formed when a cell’s capacity to degrade misfolded proteins is exceeded. Aggresome formation is not seen in vivo because the protein is normally effectively degraded (Ward et al., 1995). Aggresome formation was also observed when cells expressing wt CFTR or presenilin-1 were treated with proteasomal inhibitors, and when cells overexpressed a cytosolic GFP chimera and the P205S CFTR folding mutant, hinting that this may be a general cellular response to the presence of misfolded or overexpressed proteins. Both ubiquitinated and non-ubiquitinated proteins can form aggresomes (Johnston et al., 1998; Garcia-Mata et al., 1999; Wigley et al., 1999). Perinuclear location of the aggresome and its proximity to the Golgi apparatus can lead to the mistaken impression that proteins contained within aggresomes are located in the Golgi (Johnston et al., 1998).
Images obtained for the $tg^{la}$ long P/Q-type channel show its localization to other regions of the cell besides the perinuclear region, suggesting that only a fraction of this protein may be located in aggresomes. A recent study examining the cell surface expression of $\Delta 508F$ CFTR found an increase in the rate of internalization and/or targeted degradation of mutant proteins that make it to the plasma membrane (Heda et al., 2000). The $tg^{la}$ long channel could follow a similar pathway since some currents were detected with this mutant, indicating that a proportion of the $tg^{la}$ long calcium channels do reach the cell surface. Biotinylation studies examining the rates of internalization would be useful to resolve this issue. Although biotinylation experiments were attempted for this thesis (data not shown), the large size and highly labile nature of $\alpha_{1A}$ VDCC subunits necessitates further optimization. In contrast, recombinantly expressed $tg^{ia}$ short channels produced no inward currents, suggesting that they may be immediately targeted for degradation. This notion is supported by the imaging data, which shows a perinuclear localization for most $tg^{ia}$ short channels and therefore a possible aggresomal localization. Since the chance of aggregate formation increases with decreased protein stability (Wickner et al., 1999), the $tg^{ia}$ short protein may be less stable because of its largely truncated C-terminus (Fletcher et al. 1996). Pulse-chase experiments could be conducted to determine rates of mutant and wt $\alpha_{1A}$ protein degradation. Confirmation of aggresome formation would come from double-labeling experiments using markers specific for aggresomes, such as vimentin (IF (Intermediate filament) protein) (Johnston et al., 1998) or $\gamma$-tubulin (centrosomal marker) (Wigley et al., 1999). Density gradient fractionation could also be used to determine the exact subcellular location of the mutants in the transfected cells.
Another reason for the differences in expression between the $\alpha_{1A}$ subunit in cortical membranes and the transfected cells may be related to the use of the powerful cytomegalovirus promoter in EGFP-C2 vectors in the cell line experiments. Such vectors are important for expression of the GFP gene in mammalian cells (Cubitt et al. 1995). Overexpression of EGFP-tagged proteins in the past has been shown to result in saturation of the cellular degradation machinery and mistargeting of the expressed fusion proteins (Girotti and Banting, 1996). Such saturation could have occurred for both the wt and $tg^a$ channels in my experiments, leading to an overload of the degradation machinery and apparent equal expression of wt and mutant proteins on the Western immunoblots. Overproduction of protein would not occur in native tissue.

Another possible reason that may partially explain my results is that the mutant $\alpha_{1A}$ proteins are not targeted for degradation but rather, are trapped along the secretory pathway and are not trafficked properly to the cell membrane. Sequences in the C-termini of the closely related $\alpha_{1C}$ and $\alpha_{1S}$ subunits are known to be required for their targeting to the cell membrane (Gao et al., 2000; Proenza et al., 2000). The cell line studies in this thesis could be interpreted this way, since there was no change in protein levels between the wt and mutant channels in whole cell lysates even though there was a decrease in the currents. But the cortical membranes clearly show a decrease in $\alpha_{1A}$ protein levels in the $tg^a$ cortex versus wt. If the mutant proteins were trapped along the secretory pathway in either the ER or Golgi apparatus, they should still be detected in the cortical membrane preparations used in my thesis.

The cortical membrane results that I have obtained could also be explained as resulting from a decrease in the stability of the $tg^a$ mutant transcripts. Such a decrease
in \(tg^{ja}\) mRNA does not correlate well with the cell expression studies that were performed for this thesis. If the mutant mRNAs were degraded at a faster rate, then there would be no accumulation of protein. Two studies have examined the expression of leaner mRNA. Doyle et al. (1997) found a decrease in \(tg^{ja}\) mRNA whereas both Fletcher et al. (1996) and Lau et al. (1998) found no change in leaner mRNA levels. To confirm that \(tg^{ja}\) mRNA levels are not reduced, the rate of biosynthesis of the two mutant proteins and the rates of mRNA degradation could be examined.

Finally, the decrease in \(\alpha_{1A}\) expression in the \(tg^{ja}\) cortical membranes may be a result of selective cytotoxicity. When a protein is detected on an immunoblot or by binding assays, the results obtained reflect the expression of the protein in all cell types that are present in the preparation. If selective neuronal cell loss occurred in the \(tg^{ja}\) mouse cortex as is seen in the cerebellum, where cell death occurs in only a subset of cells and is non-uniform (Sidman et al 1965; Meier and Mackpike, 1971; Heckroth and Abbott, 1994, Abbott et al., 1996; Fletcher et al., 1996), it could explain the decrease in \(\alpha_{1A}\) and P/Q-type calcium channel expression in the \(tg^{ja}\) cortical membranes. To verify this, immunoblots of different cortical neurons could be probed with antibodies directed at neuronal markers, whose expression would be compared to \(\alpha_{1A}\) subunit expression. A decrease in both the neuronal marker and the \(\alpha_{1A}\) subunit would indicate cell loss rather than a specific reduction in \(\alpha_{1A}\) subunit expression. Interestingly, as mentioned earlier, in the cerebellum there are alterations in channel function at the single channel level with decreases in \(NP_{o}\), indicating that in addition to any observed cell loss, the channels that are expressed are not functioning properly (Dove et al., 1998).
Electrophysiological recordings on cortical neurons would determine if there are changes to cortical neuronal functions in tg<sup>a</sup> mice.

5.5 Significance of These Results for Human Pathology

A final issue concerns the potential significance of these studies. Although the murine phenotypes are not strictly related to human diseases (Weinreich and Jentsch, 2000), they can still be extremely useful in shedding light on diverse neurological disorders in humans. Owing to the severe cellular abnormalities seen in the tg<sup>a</sup> cerebellum, and the extreme motor deficiencies displayed by these animals, the tg<sup>a</sup> mouse is widely described as a cerebellar neurological mutant (Sidman et al., 1965; Meier and MacPike, 1971). Nevertheless, the tg<sup>a</sup> mouse also exhibits absence-like seizures (Fletcher et al., 1996; Lau et al., 1998) and that suggests involvement of neurons in non-cerebellar regions. The manifestation of dysfunction in such regions is certainly not unexpected, since α<sub>1A</sub> subunits are the most abundant α<sub>1</sub> subunit in the vertebrate brain (Mori et al., 1991), are expressed in many brain regions and are the major route of voltage-dependent calcium influx required for neurotransmitter release (Dunlap et al., 1995; Catterall, 1998). Studies on the tg<sup>a</sup> mutation can therefore also provide insights into deficits in cortical function.

One area where a study of the tg<sup>a</sup> phenotype could be particularly important is in human epilepsy. Almost half of all epilepsies known to have an important genetic component are thought to exhibit a multigenic profile. The identification of single mutations in mice has been a powerful tool in furthering an understanding of the role that genetics play in these more complex human epilepsies (Fletcher et al., 1996). But how could a reduction in P/Q-type calcium channels, and decreased currents, lead to
absence epilepsy? It is thought that absence epilepsy may result from cortical hyperexcitability caused by increased T-type calcium channel conductance (Tsakiridou et al., 1995; Fletcher et al., 1996; Danober et al., 1998). It is possible that in the tottering leaner mouse the loss of $\alpha_{1A}$ expression and P/Q-type calcium channels results in compensation and overexpression of the T-type VDCC, leading to epilepsy. Overcompensation occurs with the lethargic mouse which carries a mutation in its $\beta_4$ subunit, displays decreased $\beta_4$ subunit expression, and compensates by overexpressing the $\beta_{1b}$ subunit (McEnery et al., 1998). Therefore one area worth examining is the expression of T-type calcium channels in the $tg^{ia}$ mouse cortex, as compared to wt. In addition to the possible involvement of the $tg^{ia}$ cortex in absence epilepsy, Ayata et al. (2000) demonstrated that $tg^{ia}$ mice have a 10-fold resistance to cortical spreading depression (CSD), slowed propagation of CSD, and a decrease in transmitter release. A decrease in cortical excitability has been linked to FHM, a human P/Q-type calcium channelopathy (van der Kamp et al., 1997; Ayata et al., 2000). Any reduction in P/Q-type calcium channels would therefore be critical because calcium ion levels in neurons are normally strictly controlled (Moreno, 1999). Although increased calcium levels can lead to apoptosis (Nicotera and Orrenius, 1998), it has been speculated that decreased calcium concentrations can also result in apoptotic cell death (Galli et al., 1995; Fletcher et al., 1996). Apoptosis is seen in the $tg^{ia}$ mouse (Fletcher et al., 1996).

This thesis has demonstrated that there is potential reduction in P/Q-type calcium channels in the tottering leaner mouse. Experiments were done with cortical membranes by both immunobloting and MVIIC binding assays. Neither of these techniques had previously been used to assess P/Q-type channel expression in this mouse. In addition, by tagging the wt, $tg^{ia}$ long, and $tg^{ia}$ short $\alpha_{1A}$ proteins with EGFP,
their subcellular distributions could be monitored in cell culture. The expression (as detected by immunoblots) and distribution of the two mutant proteins in a heterologous cell culture system had not previously been reported.
6. FUTURE STUDIES

Various experiments could be performed in the future in order to further our understanding of the causes of the $tg^{a}$ phenotype. This thesis has shown that there is possibly a decrease in both $\alpha_{1A}$ protein and P/Q-type calcium channel levels. Experiments examining the rates of protein synthesis, folding, and degradation would clarify the basis for reduced protein expression. Second, subcellular fractionation could be performed to confirm the exact location of the leaner mutants in both mouse brain membranes and transfected cell lines. Third, biotinylation or MVIIIC binding experiments could be used to determine the internalization rates of the mutant channels, in particular the $tg^{a}$ long mutant, which produced some current in cell culture, compared to wt channels. Finally, the altered carboxy termini in the $tg^{a}$ mutants may produce or reveal a degradation signal. Mutational studies aimed at identifying such signals would be of great interest, not only for understanding the $tg^{a}$ mutation, but for investigating other membrane proteins as well.
B) tg^14 long αIA C-terminus (IVS6-stop codon)

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KSTDLTGVKGIIYAAMMMIMEEYRRQSKAKKLQLQAMREEQNRTPLMFOR
AAATCCACGGACCTGACACTGTCGTAAGATCTACGACGACCATGATGCTAGATTACAGGAGGACAGAAGCCACAAACTGACGCGTGACAGAGAACACCGACACTATGTTCCAGGCACAT

```
C) tg\textsuperscript{14} short α\textsubscript{1A} C-terminus (IVS6-stop codon)

A comparison of wt (A), tg\textsuperscript{14} long (B) and tg\textsuperscript{14} short (C) α\textsubscript{1A} cDNA and protein sequences. Grey: Intron; Boxed Italic: Altered amino acid sequences due to frameshifting; Boxed regions: Deleted regions; Boxed Grey: tg\textsuperscript{14} mutation (G to A substitution).
# APPENDIX B

## Alignment Results

SSEARCH searches a sequence database using the Smith-Waterman algorithm version 3.3t01 November 4, 1999

Please cite:

/usr/local/apache/temp/977867939-1: 449 aa
rat vs /usr/local/apache/temp/977867939-2 library searching /usr/local/apache/temp/977867939-2 library

449 residues in 1 sequences

Smith-Waterman (PGopt) (3.30 October 1999) function [BL50 matrix (15:5)], gap-penalty: -50/-1
Scan time: 0.040
The best scores are:

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s-w opt: 3079
Smith-Waterman score: 3079; **98.664%** identity in 449 aa overlap (i-449:i-449)

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| 360 | 370 | 380 | 390 | 400 | 410 | 420 |

mouse TSERSLGRYTDVDGLGTDLSMTTQSGLPKDRDQDRGRPKDRKHRPHHHHHHHHHHHHHPPAPDRERYAQE

430  440

rat  RPDTGRARAREQRWSRSPSEGREHATHRQ

| 430 | 440 |

mouse RPDTGRARAREQRWSRSPSEGREHATHRQ

449 residues in query sequences

449 residues in library sequences

Function used was Smith-Waterman

Wt mouse and rat α1A carboxy termini. Sequence alignment reveals an 98.7% amino acid identity between the two species.
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