University of Alberta

CTP:phosphocholine cytidylyltransferase expression, function and regulation during neuronal differentiation.

by

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Abstract

During differentiation neurons increase phospholipid biosynthesis to provide new membrane for neurite growth. We studied the regulation of phosphatidylcholine (PC) biosynthesis during differentiation of two neuronal cell lines: PC12 cells and Neuro2a cells. We hypothesized that in PC12 cells nerve growth factor (NGF) would up-regulate the activity and expression of the rate limiting enzyme in PC biosynthesis, CTP:phosphocholine cytidylyltransferase (CT). During neurite outgrowth, NGF doubled the amount of cellular PC and CT activity. CTβ2 mRNA increased within one day of NGF application, prior to the formation of visible neurites, and continued to increase during neurite growth.

NGF specifically activated CTβ2 by promoting its translocation from cytosol to membranes. In contrast, NGF did not alter CTα expression or translocation. In Neuro2a cells, retinoic acid treatment significantly increased CT activity and increased CTβ2 protein, coincident with neurite outgrowth but did not change CTα expression. Together, these data suggest that the CTβ2 isoform of CT is specifically up-regulated and activated during neuronal differentiation to increase PC biosynthesis for growing neurites.

The hallmark of neuronal differentiation is the formation and growth of neurites. We hypothesized that the CTβ2 isoform is up-regulated during neuronal differentiation to provide axons with a local source of phosphatidylcholine. Using immunoprecipitation techniques, we demonstrated that CTβ2 protein, but not CTα protein, is found in axons of sympathetic

neurons and retinal ganglion cells. Also, we used RNA silencing to knock down CTα and CTβ2 expression. In CTβ2-silenced PC12 cells, the numbers of primary neurites and branches were sharply attenuated. Despite having far fewer neurites, the length of neurites on CTβ2-silenced cells was significantly increased, thus, the net amount of membrane did not change. Furthermore, stimulation of neurite branch formation by treatment with LY294002, an Akt inhibitor, was completely abrogated in cells with attenuated levels of CTβ2 protein. However, CTβ2 protein was not directly phosphorylated by Akt. Together, these data suggest that CTβ2 is localized to the distal axons of neurons and is essential for proper neurite and branch formation in PC12 cells. We've identified the first phospholipid biosynthetic enzyme to be required for neurite outgrowth and branch formation.

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List of Abbreviations

Acyl CoA	acyl coenzyme A
AdoHcy	*S*-adenosylhomocysteine
AdoMet	*S*-adenosylmethionine
ATP	adenosine triphosphate
ADP	adenosine diphosphate
Ap1	activator protein 1
BP	base pair
CB	cell bodies
Cdc2	cyclin-dependent kinase 2
Cdk5	cyclin-dependent kinase 5
cDNA	complementary deoxyribonucleic acid
CDP	cytidine diphosphate
CK	choline kinase
CMP	cytidine monophosphate
CMV	cytomegalovirus
CPT	CDP-choline:1,2-diacylglycerol choline phosphotransferase
Cre-LOX	Cyclization recombination/Locus of X over P1
CTP	cytidine triphosphate
CHO58	Chinese hamster ovary cell line 58
CSF-1	colony stimulating factor 1
CT	CTP:phosphocholine cytidylyltransferase
DAG	diacylglycerol
DAx	distal axons
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethylsulfoxide
ECL	enhanced chemiluminescence
EDTA	ethylene diamine tetraacetic acid
EGF	epidermal growth factor
ERK	extracellular mitogen-activated kinase
F12-K	F12-Kaign's modification
FBS	fetal bovine serum
GAP-43	growth-associated protein 43
GFP	green fluorescent protein
HA	hemagglutinin
IB	immunoblot
IgG	immunoglobulin gamma
IP	immunoprecipitated
KB	kilobases
MEK1/2	mitogen-activated kinase/extracellular signal regulated kinase kinase ½
NCBI	National center for biotechnology information
NFkB	nuclear factor kappa beta
NGF	nerve growth factor

PA	phosphatidic acid
PAx	proximal axons
PC	phosphatidylcholine
PC12	pheochromocytoma 12 cell line
PCR	polymerase chain reaction
PE	phosphatidylethanolamine
PEMT	phosphatidylethanolamine N-methyltransferase
PI3K	phosphatidylinositol-3 kinase
PLA_2	phospholipase A_2
PLC	phospholipase C
PLD	phospholipase D
PBS	phosphate-buffered saline
PPi	pyrophosphate
PVDF	polyvinylidene fluoride
MAP	mitogen-activated protein (kinase)
mRNA	messenger ribonucleic acid
RAx	retinal axons
RGC	retinal ganglion cells
RNase	ribonucleic acidase
RT-PCR	reverse transcription polymerase chain reaction
RPA	ribonuclease protection assay
SDS	sodium dodecyl sulfate
SEM	standard error of the mean
siRNA	small interfering ribonucleic acid
PAGE	polyacrylamide gel electrophoresis
SREBPs	sterol responsive element binding proteins
TBS-T	tris-buffered saline with Tween
TEF-4	transcription enhancing factor 4
TRK	tyrosine receptor kinase
VLDL	very low density lipoprotein

Chapter 1

Introduction

1.1 Phosphatidylcholine is a key constituent of eukaryotic membranes

Phosphatidylcholine (PC) is the predominant phospholipid in eukaryotic membranes. PC is composed of a glycerol backbone esterified at the *sn*-1 and *sn*-2 positions with long-chain fatty acids and contains a choline headgroup at the *sn*-3 position (Figure 1.1) [1]. As a key component of biological membranes, PC biosynthesis is essential for cell growth and division as well as cell survival [2]. PC also has specialized roles in specific tissues. For example, PC is a primary component of lung surfactant. Surfactant is a secreted lipoprotein containing dipalmitoyl-PC that reduces surface tension within alveoli during expiration [3]. In liver, PC biosynthesis is required for secretion of bile and very low density lipoproteins [4]. PC is also a primary source of lipid signaling molecules. Phospholipase A_2, phospholipase C and phospholipase D catabolize PC to generate fatty acids, diacylglycerol and phosphatidic acid respectively (reviewed in [5]) (Figure 1.3).

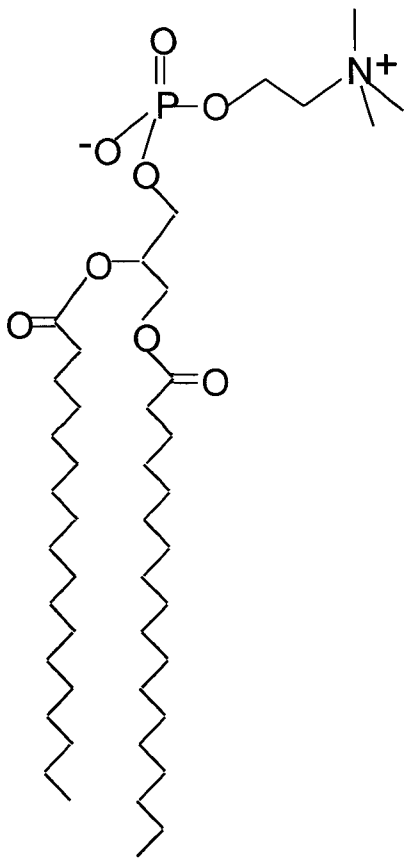
1.2 Phosphatidylcholine in the nervous system

The recent classification of choline as an essential dietary nutrient [6] underscores the importance of choline-containing biomolecules in the development and function of the nervous system. While choline has multiple metabolic fates in neurons, including synthesis of the neurotransmitter acetylcholine, phosphatidylcholine (PC) synthesis is among the most important. PC accounts for an estimated 30-35% of total phospholipids in

Figure 1.1. Structure of phosphatidylcholine (PC) and distribution of molecular species in brain. A. PC (1,2-diacyl-*sn*-glycero-3-phosphorylcholine) is the predominant phospholipid in eukaryotic membranes. **B.** Percentage distribution of the molecular species of PC in rat brain. Modified from Sastry, P.S., Prog. Lipid. Res. **24**, 69-176.

Figure 1.1

A.



B.

Fatty acid composition of PC	Percentage weight
16:0/18:1	30%
16:0/16:0	14%
18:0/18:1	12%
18:1/16:0	10%
18:0/20:4	10%
16:0/20:4	8%

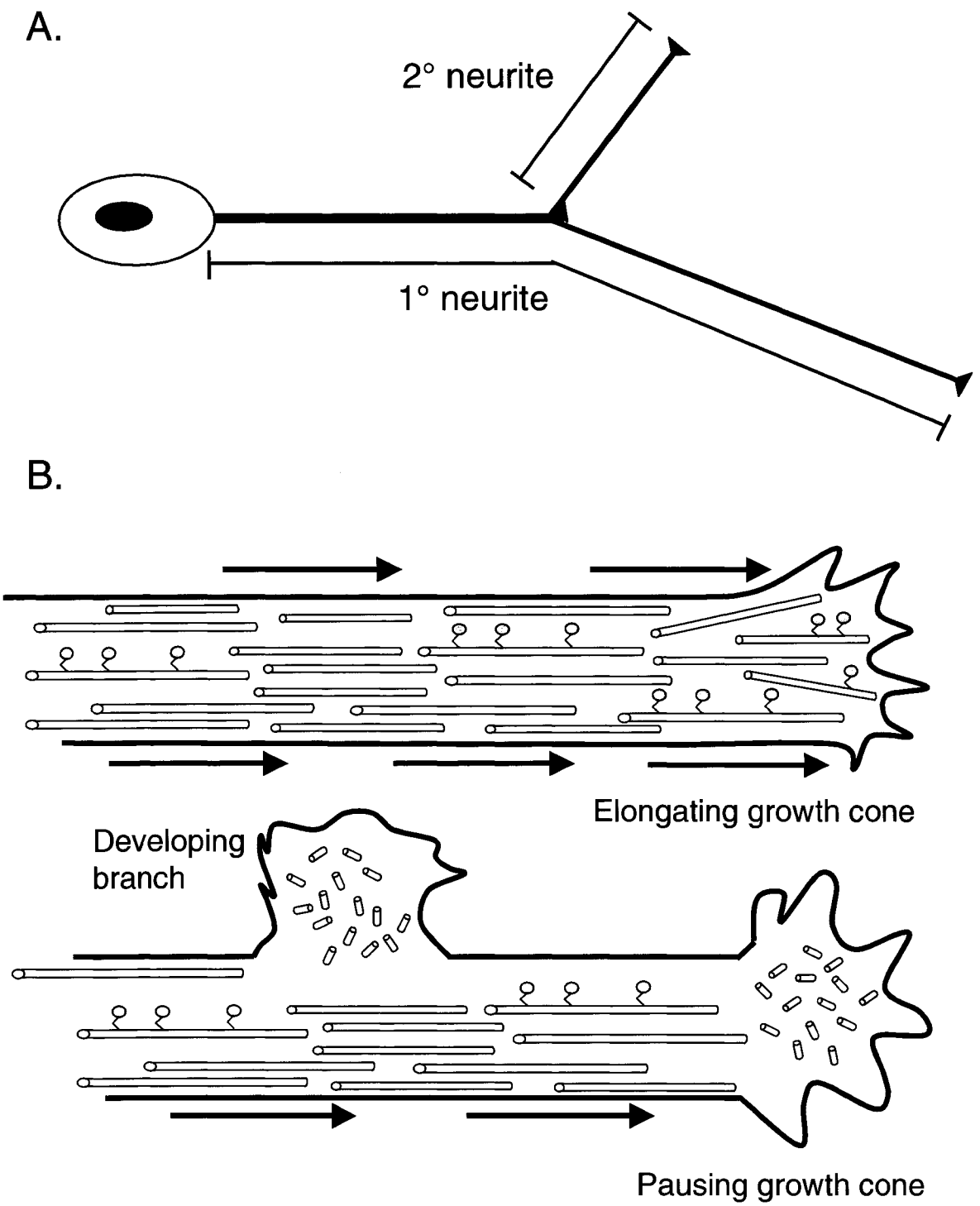
mammalian brain [7]. The predominant molecular species of PC in the brain are outlined in Figure 1.1.

During development, neurons dramatically alter their cellular morphology, by extending long processes, called neurites, from the plasma membrane. These neurites later mature into functional axons and dendrites. An elaborate network of neurites creates a surface area many times larger than that of the cell body itself. Thus, during neurite outgrowth, it is predictable that PC biosynthesis must accelerate to accommodate the demand for new membrane. Indeed, PC biosynthesis increases during neurite outgrowth in rat pheochromocytoma-derived PC12 cells and [8] cultured neurons [9]. In PC12 cells, Wurtman *et al.* have shown that substrate availability, namely diacylglycerol [8, 10] and cytidine [11, 12], also regulate PC biosynthesis. To date, the role of CTP:phosphocholine cytidylyltransferase (CT), which catalyzes the rate-limiting step in the cytidine diphosphate (CDP)-choline pathway of PC biosynthesis has not been evaluated in the nervous system.

Since PC serves as both a key membrane component and as a reservoir for lipid second messengers and sphingomyelin biosynthesis, choline deficiency, with subsequent PC depletion, triggers apoptosis in neurons [13] and neuronal cell lines [14]. In contrast, dietary choline supplementation in rats accelerates PC synthesis and promotes memory formation and axonal growth [14].

Figure 1.2. Neurite branching in PC12 cells. A. Classification of primary and secondary neurites is determined by their position relative to the cell body. Primary neurites project from the cell body while secondary neurites project from primary neurites. Distal to the branch site, the continuing primary neurite is identified by its less aberrant course from the direction of the primary neurite prior to the branching event. **B.** Schema of the growth cone or branch point of a neurite. Within the neurite, microtubules have the same polarity and provide a structural framework for delivery of vesicles (shown in green) from the cell body. Within the growth cone or branch point, microtubules shorten and lose their polarity resulting in disruption of organized vesicle transport. Modified from Kalil, *et al.*, (2002) J. Neurobiology, **44**.

Figure 1.2



1.3 Phospholipid biosynthesis in axons

While the lipid composition of axons is distinct from that of neuronal cell bodies, PC is the predominant phospholipid within both regions of the cell. In rat dorsal root ganglion neurons, PC comprises 29% and 27% of the total phospholipid in the cell bodies and neurites respectively [15]. Until recently, conventional theory held that growing axons could not synthesize their own phospholipids *in situ* to accommodate the demand for new membrane. However, the activities of PC biosynthetic enzymes have been detected in rat brain synaptosomes [16], squid axoplasm [17] and distal axons of sympathetic neurons [18].

Posse de chaves *et al.* have reported that at least 50% of the PC in axon membranes is derived from biosynthesis within the axon [18]. Moreover, PC synthesis in the axon is not redundant. When sympathetic neurons are treated with alkylphosphocholines, inhibitors of PC biosynthesis, axonal growth is completely stalled [19]. Thus, production of PC within axons is critical for normal axon growth.

In addition to PC, the biosynthesis of phosphatidylethanolamine, phosphatidylserine and sphingomyelin has been demonstrated in the axons of sympathetic neurons [18]. Accordingly, the enzymes of these lipid biosynthetic pathways must be localized to axons; however, the specific isoforms of these enzymes localized within axons are not yet known. One exception is a ganglioside-specific sialyltransferase, GM3 synthase, which has been identified within the axons of hippocampal neurons [20].

1.4 Cellular models of neuronal differentiation

Since its discovery in 1976 [21], the PC12 cell line remains an invaluable model for studies of neuronal differentiation. In response to nerve growth factor, PC12 cells stop proliferating and differentiate into sympathetic-neuron like cells [21, 22] which become electrically excitable, able to synthesize and secrete acetylcholine and form functional cholinergic synapses [23].

The morphological hallmark of neuronal differentiation is the sprouting and growth of neurites: long, branched extensions of the plasma membrane. In neurons, these later mature into functional axons and dendrites. During development, neurites branch repeatedly to create an extensive network. For classification purposes, neurites are named by their location relative to the cell body. Primary neurites project directly from the cell body. Secondary neurites, or branches, project from primary neurites (Figure 1.2A). Tertiary neurites project from secondary neurites and so on. This anatomical distinction is important and reflects the current thought that primary neurite outgrowth and branch formation are distinct processes that are separately regulated.

In PC12 cells, like neurons, neurite outgrowth greatly increases the surface area of the cell, thereby producing a strong demand for new membrane synthesis. Within 2 days of NGF treatment, PC12 cells extend visible neurites and, with continual NGF exposure, create an extensive arborization of primary neurites and branches. It is therefore not surprising that PC biosynthesis increases during neurite outgrowth of PC12 cells [8].

1.5 Metabolic Fates of PC during neuronal differentiation

During neuronal differentiation, PC plays both structural and signaling roles. For example, in PC12 cells, neurite outgrowth requires activation of phospholipase D (PLD), a PC catabolizing enzyme, via the MAP kinase signaling cascade [24]. PLD catabolizes PC to yield phosphatidic acid. Phosphatidic acid can act as a second messenger or be converted to diacylglycerol by phosphatidic acid phosphohydrolase. PLD is found in the neurites of PC12 cells, and is particularly enriched within growth cones at the distal end of neurites [24]. It is unknown whether PLD activation alters PC levels within the growth cones of PC12 cells; however, PLD-dependent catabolism of PC has been reported in mouse and human neuroblastoma cell lines [25]. Since NGF-dependent activation of PLD, via the MAP kinase signaling cascade, is an absolute requirement for neurite growth, there is likely considerable turnover of PC within growth cones.

In addition, PC donates its phosphocholine group to produce sphingomyelin. Sphingomyelin, a sphingolipid, is particularly enriched in the nervous system [7]. Sphingomyelin can be synthesized in the distal axons of sympathetic neurons [18] and is essential for the formation of detergent-insoluble glycolipid complexes within the axonal membrane [26]. In short, membrane biogenesis, PC turnover within the growth cone and sphingomyelin biosynthesis all contribute to the high demand for PC within growing neurites.

1.6 Neurite Outgrowth and Branch Formation

Neurites have discrete microtubule and actin-based cytoskeletal domains. Microtubules are oriented within the long axis of neurites and serve both as structural framework and supply network – delivering materials from the cell body to axons and dendrites [27]. In contrast, actin is enriched specifically within the growth cones of axons [27]. Growth cones are highly dynamic, motile structures at the distal tips of axons which respond to environmental cues to direct growth.

Successful neurite arborization requires that a delicate balance be struck between neurite growth and branch formation [28]. Time-lapse recording of the axons of sensory neurons showed that axons pause, for up to several hours at the site of future branch points [29]. During this pause, microtubules become splayed and disorganized at the site of the branch point. Eventually, the microtubule network reorganizes and short microtubules project into the developing neurite (Fig. 1.2B, reviewed in [28]). Although the signaling cascades involved in neurite branching are poorly understood, several proteins have been implicated in neurite branching. For example, the phosphatidylinositol 3- kinase (PI3-K) pathway is involved in neurite outgrowth and branch formation [30]. In this pathway, NGF-dependent activation of one of its membrane-associated receptors, trkA results in recruitment of PI3-K to the plasma membrane [31]. Although PI3-K has multiple downstream effectors; one of them, the serine/threonine kinase Akt,

has been strongly implicated in the regulation of neurite branch formation [32] [33].

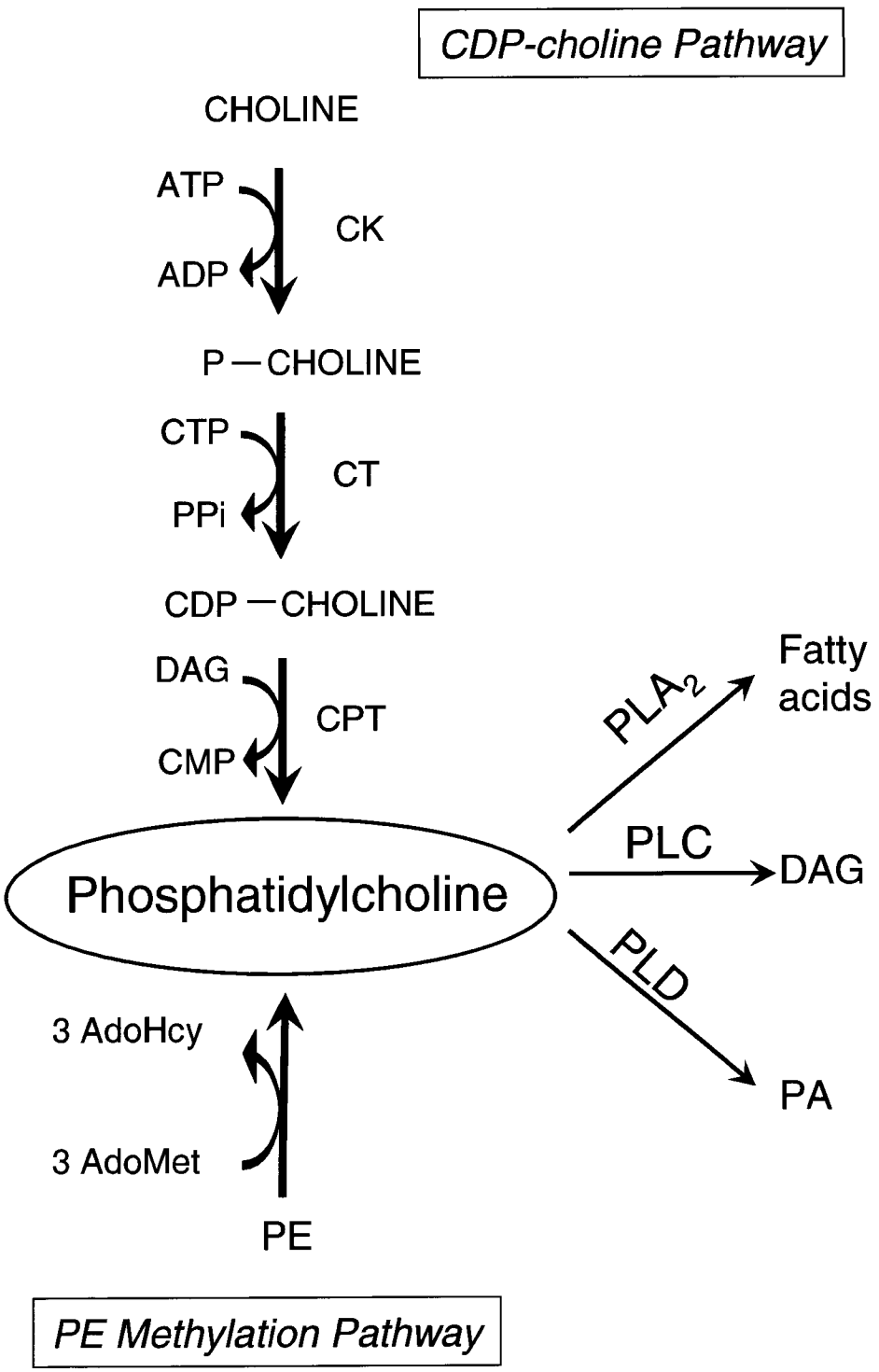
Also, the microtubule-binding protein, Raspotlin has been studied in PC12 cells [34, 35]. Rapostlin is required for branch formation and in its absence, PC12 cells produce far fewer branches [34].

1.7 PC biosynthetic pathways

In 1956, Kennedy and Weiss described a novel enzymatic reaction whereby phosphocholine was converted to CDP-choline using cytidine trinucleotide as a cofactor [36, 37]. This was the first description of the reaction catalyzed by CTP:phosphocholine cytidylyltransferase (CT) [36]. This seminal work established the CDP-choline or Kennedy pathway of PC biosynthesis. In this pathway, choline, typically from a dietary source, is transported into the cell and phosphorylated by choline kinase to produce phosphocholine. Phosphocholine and cytidine trisphosphate are converted to CDP-choline in a reaction catalyzed by CT. In the final reaction of the pathway, CDP-choline:1,2-diacylglycerol cholinephosphotransferase catalyzes the transfer of the choline moiety of CDP-choline to diacylglycerol to yield PC [36] [38] (The PC biosynthetic pathways are shown in Figure 1.3). A second PC biosynthetic pathway exists in liver. The phosphatidylethanolamine (PE) methylation pathway is characterized by the sequential methylation of PE to generate PC (reviewed in [39]).

Figure 1.3. Overview of phosphatidylcholine metabolism. PC is synthesized by two pathways: the CDP-choline pathway and the sequential methylation of phosphatidylethanolamine in the PE methylation pathway. PC can be catabolized by several phospholipases to produce lipid second messenger molecules. Abbreviations: AdoHcy, *S*-adenosylhomocysteine; AdoMet, *S*-adenosylmethionine; CK, choline kinase; CT, CTP:phosphocholine cytidylyltransferase; CPT, CDP-choline:1,2-diacylglycerol cholinephosphotransferase; DAG, *sn*-1,2-diacylglycerol; PA, phosphatidic acid; PE, phosphatidylethanolamine PEMT, phosphatidylethanolamine *N*-methyltransferase; PLA_2, phospholipase A_2; PLC, phospholipase C; PLD, phospholipase D.

Figure 1.3



1.8 CTP:phosphocholine cytidylyltransferase catalyzes the rate-limiting step of the CDP-choline pathway.

Within the CDP-choline pathway, several lines of evidence suggest that the formation of CDP-choline by CT is the rate-limiting step. Firstly, measurements of aqueous intermediates demonstrated that CDP-choline is ten-fold less abundant than choline and 100 times less abundant than phosphocholine [40]. The relative accumulation of choline and phosphocholine suggest that the formation of CDP-choline (catalyzed by CT) governs the flux of substrates through the pathway. Furthermore, pulse chase studies with HeLa cells [40] and hepatocytes [41], have shown that [methyl-^{3}H]choline is rapidly incorporated into phosphocholine and then quantitatively converted to PC. The amount of radiolabelled CDP-choline was negligible, consistent with its synthesis limiting the rate of PC biosynthesis. One important caveat must be addressed. Spence *et al.* have demonstrated in electropermeabilized C6 glioma cells that endogenous phosphocholine and CDP-choline, rather than being freely diffusible, were channeled toward PC biosynthesis in an energy and calcium-dependent manner [42] [43]. This phenomenon of substrate channeling within multienzyme complexes has been described for many biosynthetic pathways [44] and suggests that the intermediates of a given pathway are compartmented. If this is the case for the CDP-choline pathway, measurements of intermediate pool sizes may not reflect relative enzymatic rates and cannot accurately predict the rate-limiting step of a biosynthetic pathway.

1.9 CTP:phosphocholine cytidylyltransferase isoforms

In 1987, CT protein was purified from rat liver [45] and subsequently cloned from *Saccharomyces cerevisiae* by complementation of yeast lacking the enzyme [46]. *Pcyt1a*, the murine gene encoding CTα was identified in 1997 [47]. In mice, *Pcyt1a* is on chromosome 16, contains nine exons and eight introns and spans approximately 26 kilobases [47]. The exons of *Pcyt1a* are organized in association with the functional domains of CTα protein. Exon 1 is untranslated, exon 2 encodes seventy three amino acid residues, including a nuclear localization signal, exons 4-7 encode the catalytic domain, exon 8 encodes the α-helical membrane-binding domain and exon 9 encodes the phosphorylation domain within the carboxyl terminus (Figure 1.4) [47].

In 1998, Lykidis *et al.* identified a second gene, *Pcyt1b,* encoding two CTβ isoforms: CTβ1 and CTβ2 in humans, CTβ2 and CTβ3 in mice [48] [49] [50]. In both mice and humans, *Pcyt1b* is found on the X chromosome. In mice, the two CTβ transcripts, CTβ2 and CTβ3, differ in their initiating exons: the CTβ2 mRNA initiates at exon 1 while the CTβ3 transcript initiates at exon 2 [51] (Figure 1.5).

Similar to *Pcyt1a*, exons of the *Pcyt1b* gene are organized in the context of the functional domains of CTβ proteins. Exons 4-7 encode the catalytic domain, exon 8 encodes the lipid-binding domain and exon 9 encodes the phosphorylation domain [51] .

In summary, three CT isoforms have been identified in rodents: CTα, CTβ2 and CTβ3. CTα is a 41 kDa protein composed of 367 amino acids. CTβ2 has

Figure 1.4. Structure of the murine *Pcyt1a* gene. The introns are numbered with roman numerals with their sizes (kB) listed beneath their numbers. The exons, shown in light blue, are aligned with their corresponding cDNA organization by the dotted lines. The amino acid residue positions are listed beneath the cDNA organization along with their corresponding functional domains. Modified from Tang *et al.*, J. Biol. Chem. **272**, 13148.

Figure 1.4

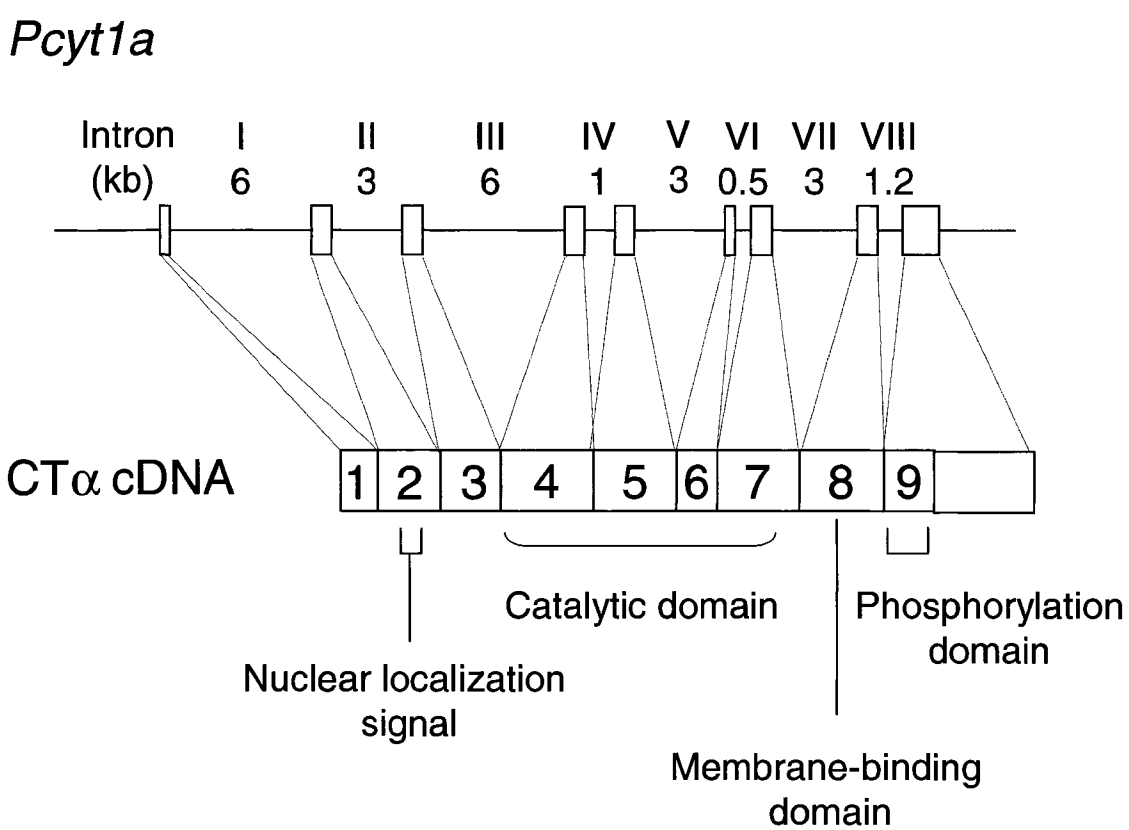
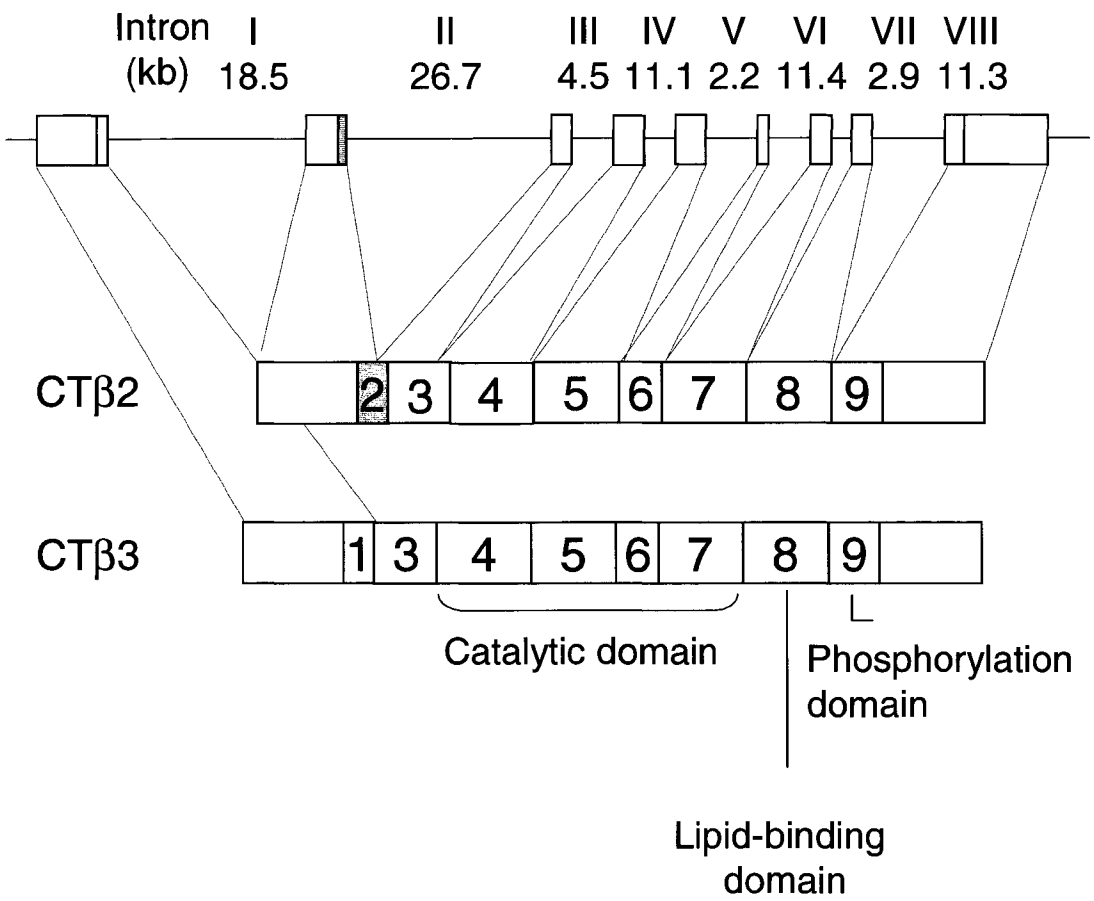


Figure 1.5. Structure of the murine *Pcyt1b* gene. The introns are numbered with roman numerals with their sizes (kb) listed beneath them. The exons, shown in light blue, are aligned with the corresponding cDNA by the dotted lines. The green and dark blue components within exons 1 and 2 indicate alternative transcriptional start sites which generate CTβ2 and CTβ3 mRNAs respectively. The amino acid residue positions are listed beneath the cDNA along with their corresponding functional domains. Modified from Karim *et al.*, 2003, Biochim. Biophys Acta, **1633**: 2.

Figure 1.5

Pcyt1b



a predicted size of 42 kDa and contains 369 amino acids. CTβ2 and CTβ3 differ only by 28 amino acids within their amino termini.

1.10 Functional Organization of CT protein

To date, CT othologs have been characterized from *Caenorhabditis elegans* [52], *Saccharomyces cerevisiae* [53], *Streptococcus pneumoniae* [54], [55], *Drosophila melanogaster* [56], rat [57] and mouse [48], [49]. Characterization of CT protein from several species led to the identification of four functional domains: a nuclear localization signal, catalytic domain, membrane-binding domain and a phosphorylation domain (Figures 1.4, 1.5). CTα has a nuclear localization signal [58] and immunofluorescence studies have localized CTα both to the nucleus and endoplasmic reticulum [58] [59]; however the role of CTα within the nucleus is poorly understood. Ridgway *et al.* recently reported that CTα is involved in regulating proliferation of the nucleoplasmic reticulum [60]. CTα may also be localized to the nucleus to regulate chromosomal replication. In cells overexpressing the oncogene B cell lymphoma-2, CTα expression was required to prevent chromosome duplication to a tetraploidy state [61]. In contrast, CTβ2 lacks a nuclear localization signal and immunofluorescent studies in murine macrophages, HeLa cells and CHO58 hamster ovary cells have localized CTβ2 to the endoplasmic reticulum and Golgi complex [49].

The catalytic domain of CT is homologous to that of the nucleotidyltransferase superfamily which, although diverse in function, use

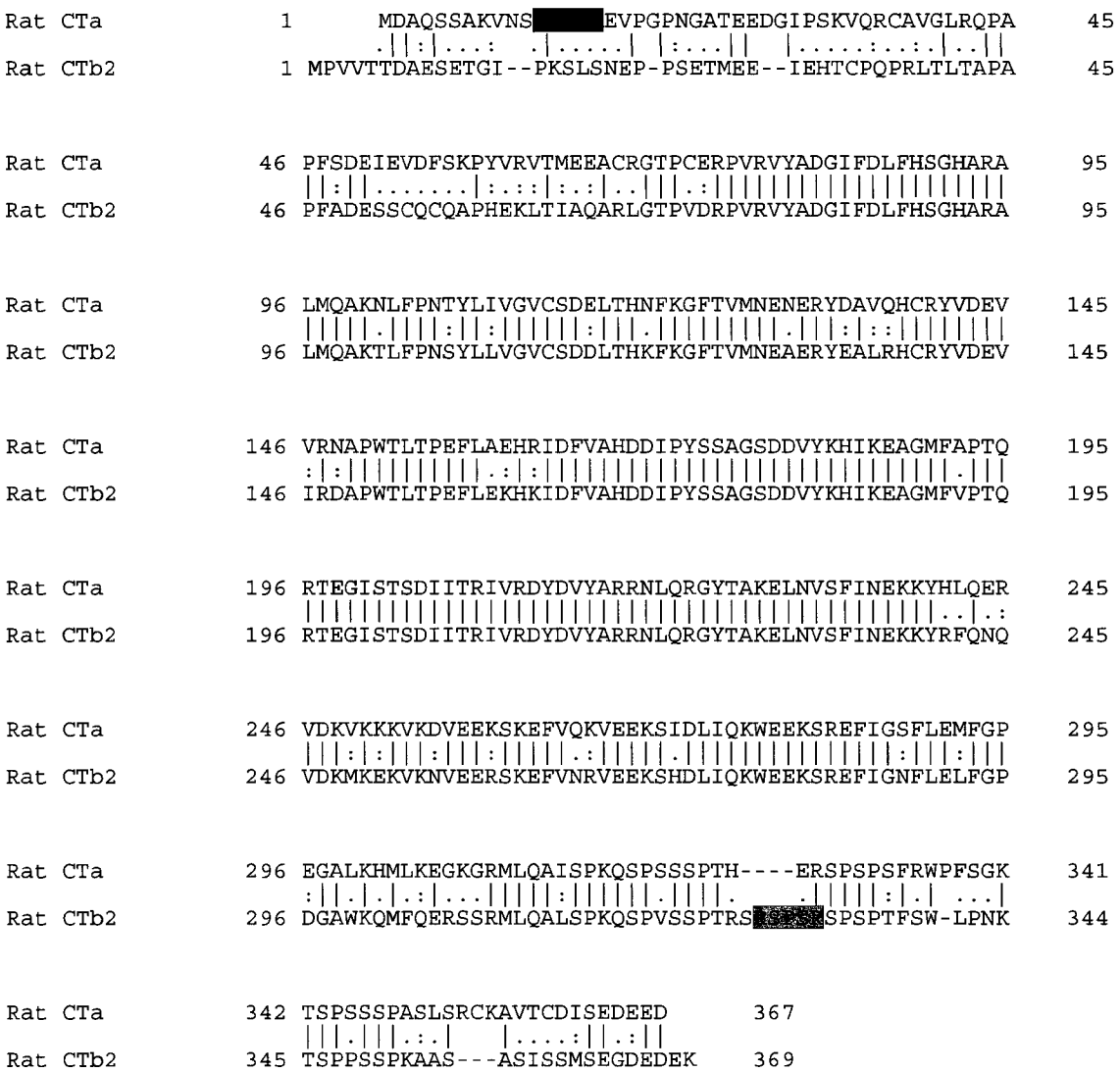
nucleotides as substrates [62]. For example, cytidylyltransferases use CTP as a substrate. Within the CT protein, the catalytic domain mediates the conversion of phosphocholine and CTP to CDP-choline. There are two highly conserved motifs within the CT isoforms: HXGH and RTEGISTS. Site-directed mutagenesis studies of CTα protein have identified roles for His 89 and His 92 (within the HXGH motif) [63, 64] and arginine 196 (within the RTEGISTS motif) [65] in CTP binding and identified lysine 122 as a potential contact site for phosphocholine [65]. Subsequently, the crystal structure of a CT homolog, CTP:glycerol-3-phosphate cytidylyltransferase, from *Bacillus subtilis*, confirmed that CTP interacts with both the HXGH and RTRGISTS domains [66]. Since the HXGH and RTEGISTS motifs, as well as Lys 122 are conserved in CTβ isoforms [49] (Figure 1.6), they are presumed to have similar roles in substrate binding; however, no such analysis has been done.

CT proteins interact with membranes via their membrane-binding domains. The membrane-binding domain is composed of an amphipathic helix containing three 11-mer repeats [67]. In a helical wheel conformation, the repeats align with hydrophobic residues forming one face of the wheel. Intercalation of the hydrophobic residues within the membrane may be the basis of membrane-association of CT [68]. This lipid-binding domain is highly conserved among all CT isoforms underscoring the importance of lipid association in CT activity.

Within their carboxyl termini, both CTα and CTβ2 proteins contain a number of putative phosphorylation sites. CTα contains 16 serine residues, thirteen of

Figure 1.6. Comparison of the predicted rat CTα and CTβ2 protein sequences. Global sequence alignment of predicted rat CTα and CTβ2 protein sequences (Accession numbers: Q9QZC4 and AAH85713). The nuclear localization signal (CTα) is highlighted in red. The conserved catalytic motifs are highlighted in yellow. The cdk5 consensus sequence (RSPSR) within the CTβ2 sequence, is highlighted in dark green.

Figure 1.6



which are known to be phosphorylated [69]. In contrast, CTβ2 contains 22 potential phosphorylation sites [49]. Within their respective phosphorylation domains, CTα and CTβ2 share two identical motifs (known to be phosphorylated in CTα): SSPTR and RSPS (Figure 1.6). However, CTβ2, alone contains an RSP*X*R motif that directs phosphorylation by cyclin-dependent kinase 5 [49]. Phosphorylation of CT isoforms is further discussed in the section on regulation of CT activity.

In a two-dimensional context, CT proteins are described as having four functional domains; however, in the assembled protein, these domains are unlikely to function independently of one another. Mass spectroscopy of proteolyzed CT fragments revealed that an ~80 amino acid core within the catalytic domain of CTα is protease-inaccessible both in its membrane-associated and soluble forms. While burial within secondary structure may partially account for protection of the catalytic domain, three-dimensional modeling identified several protected sites on the surface of the CT protein. Bogan *et al.* suggest interactions between the amino terminus of CTα and the membrane-binding domain protect both domains from proteolytic degradation [70]. Since CTα and CTβ isoforms differ significantly within their amino termini, it is unknown whether CTβ2 has a similar inter-domain contact.

CTα protein domains also contribute to the quaternary structure of the enzyme. As discussed above, CT forms a homodimer in both its membrane-associated and soluble forms [71]. Interactions within the N domain [72] and catalytic domain of CTα protein facilitate dimerization [52, 72, 73]. While the

exact role of dimerization is unknown, chemical cross-linking data strongly suggest that membrane association alters the quaternary configuration of the homodimer [72]. These studies are particularly relevant in elucidating isoform-specific roles for CT since the cysteine residues involved in CTα homodimerization are not conserved in the CTβ2 isoform (Figure 1.6).

1.11 Cellular and Tissue Distribution of CT isoforms

CT isoforms have strikingly different tissue distributions. CTα is expressed in virtually all tissues and has its highest level of expression in liver. CTβ2 and CTβ3, which are transcribed from alternate start sites within the murine *Pcyt1b* gene, are both enriched in brain [49, 51]. In embryonic mouse, CTβ2 mRNA is particularly enriched in hippocampus, striatum and pons [51]. CTβ2 mRNA levels are low in most tissues but it is most abundant in the gonads, lung and brain [74]. Specifically, CTβ2 comprises ~ 30% of the CT mRNA transcripts expressed in brain and only ~5% of the CT mRNA transcripts in lung [74]. Whereas CTβ2 is expressed in both embryonic and adult mouse brain, CTβ3 is found only in adult brain. Despite the abundance of CTβ3 mRNA in adult mouse brain, there is far less CTβ3 protein than CTβ2 protein in brain [51].

Since CTα is expressed in virtually all cell types, the enhanced expression of CTβ2 in brain suggests a role for CTβ2-derived PC in neuronal development.

Interestingly, in neurons, up-regulation of CT expression or activity is associated with growth of axons from neurons. In rat hippocampal neurons, CTβ2 mRNA is up-regulated by arginine vasopressin $_{4\text{-}8}$, a metabolite of arginine vasopressin that promotes hippocampal axon growth and facilitates memory acquisition [75]. Also, in hippocampal neurons, treatment with glucosylceramide increased CT activity coincident with increased axonal growth [76].

1.12 Studies of CTα and CTβ2 in genetically modified animals

Since PC is required for cell survival, prior to the discovery of a second CT-encoding gene, it was assumed that disruption of CT expression would confer a lethal phenotype. For this reason, the *Pcyt1a* gene, encoding CTα, was first disrupted within specific cell lineages rather than within the whole animal (reviewed in [50]).

A Cre-LOX recombination strategy was used to generate mice with a targeted disruption of the *Pcyt1a* gene in macrophages [77] and liver [78]. Within macrophages, a deficiency of CTα protein led to increased susceptibility to free cholesterol-induced cell death [77]. Interestingly, in macrophages deficient in CTα protein, CTβ2 expression was up-regulated, suggesting that there is a coordinated regulation of CT isoform expression in macrophages [77].

Within liver, the CDP-choline pathway accounts for approximately 70% of PC biosynthesis while the sequential methylation of

phosphatidylethanolamine to PC (catalyzed by PEMT) accounts for the remaining 30% of PC biosynthesis. PC is the predominant phospholipid on the surface monolayer of all plasma lipoproteins (reviewed in [79]) and impairment of PC biosynthesis alters very low density lipoprotein (VLDL) secretion by the liver [80]. In mice lacking hepatic CTα protein, high density lipoprotein and VLDL secretion were sharply attenuated [78], thereby implicating CTα in the production of PC for lipoprotein secretion. Although CTβ2 is normally expressed in liver and, notably, was up-regulated in the livers of mice lacking CTα [78], its role in hepatic PC metabolism and lipoprotein secretion has not been evaluated.

Jackowski *et al.* have disrupted CTα and CTβ2 expression in mice to investigate their cellular and tissue-specific roles in PC metabolism. Using a Cre-lox recombination strategy, exons 5 and 6 of *Pcyt1a* were deleted to generate a mouse lacking CTα protein. Since *in situ* hybridization of CT mRNAs within normal mouse embryos demonstrated that CTα is the predominant transcript [81], it was anticipated that disruption of *Pcyt1a* would confer a lethal phenotype. *Pcyt1a* $^{-/-}$ zygotes stalled at embryonic day 3.5 and failed to implant [81]. These observations suggest that CTα is the major CT isoform in PC biosynthesis during early embryonic development. *Pcyt1* $^{+/-}$ heterozygote embryos did up-regulate both CTβ2 and phosphatidylethanolamine-N-methyltransferase transcripts with no loss of PC biosynthesis [81] providing additional evidence that expression of CT isforms is coordinately regulated.

Jackowski *et al.* have also generated mice with a disruption in the second exon of *Pcyt1b*, thereby selectively disrupting CTβ2 expression ($CT\beta2^{-/-}$) (Figure 1.5). The most striking phenotype of the $CT\beta2^{-/-}$ mice was severe ovarian dysfunction resulting in sterility. Specifically, the ovaries of female $CT\beta2^{-/-}$ mice lacked ovarian follicles and had interstitial stromal cell hyperplasia [74]. Also, male $CT\beta2^{-/-}$ mice showed progressive testicular degeneration [74].

In striking contrast, despite the abundance of CTβ2 in nervous tissue, brains of $CT\beta2^{-/-}$ mice had no gross pathology nor any significant decrease in PC content compared to those of wildtype mice [74]. However, neurons from the brains of $CT\beta2^{-/-}$ mice were not visualized at the cellular or molecular level for defects in neuronal morphology or PC metabolism. Also, since non-neuronal cells far outnumber neurons within the brain, it is possible that subtle alterations in neuronal PC content may not be appreciated in measurements of whole brain PC.

1.13 Tissue Specific Roles of CT isoforms

PC is a key constituent of lung surfactant. Surfactant is synthesized and secreted by alveolar type II epithelial cells to reduce surface tension within alveoli during expiration. Insufficient surfactant production is associated with neonatal respiratory distress syndrome and can contribute to acute lung injury. During development, alveolar type II epithelial cells up-regulate PC production in order to provide sufficient PC for surfactant production.

Although several CT isoforms are detectable in mouse lung, quantitative real-time PCR has shown that CTα mRNA transcripts are 10 X more abundant than either CTβ2 or CTβ3 transcripts [74]; thus, CTα is the most likely isoform involved in surfactant PC production. Also, over-expression of CTα in fetal type alveolar type II epithelial cells increased PC production [82]. Recently, Mallampalli *et al.* reported that oxysterols decreased PC production for surfactant through ERK-mediated phosphorylation of CTα [83].

Role of CT isoforms in gonadal function

In Drosophila, *Cct1*, encodes a CT ortholog involved in ovarian follicle cell patterning [84]. This observation is particularly striking since, unlike in mammalian membranes, PC is not the most abundant phospholipid in insect membranes. Subsequently, studies in CTβ2$^{-/-}$ mice identified a novel role for CTβ2 in mammalian ovarian and testicular function as described above [74]. Given the conserved role of CT in ovarian function from *Drosophila melanogaster* to mouse, CT likely has a universal role in gonadal function.

1.14 Regulation of CT activity

Translocation of CT proteins between cytosol and membranes

CT is an amphitropic enzyme existing in a soluble form with low activity, and an active, membrane bound form (reviewed in [68]). Translocation of CT from cytosol to membranes is a major mode of regulation of CT activity [85] [86] and results in activation of CT with up to an 80 fold increase in its catalytic rate [73]. Translocation of CTα to membranes is stimulated by numerous

lipids including fatty acids, anionic phospholipids and diacylglycerols [87] [41, 88] [89]. The lipid binding domains of CTα and CTβ2 are very similar and so far, oleate has been identified as a lipid activator of CTβ2; however, exhaustive studies of lipid regulation of CTβ2 have not been done [49].

Davies *et al.* categorized the lipid activators of CT into two classes: class I activators include anionic phospholipids and fatty acids; class II activators include diacylglycerols and phosphatidylethanolamine [90]. The negatively charged head groups of anionic phospholipids are thought to interact with the basic residues of the amphipathic helix [91]. The class II lipid activators are thought to promote CT association with membranes via generation of negative curvature stress. The monolayer negative curvature strain induced by class II lipid activators is seen by their ability to promote hexagonal II phase in pure lipid mixtures with PC. Thus, in a biological bilayer conformation, class II lipids have increased stored curvature strain energy. Insertion of CT's lipid-binding domain into this "stressed" membrane may decrease the membrane's stored curvature strain energy [90].

Role of CTα phosphorylation

Phosphorylation is a major modality of enzyme regulation. Similar to many other enzymes, CT isoforms contain a large number of putative phosphorylation sites; however the role of phosphorylation in CT regulation is poorly understood. Within its phosphorylation domain, CTα contains sixteen serine residues which can be phosphorylated [69]. Of the sixteen serine residues within CTα that can be phosphorylated *in vivo*, seven serine

residues are found within consensus sequences for proline-directed kinases, another two serines are found within protein kinase C consensus sequences and another was found within a putative casein kinase II consensus phosphorylation site [92]. Although CT phosphorylation *in vitro* was catalyzed by casein kinase II, cdc 2 kinase, protein kinases C α and βII and glycogen-synthase kinase-3 [93], none of these kinases was reported to have a physiologic role in CTα phosphorylation *in vivo*. Indeed, CTα protein lacking its entire phosphorylation domain or containing substitutions at any one of its sixteen serine residues has no appreciable decrease in PC biosynthesis [69].

There is some indication that phosphorylation affects translocation of CT from cytosol to membranes. Soluble CT is highly phosphorylated while membrane-associated CT is less phosphorylated. Furthermore, treatment with okadaic acid, a phosphatase inhibitor, decreased translocation of CT from cytosol to membranes [94]. However, Houwelling *et al.* have demonstrated that CTα translocation from cytosol to membranes precedes dephosphorylation [95]. Thus, the exact role of phosphorylation in regulation of CT activity remains poorly understood.

Some insight has been gained from studies on insulin and epidermal growth factor (EGF) signaling in HeLa cells. Both insulin and EGF stimulated CT phosphorylation after oleate-stimulated dephosphorylation and translocation of CT to membranes [96]. Also, CT is a substrate for $p44^{mpk}$ MAP kinase, a proline-directed kinase, *in vitro* [96] and CT phosphorylation is reduced in the presence of olomoucine, an inhibitor of proline-directed kinases [97].

In surfactant production, extracellular-mitogen activated kinase (ERK) has been identified as a direct mediator of CTα-mediated PC biosynthesis. Furthermore, Mallampalli *et al.* recently reported that CTα contains a novel ERK-docking domain within its membrane-binding domain and they identified a nearby serine residue as a potential phosphorylation site for ERK [83].

Phosphorylation of CTβ2

Like CTα, CTβ2 contains many putative phosphorylation sites within its carboxyl terminus [49]; however, the role of phosphorylation in regulation of CTβ2 has not been studied. When a CTβ2-encoding cDNA is expressed in COS cells, CTβ2 protein is highly phosphorylated; however, the kinases responsible for its phosphorylation have not been identified. Both CTα and CTβ2 contain a large number of putative phosphorylation sites and, in fact, sixteen serines are conserved between them (Figure 1.6). As previously mentioned, CTβ2, alone contains a consensus site for cyclin-dependent kinase 5 (cdk5) phosphorylation [49] (Figure 1.6). Cdk5 is a member of the cyclin-dependent kinase family. Cdks are proline-directed threonine and serine kinases which orchestrate progression through the cell cycle (reviewed in [98]). Cdk5 is the sole isoform enriched in brain and has been implicated in neuronal migration [99] and axon growth [100] in neurons as well as PC12 cells [101] Also, Cdk5 is activated during regeneration of facial nerve axons after injury [102] thereby suggesting it plays a role in nerve recovery after injury.

1.15 Transcriptional regulation of *Pcyt1a*

Predictably, regulation of *Pcyt1a* and *Pcyt1b* gene expression is another contributor to the overall regulation of CT expression and activity. To date, elevation of CT mRNA and/or protein has been observed following partial hepatectomy [103], and in response to colony-stimulating factor or treatment with an arginine vasopressin metabolite (CTβ2) [75]. Once the murine *Pcyt1a* gene was cloned, its proximal promoter was studied to identify putative transcriptional regulatory elements [47]. The proximal promoter lacks a TATA box but contains a GC-rich sequence. There are numerous putative transcription factor binding sites in the proximal promoter, including the Sp family of transcription factors as well as transcriptional enhancer factor 4 (TEF-4), Ap1 and NFκB.

Three functional *cis*-acting elements containing multiple Sp-binding sites have been identified within the proximal promoter of *Pcyt1a* [104]. The role of Sp1 in *Pcyt1a* transcription is perhaps best understood in the context of the cell cycle. PC is the most abundant phospholipid in eukaryotic membranes and its synthesis greatly increases during the G0-G1 phase of the cell cycle in preparation for cell division [105]. In C3H10T1/2 fibroblasts, CTα mRNA synthesis up-regulates during the S phase of the cell cycle [105]. Banchio *et al.* have demonstrated that Sp1 is involved in the activation of *Pcyt1a* expression during the S phase of the cell cycle [106]. Interestingly, cyclin-dependent kinase 2, a member of the cyclin-dependent kinase superfamily

involved in cell cycle regulation, phosphorylates Sp1 to enhance its binding to the *Pcyt1a* promoter [107].

Much less is known about the roles of Sp2 and Sp3 in regulation of *Pcyt1a* transcription. In Drosophila SL2 cells, Sp2 represses *Pcyt1a* transcription; in fibroblasts, Sp2 stimulates *Pcyt1a* transcription [108]; however, the two cell lines differ in their basal production of Sp factors. Thus, it is postulated that the relative abundance of Sp transcription factors and their cellular context govern the expression of CTα mRNA [108].

A role for TEF-4 in *Pcyt1a* transcription has also been described. Using a yeast one-hybrid screening method, Sugimoto *et al.* identified TEF-4 as the transcriptional factor binding to an enhancer element (Eb) within the murine *Pcyt1a* proximal promoter [109]. TEF-4 is postulated to act as a dual transcriptional modulator: it suppresses *Pcyt1a* transcription via binding to the Eb element and activates *Pcyt1a* transcription via interactions with the basal transcriptional machinery. Subsequently, studies in COS-7 cells identified another transcription factor, Ets-1, as an enhancer of TEF-4 and an activator of *Pcyt1a* transcription [110].

Lipid regulation of CT activity has been well described and lipids can also directly regulate CT isoform expression. Sphingosine is a bioactive metabolite of sphingomyelin catabolism. In lung alveolar epithelial cells, sphingosine inhibited CTα mRNA expression via a negative regulatory element within the *Pcyt1a* promoter [111].

Sterol responsive element binding proteins (SREBPs) are a class of transcriptional factors that regulate the expression of cholesterol and fatty acid biosynthetic enzymes (reviewed in [112]). Interestingly, the *Pcyt1a* promoter contains a sterol responsive element and SREBP1a/SREBP2 stimulate CTα mRNA expression [113] as does sterol depletion of CHO cells, THP-1-derived macrophages [114] and murine alveolar type II epithelial cells [115]. However, Ridgway *et al.* have shown in CHO cells that SREBP-dependent activation of CT activity is dependent on fatty acid synthesis rather than up-regulation of CTα mRNA expression [113, 116], thus, SREBPs likely modulate CT at both the transcriptional and post-translational level.

1.16 Transcriptional Regulation of *Pcyt1b*

To date, promoters for CTβ2 and CTβ3 mRNAs have not been cloned thus nothing is known about the transcriptional regulation of CTβ isoforms. However, when Sp3 is over-expressed in murine fibroblasts, both CTα and CTβ2 mRNAs are up-regulated [117], thereby implicating Sp3 in the regulation of CTβ2 expression. As previously discussed, CTβ isoforms are preferentially expressed in brain, lung and gonadal tissues. Thus, identification of transcriptional elements and associated transcription factors involved in CTβ isoform expression may offer insight into the tissue-specific functions of CTβ isoforms.

There is also a precedent for growth factor-dependent regulation of CT expression. Colony stimulating factor 1 (CSF-1) is a trophic factor required for

both proliferation and survival of bone-derived macrophages. In a murine macrophage cell line, BAC1.2F5, CTα mRNA increased four-fold in response to CSF-1 [118]. Interestingly, CSF-1 also increased CTα mRNA stability, identifying an additional mechanism for regulation of CT isoform expression.

1.17 CT isoforms and cell survival

Since PC is the predominant phospholipid in eukaryotic membranes, it is predictable that interference with PC biosynthesis would impair cell growth and division. In fact, PC biosynthesis is also required for cell survival. Indeed, depletion of cellular PC in a CHO cell line with a conditional mutation in CTα [119] triggered apoptosis [120]. Also, direct inhibition of CT activity by ceramide [121] and antineoplastic alklyphospholipid [122] [123] as well as bacterial infection with *Streptococcus pneumoniae* [124] resulted in apoptosis. Although several apoptotic triggers have been identified, the link between CT activity, inhibition of PC biosynthesis and apoptosis is poorly understood (reviewed in [2]). One exception is the mechanism of farnesol-induced apoptosis. In CHO cells, Lagace *et al* found that farnesol treatment resulted in caspase-mediated cleavage of the nuclear localization signal from CTα and export of CTα from the nucleus to the cytoplasm [125]. It should be noted that farnesol is also an inhibitor of choline phosphotransferase, the enzyme that catalyzes the final step in the CDP-choline pathway; thus, apoptosis may be associated with multiple defects in PC biosynthesis.

Depletion of cellular PC also induces apoptosis in neuronal cell lines as well as terminally differentiated (non-dividing) hippocampal and cortical neurons [14] New evidence further supports the hypothesis that inhibition of PC biosynthesis rather than acceleration of PC turnover mediates neuronal death. Impairment in PC biosynthesis is also associated with several neurodegenerative, glycolipid storage disorders. In a mouse model of Sandhoff disease, characterized by accumulation of ganglioside, GM2, in brain [126], PC mass and CT activity were sharply decreased; however, neither CTα nor CTβ2 expression were altered, suggesting a post-translational regulation of CT activity [127]. Interestingly, in neurons of the Sandhoff disease mouse model, decreases in CT activity and PC biosynthesis correlated with impaired axon and dendritic growth [128] similar to the effect of alkylphosphocholines (PC synthesis inhibitors) on axonal extension in rat sympathetic neurons [19].

The converse phenomenon has also been observed. In Gaucher disease, a metabolic disorder characterized by an accumulation in glucosylceramide, axonal growth accelerates coincident with increases in CT activity and PC biosynthesis [76]. Also, glucosylceramide was identified as a direct activator of CT activity *in vitro*; thereby suggesting a novel role for gangliosides as lipid regulators of CT activity [76]. In PC12 cells, neurite growth is also influenced by glucosylceramide levels; however, this effect was attributed to prevention of PI-3 kinase signaling [129].

PC biosynthesis may also be directly neuroprotective. Gasull *et al.* reported that excitotoxic neuronal cell death dramatically inhibited PC biosynthesis with a concomitant rise in extracellular choline [130]. Moreover, choline-deficiency and inhibition of PC biosynthesis leads to apoptosis in cultured hippocampal neurons [13].

A large body of clinical and molecular data suggests that CDP:choline (also referred to as citicholine) is neuroprotective against many neurotoxic insults. CDP-choline has been used extensively in European markets to treat both acute and chronic cerebrovascular disorders. In fact, a Cochrane meta-analysis of thirteen double-blind, placebo-controlled clinical trials demonstrated a positive effect of CDP-choline on memory and behaviour. However, due to the short duration of the clinical trials, little can be concluded about long-term effect of CDP-choline on cognitive and behavioural function [131]. Recently, Teather *et al.* reported that long-term CDP-choline supplementation (500 mg/kg/day) ameliorated memory impairment in rats reared in impoverished environments [132].

In rodent models of focal and global cerebral ischemia, treatment with CDP-choline significantly reduced infarct volume and cerebral edema [133]. Pretreatment with CDP-choline dramatically reduced excitotoxic cell death in cerebellar granule cells [134] and decreased hippocampal damage associated with *Streptococcus pneumoniae*-mediated meningitis [124].

Several studies have reported the mechanism whereby CDP-choline is taken up into cells. Extracellularly, CDP-choline is hydrolyzed to CDP and

choline, taken up and rephosphorylated into CTP and phosphorylcholine and re-synthesized into CDP-choline [135]. While the neuroprotective mechanism of CDP-choline remains poorly understood, Hurtado *et al.* report a coincident decrease in loss of glutamate after ischemia in cultured cortical neurons [136]. Thus, in stroke and acute neural ischemia, CDP-choline supplementation may accelerate PC biosynthesis to prevent glutamate excitotoxity

In summary, the importance of choline and its metabolites in neuronal survival and recovery from injury has been clearly established. Despite this clear role, the source and metabolism of CDP-choline within neurons is poorly understood.

1.17 Rationale and Objectives

During neuronal differentiation, neurite outgrowth requires an increased supply of PC [8]. Like most other tissues, in neurons, the vast majority of cellular PC is provided by the CDP-choline pathway wherein the rate-limiting step, formation of CDP-choline, is catalyzed by CTP:phosphocholine cytidylyltransferase (CT) [40]. To date, three CT isoforms, CTα, CTβ2 and CTβ3, have been identified in rodents [49], [50] [51]; however, little insight has been gained into their individual contributions to PC metabolism. Our main objective is to identify how CT contributes to neuronal PC biosynthesis and thus to the process of neurite outgrowth and cellular differentiation.

In Chapter 2, we examined CT activity and CT isoform expression during neuronal differentiation. We hypothesized that CT expression must be up-regulated to accommodate the demand for PC biosynthesis during neurite growth. Furthermore, since CTβ2 is enriched in neuronal tissues [49, 75], we hypothesized that there is a specific up-regulation of CTβ2 expression in differentiating neuronal cells. To test this hypothesis, we examined CT activity as well as CT isoform mRNA and protein abundance in two neuronal cell lines: pheochromocytoma-derived PC12 cells and mouse neuroblastoma cells.

Since CTα and CTβ2 are both expressed in neurons, what specific function does CTβ2 fulfill that would necessitate its up-regulation during neurite outgrowth? There is strong evidence that phospholipids, including PC, are synthesized both in cell bodies as well as in axons [9, 17, 18, 137]. We hypothesized that CTα and CTβ2 are targeted to distinct subcellular locations

(i.e. cell body, proximal axons or distal axons) within neurons. Thus, in Chapter 3, the subcellular distribution of CT isoforms within sympathetic neurons was examined. Also RNA silencing and recombinant cDNA strategies were used to either "knockdown" or over-express CT isoforms in differentiating PC12 cells and determine their individual contributions to neurite formation and growth.

In Chapter 4, the role and regulation of CTβ2 activity in neurite formation and growth is investigated. In PC12 cells, both Akt and cyclin-dependent kinase 5 have been implicated in neurite outgrowth and branch formation. We examined how these kinases affect CTβ2-dependent neurite branching and examined whether CTβ2 is phosphorylated by either kinase.

Studies of CT expression and function in neuronal differentiation are particularly valuable in light of the well documented therapeutic benefits of CDP-choline in the treatment of ischemic stroke and other neurodegenerative conditions [131].

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Chapter 2

Enhanced expression and activation of CTP:phosphocholine cytidylyltransferase β2 during neurite outgrowth.

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2.1 Abstract

During differentiation neurons increase phospholipid biosynthesis to provide new membrane for neurite growth. We studied the regulation of phosphatidylcholine (PC) biosynthesis during differentiation of two neuronal cell lines: PC12 cells and Neuro2a cells. We hypothesized that in PC12 cells nerve growth factor (NGF) would up-regulate the activity and expression of the rate limiting enzyme in PC biosynthesis, CTP:phosphocholine cytidylyltransferase (CT). During neurite outgrowth, NGF doubled the amount of cellular PC and CT activity. CTβ2 mRNA increased within one day of NGF application, prior to the formation of visible neurites, and continued to increase during neurite growth.

When neurites retracted in response to NGF withdrawal, CTβ2 mRNA, protein and CT activity decreased. NGF specifically activated CTβ2 by promoting its translocation from cytosol to membranes. In contrast, NGF did not alter CTα expression or translocation. The increase in both CTβ2 mRNA and CT activity was inhibited by U0126, an inhibitor of mitogen-activated kinase/extracellular signal-regulated kinase kinase 1/2 (MEK1/2). In Neuro2a cells, retinoic acid significantly increased CT activity (by 54%) and increased CTβ2 protein, coincident with neurite outgrowth but did not change CTα expression. Together, these data suggest that the CTβ2 isoform of CT is specifically up-regulated and activated during neuronal differentiation to increase PC biosynthesis for growing neurites.

2.2 Introduction

The differentiation of neuronal cell lines is characterized by a halt in proliferation, and the production of neurites. The rapid growth of neurites places a heavy demand on the differentiating cell for new membrane components. It is therefore not surprising that studies in rat pheochromocytoma (PC12) cells have shown that phosphatidylcholine (PC) biosynthesis increases during neurite outgrowth [1]. Likewise, sympathetic neurons also increase phospholipid biosynthesis during axonal growth [2]. PC, the predominant phospholipid in mammalian membranes, is synthesized primarily by the Kennedy pathway in which choline is phosphorylated by choline kinase to phosphocholine which is converted to CDP-choline and subsequently to PC [3]. In this pathway, the conversion of phosphocholine to CDP-choline, catalyzed by CTP:phosphocholine cytidylyltransferase (CT), is the rate-limiting step. To date, two murine/human genes encoding CT isoforms have been identified: *Pcyt1a* and *Pcyt1b*.

CTα, the product of *Pcyt1a*, is expressed in all tissues. CTβ2 and CTβ3, which are splice variants of murine *Pcyt1b*, are both enriched in brain compared to other tissues [4], [5]. However, whereas CTβ2 is expressed in both embryonic and adult mouse brain, CTβ3 is found only in adult brain [5]. Orthologs of CTα and CTβ2 have been cloned from rat and have similar tissue distributions to those in mouse [6]. Interestingly, in rat hippocampus, CTβ2 mRNA is up-regulated by arginine vasopressin$_{4\text{-}8}$, a metabolite of

arginine vasopressin known to enhance memory and promote neurite growth in hippocampal neurons [6].

PC12 cells are widely used for studies on neurite growth that is induced by nerve growth factor (NGF) [7]. NGF elicits neurite outgrowth via activation of mitogen activated protein kinase (MAP kinase) [8]. MAP kinase signaling has also been implicated in ganglioside-dependent neurite outgrowth in murine Neuro2a cells [9] which rapidly differentiate in response to retinoic acid.

We hypothesized that in differentiating neuronal cells PC biosynthesis is stimulated by an increase in CT activity and/or expression. To test this hypothesis, we examined PC biosynthesis, CT activity and CT isoform expression in PC12 cells and in Neuro2a cells. The results demonstrate a role for CT in neuritogenesis. Specifically, we observed a striking induction in expression of the CTβ2 isoform during neurite outgrowth.

2.3 Materials and methods

Materials. The MEK inhibitor, U0126, was obtained from Promega. *All-trans* retinoic acid was from Sigma. All cell culture media and reagents were from Life Technologies, Inc.

Cell culture. PC12 cells were obtained from the American Type Cell Culture Collection. Cells were maintained in F12-K medium supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum (FBS) at 37°C in a humidified atmosphere containing 5% CO_2. For differentiation experiments, cells were seeded on collagen-coated 35 mm dishes at a density of 1 x 10^5 cells/dish. Cells were incubated overnight and then 50 ng/ml 2.5S NGF (Alomone Labs) was added. For NGF-withdrawn conditions, the cells were grown in NGF-containing medium for 5 days after which the medium was removed and replaced with control medium lacking NGF. All cells were harvested on day nine. The MEK inhibitor, U0126, was dissolved in dimethyl sulfoxide and added to the medium to give a final concentration of inhibitor of 50 μM. The control medium contained the same volume of vehicle. The murine neuroblastoma cell line, Neuro2a, was a generous gift from Dr. David Williams (State University of New York, Stonybrook). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM)/F12 medium (1:1 ratio) containing 10% heat-inactivated FBS. For differentiation experiments, cells were plated on 35 mm dishes at a density of 1.5 x 10_5 cells per dish in DMEM containing 2% fetal bovine serum. Following an overnight incubation,

cell medium was replaced with DMEM with 2% FBS containing 20 μM retinoic acid (Sigma). After incubation for 48 h, cells were harvested in homogenization buffer A [50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 2 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride and 100 μg/ml each of leupeptin and aprotinin] and treated as described for PC12 cells.

Determination of Phospholipid Mass and in vitro *CT activity assays*. Cells were collected in 1 ml of homogenization buffer A. The cells were counted using a hemacytometer and then sonicated for 20 s at 4°C. Cell lysates were centrifuged at 7,000 x *g* for 5 min to pellet nuclei and unbroken cells. Aliquots of supernatant were used for lipid extractions, *in vitro* CT activity assays and immunoblotting. Lipids were extracted as described by Sundler [10] and separated by thin layer chromatography (TLC) on Silica gel G60 plates. Phospholipids were separated using TLC with chloroform/methanol/acetic acid/formic acid/water (70:30:12:4:1) as a developing solvent. After TLC, and visualization with iodine vapor, the bands of interest were scraped and analyzed. PC mass was determined by measuring the phosphorous content [11].

For fractionation of membranes and cytosol, an aliquot of the cell lysate was centrifuged at 470,000 x *g* for 30 min. The supernatant corresponded to the cytosol fraction; the pellet (microsomal fraction) was re-suspended in homogenization buffer A. All protein concentrations were measured using the Bio-Rad protein assay with bovine serum albumin used as a standard. CT

activities in the total homogenate, soluble and membrane fractions were determined in the presence of PC:oleate vesicles by monitoring the conversion of phospho[^{3}H]choline to CDP-[^{3}H]choline as previously described [12].

RNA Preparation and Reverse Transcriptase-Mediated PCR. Total poly (A) RNA was isolated using the TRIZOL (Life Technologies Inc.) reagent as described by the manufacturer. One μg of total RNA was reverse-transcribed using a first strand cDNA synthesis kit (Superscript II, Life Technologies) according to the manufacturer's protocol. Aliquots of the reverse transcribed product were used for PCR amplification with the following sequence-specific primers for CTα: AGTGGAGGAGAAGAGCATCG (sense) and GGAAGTCTTGCCAGAGAAGG (antisense); choline kinase α: GTGAATGGATGGCTCCACTC (sense) and ACCTTCAAAGCCTGGTCATG (antisense); cyclophilin TCCAAAGACAGCAGAAAACTTTCG (sense) and TCTTCTTGCTGGTCTTGCCATTC (antisense); CTβ3: AAATGCAGCATGGACAAGG (sense) and TTTGCTTGCATAAGTGCCC (antisense). Two separate primer pairs were used for PCR amplification of CTβ2: pair 1, CCAAGGAGCTGAATGTCAGC (sense) and GCACTTGTTAACCAGGCACC (antisense); pair 2, GCCATGCCAGTAGTTACCACT (sense) and TTTGCTGCATAAGTGCCC (antisense). All PCR amplifications used the following program: 94°C for 1 min, 60°C for 1.5 min and 72°C for 1.5 min for a total of 32 thermocycles (40

cycles for CTβ3) which was within the linear response range for each primer pair. The primers generated amplicons of the following sizes: CTα: 232 bp; CTβ2: 452 bp (pair 1) and 302 bp (pair 2); CTβ3 (218 bp); choline kinase α (200 bp); and cyclophilin: 380 bp. All amplified products were resolved on 1.5% agarose gels and stained with ethidium bromide. The amounts of the products generated with the CTα, choline kinase α, CTβ2 and CTβ3 primer pairs were normalized to the amount of cyclophilin product.

RNase Protection Assays. Ribonuclease protection assays were performed using a RPA III™ kit (Ambion) according to the manufacturer's protocol. Briefly, 5 μg of total RNA was hybridized to [^{32}P]-labeled antisense riboprobes corresponding to CTα, CTβ2 or cyclophilin. Following an overnight hybridization, the RNA was digested with a singlestrand-specific RNase (Ambion) and then separated on a 5% denaturing polyacrylamide gel and detected by autoradiography. Riboprobe-protected fragments were predicted to be 200bp (CTα), 200bp (CTβ2) and 250bp (cyclophilin).

Immunoblots. Anti-human CTβ2 and anti-human CTα rabbit polyclonal antibodies were generous gifts from Dr. S. Jackowski (St. Jude Children's Research Hospital, Memphis, TN). The CTβ2 antibody was raised against a peptide corresponding to amino acids 347-365 of CTβ2 [4]. The CTα antibody was raised against a peptide corresponding to amino acids 1-17 of CTα [4]. Equal amounts of protein in cell lysates were separated by electrophoresis on

10% polyacrylamide gels containing 0.1% SDS and transferred onto Immobilon-P transfer membranes (Millipore). Ponceau S stain was used to compare protein loading in all lanes of the gel. The membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 (TBS-T) and then incubated overnight with either anti-CTα (dilution 1:1,000) or anti-CTβ2 (dilution 1:1,000) antibodies. The blots were washed with TBS-T for 1 h and incubated with secondary antibody, horseradish peroxidase linked to antirabbit IgG (1/2500 dilution) for 1 h. After incubation the blot was washed with TBS-T and visualized with Amersham ECL reagents.

2.4 Results

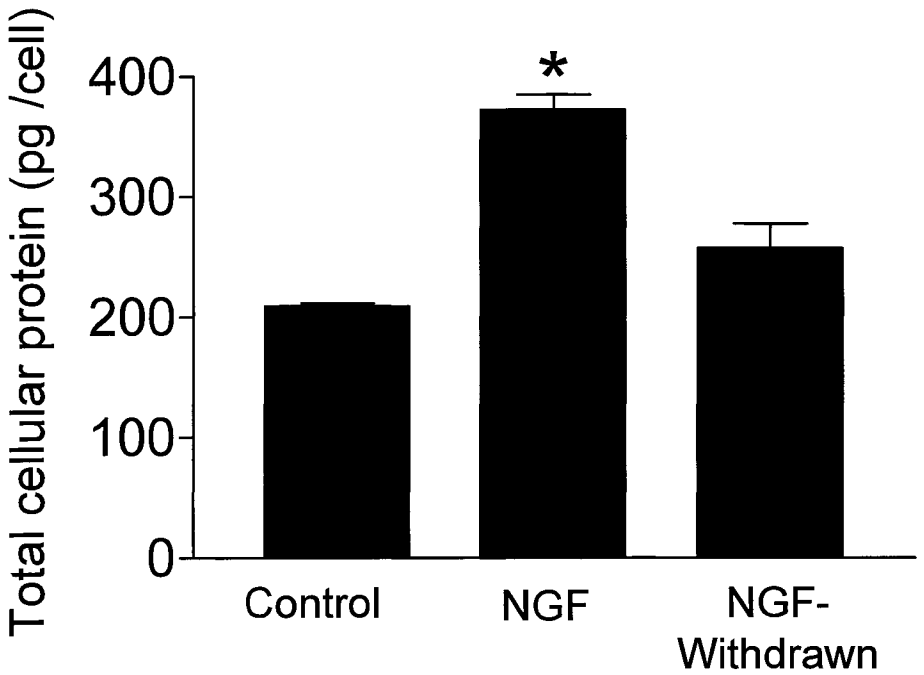
2.4.1 NGF increases cellular protein and phosphatidylcholine levels in PC12 cells. PC12 cells require NGF for differentiation to a neuronal phenotype, however, this differentiation is reversible. Once NGF is removed from the culture medium, the cells retract their neurites and resume proliferation. In our experiments, PC12 cells were incubated with medium containing 50 ng/ml NGF for 9 days (NGF), incubated with 50 ng/ml NGF for 5 days then cultured in medium lacking NGF for 4 more days (NGF-Withdrawn) or incubated without NGF throughout the experiment (Control). As expected, untreated cells continued to proliferate while NGF-treated cells stopped proliferating and started to extend neurites by day 3 of NGF treatment. NGF-withdrawn cells extended neurites during NGF treatment but the neurites retracted when NGF was removed from the culture medium.

Previous studies have shown that incubation of PC12 cells with NGF significantly increases cellular protein content as well as PC content [1]. We used these two parameters, along with the presence of neurites, to confirm that the PC12 cells were responding appropriately to NGF treatment. After 9 days of NGF treatment, the amount of protein per cell increased by 78% compared to that of control cells (373 pg/cell vs. 209 pg/cell) (Fig. 2.1A). Prior to NGF withdrawal on day 5, NGF-withdrawn cells had significantly higher cellular protein compared to that in control cells; however, at day 9, when the cells had been deprived of NGF for 4 days, cellular protein was no longer significantly different from that of control cells (258 pg/cell) Figure 2.1B

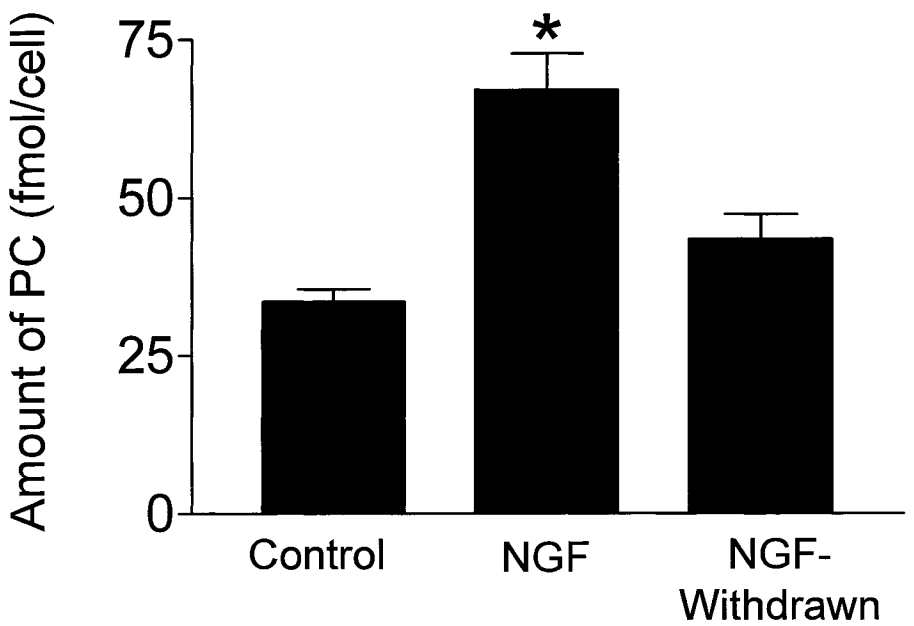
Figure 2.1. Cellular protein and phosphatidylcholine are increased in NGF-treated PC12 cells. PC12 cells were incubated with 50 ng/ml NGF for 9 days (NGF), incubated for 5 days with NGF after which medium lacking NGF was present for 4 days (NGF withdrawn), or incubated without NGF for 9 days (Control). Cells were harvested and lipids were extracted from cell lysates. The amount of cellular protein (**A**) and cellular phosphatidylcholine (PC) (**B**) were measured in each treatment group. Data represent means ± SEM (n = 6) of three independent experiments with duplicate dishes. * Control vs. NGF, $P < 0.001$.

Figure 2.1

A.



B.



demonstrates that treatment with NGF doubled the PC content per cell ($P < 0.001$). In contrast, the level of PC in the NGF-withdrawn cells was not significantly different from that in control cells. These results confirm that the increase in cellular protein and PC content is dependent on NGF and coincident with neurite outgrowth.

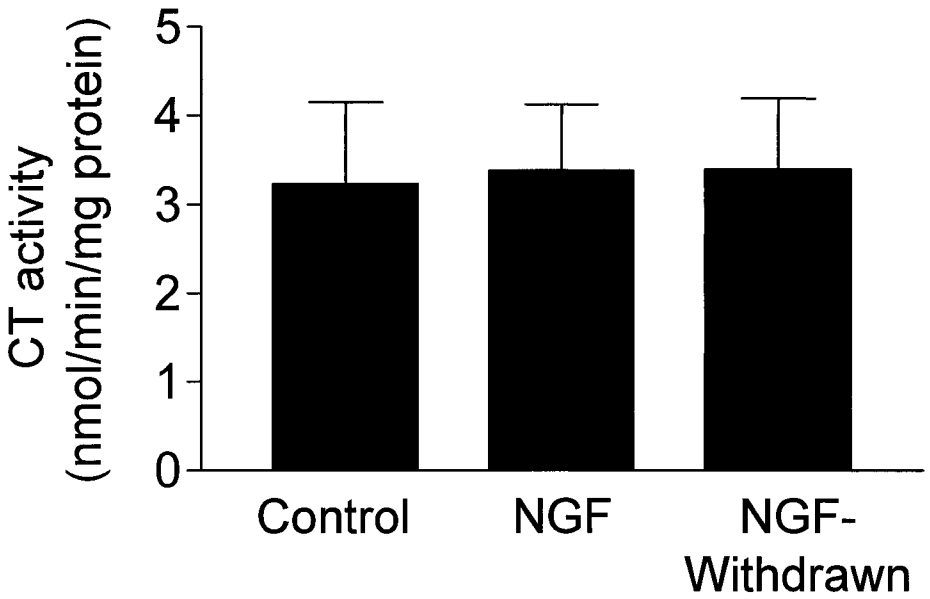
2.4.2 NGF stimulates CT activity in PC12 cells.

Consistent with previous studies [1], NGF did not significantly increase CT activity *in vitro* when the activity was normalized to mg protein (Fig. 2.2A, 3.24 nmol/min/mg protein and 3.39 nmol/min/mg protein in control and NGF-treated cells, respectively). Nor did NGF withdrawal alter CT activity. However, since NGF-dependent differentiation substantially increased the amount of protein per cell (Fig. 2.1A), presumably due to neurite outgrowth, we also normalized CT activity to cell number. Fig. 2.2B shows that NGF significantly increased CT activity per cell by greater than 100% ($P < 0.001$, Fig. 2.2B). Furthermore, in cells that were treated with NGF and in which NGF was subsequently withdrawn, CT activity returned to a level no longer significantly different from that in control cells. These results suggest that the increase in CT activity is dependent on NGF and coincides with neurite outgrowth. CT, an amphitropic enzyme, is activated by translocation from a soluble form to a membrane-associated form [13, 14]. We assayed soluble and membrane fractions of PC12 cells to determine whether or not NGF increased CT activity by promoting CT translocation to membranes. As shown in Fig. 2.3A, NGF

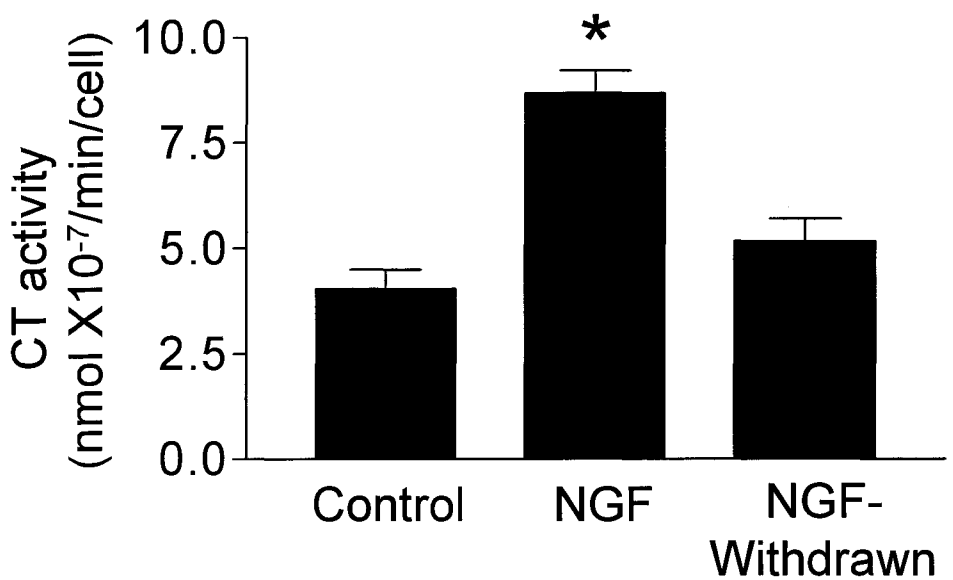
Figure 2.2. CT activity is stimulated by NGF in PC12 cells. Cells were incubated with 50 ng/ml NGF for 9 days (NGF), incubated for 5 days with NGF, then cultured for 4 more days in medium lacking NGF (NGF-withdrawn) or incubated without NGF for 9 days (Control). CT activity was measured in cell lysates and normalized to protein (**A**) or cell number (**B**). Data represent means ± SEM of 4 independent experiments with triplicate dishes. * Control vs. NGF, $P < 0.001$.

Figure 2.2

A.



B.



significantly increased CT activity in the membrane fraction compared to that of control cells ($P < 0.01$). CT activity in membranes from NGF-withdrawn cells was not significantly different from that of control membranes. NGF did not significantly alter CT activity in the soluble fraction (Fig. 2.3B). Together, these results suggest that NGF activates CT by increasing its activity on cellular membranes. However, since we did not observe a corresponding decrease in soluble CT activity (Fig. 2.3B), we hypothesized that NGF might also up-regulate CT expression.

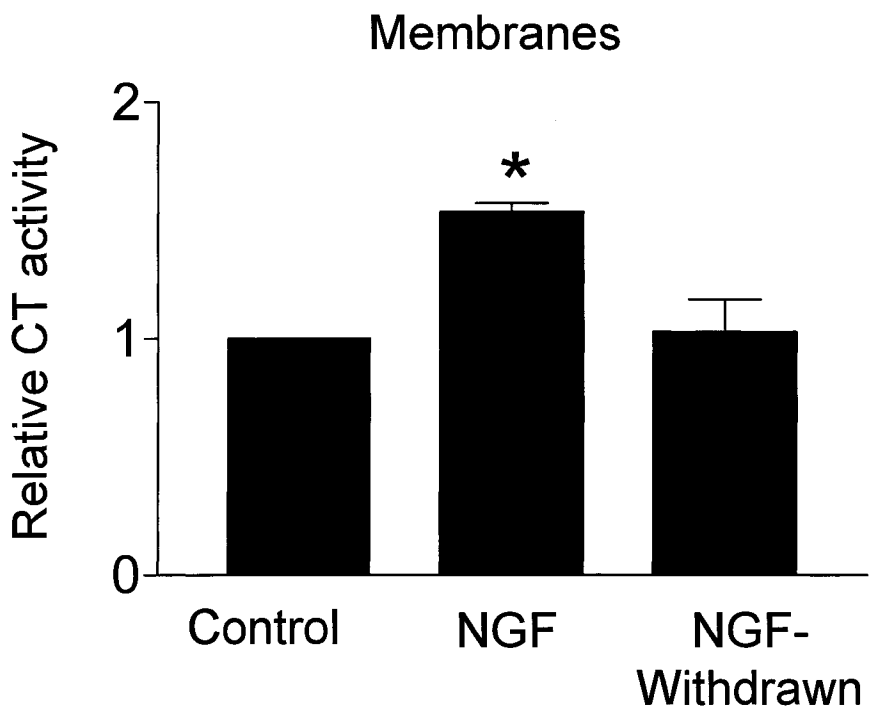
2.4.3 NGF induces CTβ2 mRNA expression.

To determine whether or not NGF increased expression of the different CT isoforms, we assessed the mRNA abundance of the three known rodent isoforms of CT (CTα, CTβ2 and CTβ3) by semi-quantitative RT-PCR. As shown in Fig. 2.4A, when PC12 cells were treated with NGF for 9 days, CTβ2 expression dramatically increased compared to that in cells not treated with NGF. In NGF withdrawn cells CTβ2 mRNA was barely detectable. Furthermore, NGF increased CTβ2 expression as early as one day after application, and CTβ2 mRNA abundance increased with prolonged NGF exposure (Fig. 2.4B). In contrast, NGF did not alter CTα mRNA expression or choline kinase α mRNA expression. (Fig. 2.4A). Since neither the murine nor the rat cDNA encoding CDP-choline:diacylglycerol cholinephosphotransferase has been cloned, we could not examine if NGF affected its expression. We also used a ribonuclease protection assay to

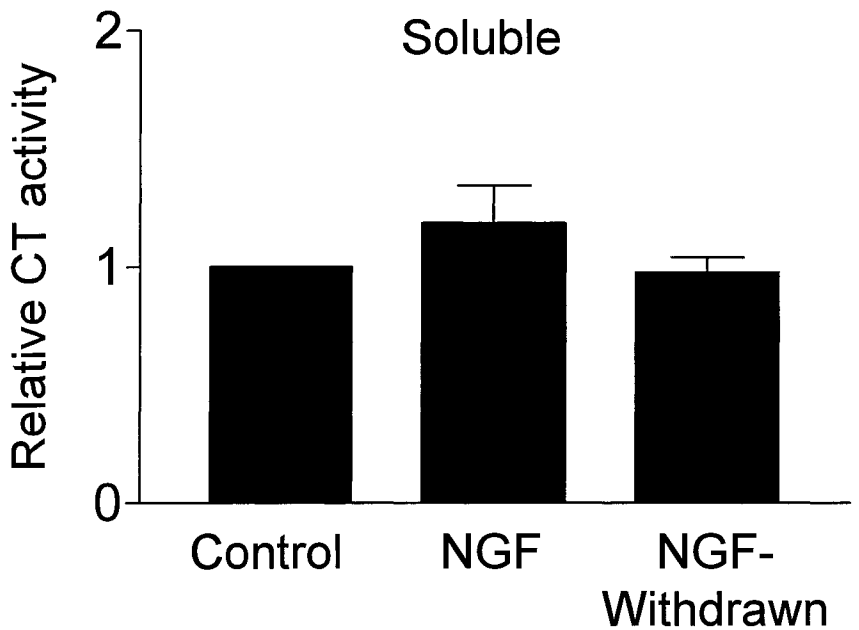
Figure 2.3. NGF increases membrane-associated CT activity but not soluble CT activity in PC12 cells. Cells were incubated as in Fig. 2.2. Cell lysates were centrifuged at 470,000 X *g* for 30 min. CT activity was measured in the pellet (membranes, panel **A**) and the supernatant (soluble fraction, panel **B**). CT activity was 0.309 fmol/min/cell in membranes of control cells and 1.27 fmol/min/cell in the soluble fraction of control cells. Data represent means ± SEM of three independent experiments with triplicate dishes. * Control vs. NGF, $P < 0.01$.

Figure 2.3

A.



B.



obtain a more quantitative assessment of whether or not NGF affected CTα or CTβ2 expression. As shown in Fig. 2.5A, CTβ2 mRNA was barely detectable in PC12 cells incubated without NGF (lane 2). However, CTβ2 mRNA was dramatically increased in cells treated with NGF for 7 days (lane 3). In contrast, and consistent with the RT-PCR results (Fig. 2.4A), CTα mRNA abundance was unaffected by NGF treatment. The approximately equal abundance of the 100 bp ribonuclease-protected cyclophilin mRNA fragment in all samples confirmed that equivalent amounts of RNA were used in the hybridization reactions.

Recently, a novel isoform of CTβ has been identified, CTβ3, which is predicted to be a splice variant of CTβ2 [5]. Like CTβ2, CTβ3 mRNA is found in mouse brain [5]. We used RT-PCR to determine whether PC12 or Neuro2a cells expressed CTβ3 mRNA. As shown in Fig. 2.5B, CTβ3 mRNA was undetectable in PC12 cells after 40 PCR cycles. For this reason, we conclude that CTβ3 plays little or no role in the NGF-dependent increase in CT activity in PC12 cells.

2.4.4 NGF increases the amount of CTβ2 protein and promotes translocation to membranes.

Since NGF treatment dramatically increased CTβ2 mRNA abundance, we hypothesized that NGF also increases the amount of CTβ2 protein. Fig. 2.6A demonstrates that NGF markedly increased the amount of CTβ2 protein compared to that in control cells. The anti-CTβ2 antibody produces a

Figure 2.4. CTα, CTβ2 and choline kinase mRNA expression in NGF-treated PC12 cells. A. Cells were incubated as in Fig. 2.2. On day 9, total RNA was isolated. 1μg of RNA was reverse transcribed, then sequence–specific primers for CTα, CTβ2, CTβ3, choline kinase α and cyclophilin were used in PCR amplifications to detect their relative abundance. **B.** PC12 cells were incubated without NGF for 9 days (Control), or incubated with 50 ng/ml NGF for 1, 2 or 5 days and total RNA was isolated. 1 μg of total RNA was reverse transcribed and CTβ2- and cyclophilin-specific primers were used in PCR amplifications to detect their relative abundance. Results were similar in two independent experiments.

Figure 2.4

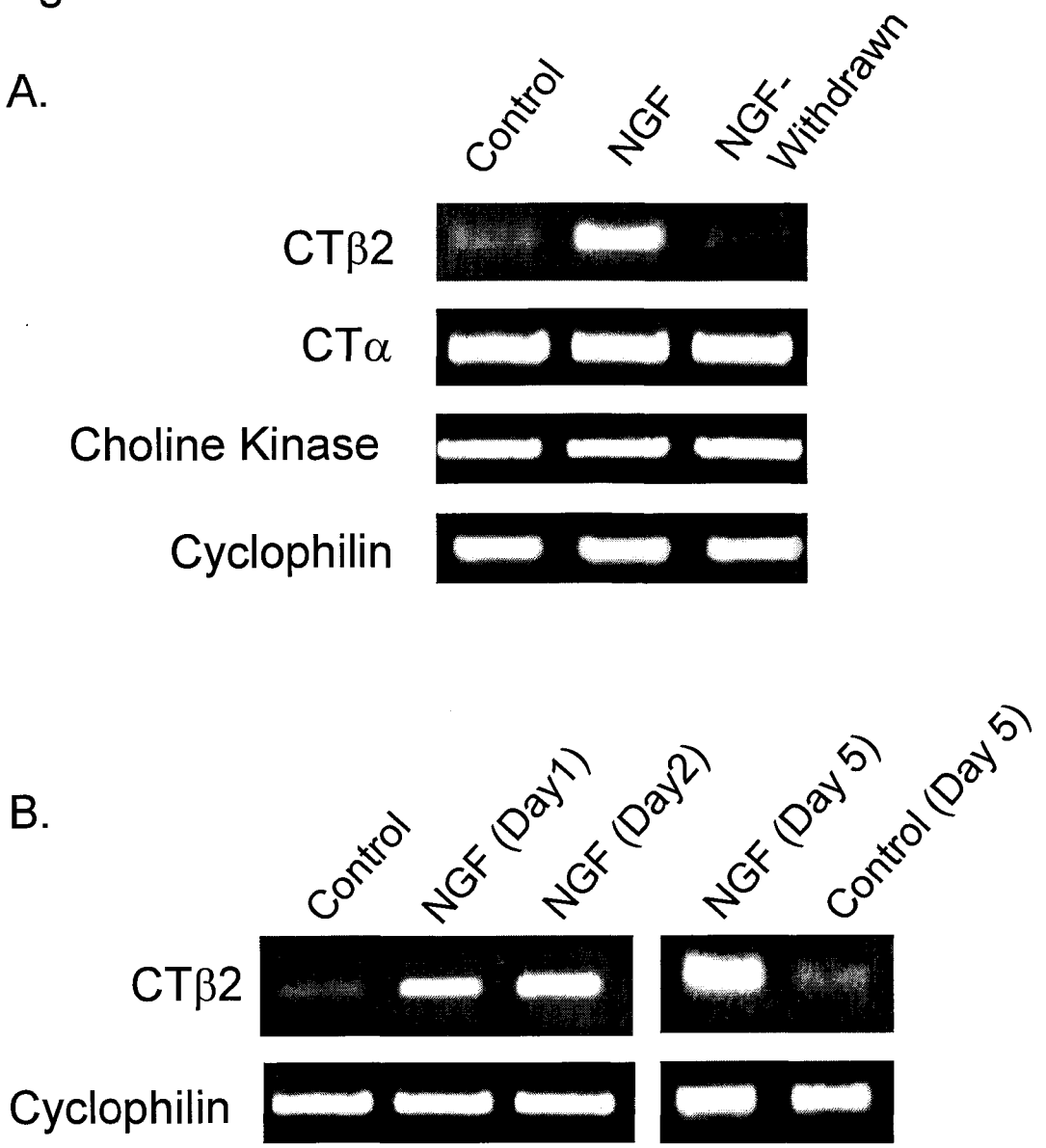
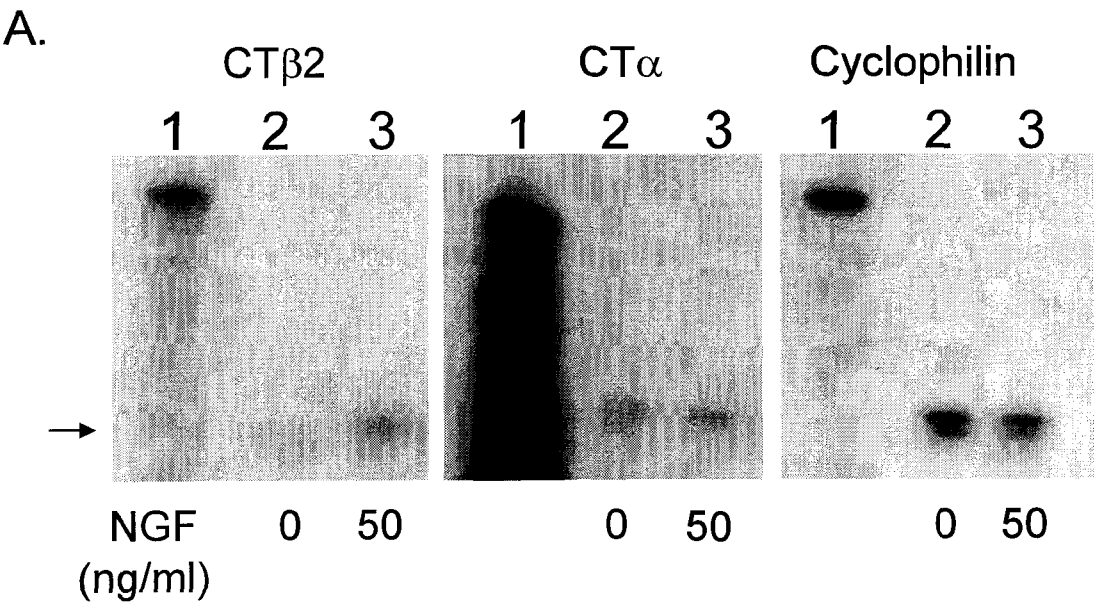
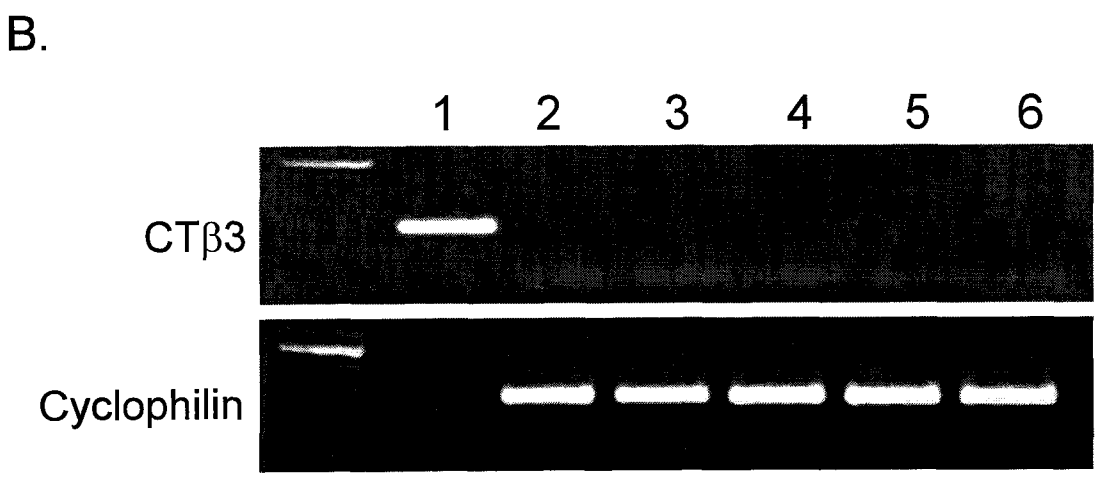


Figure 2.5. CT isoform expression in NGF-treated PC12 cells.

A. Ribonuclease protection assay in NGF-treated PC12 cells. PC12 cells were cultured for 7 days in the absence (lane 2) or presence (lane 3) of 50 ng/ml NGF. Total RNA (5 μg) was incubated with CTα–, CTβ2- and cyclophilin-specific ribonucleotide antisense probes. Hybridization of the antisense probes to the target RNA gave rise to protected mRNA fragments of the following sizes: CTβ2, 193 nucleotides; CTα, 200 nucleotides; cyclophilin, 103 nucleotides. Lane 1 shows undigested ribonucleotide antisense probe specific CTβ2, CTα or cyclophilin as indicated. **B.** PC12 cells were incubated as described in Fig 2.2. Neuro2a cells were plated and incubated for 48 h in the presence or absence of 20 μM retinoic acid. Total RNA was isolated as described for Fig. 2.4. Primers for CTβ3 and cyclophilin were used in PCR amplifications to detect their relative abundance. cDNA templates used in the PCR reactions were as follows: lane 1, plasmid containing full-length CTβ3 cDNA; lane 2, PC12 cells incubated without NGF; lane 3, NGF-treated PC12 cells; lane 4, NGF-withdrawn PC12 cells; lane 5, Neuro2a cells incubated without retinoic acid; lane 6, retinoic acid-treated Neuro2a cells. Results were similar in two independent experiments.

Figure 2.5





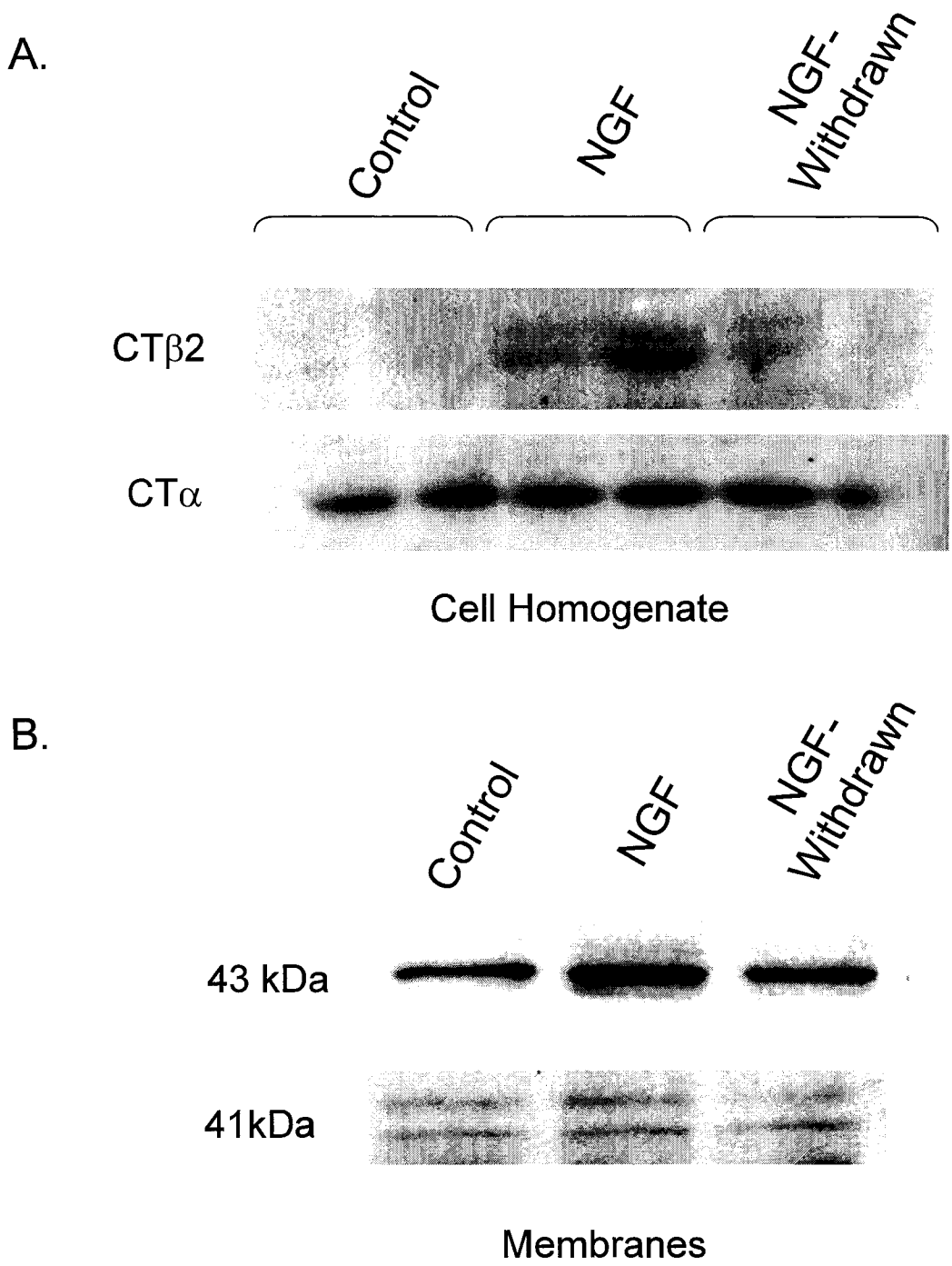
characteristic doublet which corresponds to the predicted 43 kDa CTβ2 protein and a larger, phosphorylated form of CTβ2 [4]. Moreover, when NGF was removed from the culture medium, and the PC12 cells retracted their neurites, the amount of CTβ2 protein decreased to virtually undetectable levels. In contrast, NGF treatment did not alter the amount of CTα protein (Fig. 2.6A). These results suggest that the increase in the amount of CTβ2 protein also depends on NGF and coincides with neurite outgrowth, consistent with the observed increases in PC content and CT activity.

Since CT activity increased in the membrane fraction of NGF-treated PC12 cells, we hypothesized that NGF not only up-regulates the expression of CTβ2 specifically, but also activates CTβ2 by promoting its translocation from the soluble reservoir to membranes. Immunoblot analysis (Fig. 2.6B) demonstrated that membranes of NGF-treated PC12 cells contained more CTβ2 than did control or NGF-withdrawn cells. In contrast, membranes of NGF-treated cells did not show an appreciable increase in CTα immunoreactivity. The anti-CTα antibody revealed a doublet of immunoreactive bands in membranes from untreated, NGF-treated and NGF-withdrawn PC12 cells, as well as in the positive control (10 μg of protein from a lysate of a McArdle 7777 rat hepatoma cells which overexpress CTα, data not shown). The lower band corresponds to the predicted size of CTα (41kDa) but the origin of the second, larger immunoreactive band in all of the samples is not clear. We conclude that NGF increases CT activity in two specific ways. First, NGF upregulates CTβ2, but not CTα, expression.

Figure 2.6. NGF increases the amount of CTβ2, but not CTα, protein.

Cells were incubated as described in Fig 2.2. Proteins (25μg) from cell lysates (**A**) or membranes (**B**) were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Amounts of CTα and CTβ2 protein were assessed by immunoblotting with antibodies against CTα or CTβ2. Results were similar in two independent experiments.

Figure 2.6



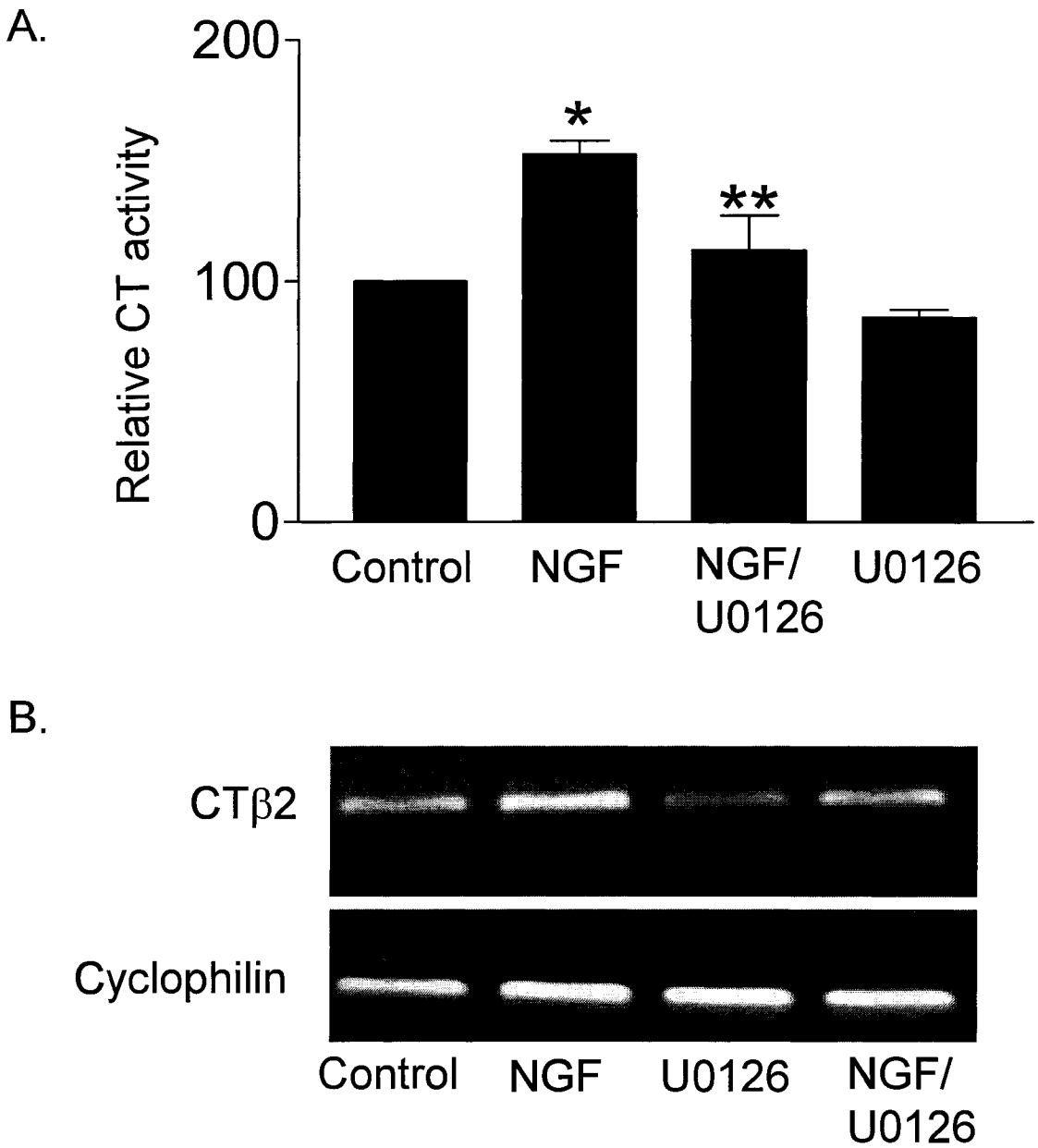
Second, NGF specifically activates CTβ2, but not CTα, by promoting the association of CTβ2 with membranes.

2.4.5 U0126 inhibits the NGF-dependent increase in CT activity and CTβ2 expression.

NGF signals through multiple intracellular cascades to promote neuronal survival and differentiation. Among these signaling cascades, MAP kinase is essential for neurite outgrowth [8, 15]. In this pathway, NGF activates Ras, which activates mitogen-activated kinase/extracellular signal-regulated kinase kinase (MEK) which subsequently activates MAP kinase. We, therefore, hypothesized that NGF signals through the MAP kinase pathway to induce CTβ2 expression and activation. We treated PC12 cells with U0126, an inhibitor of MEK1/2, which inhibits the MEK-dependent activation of MAP kinase, and examined its effect on CT activity and CTβ2 expression in NGFdifferentiated PC12 cells. Previous studies have shown that when NGF-treated PC12 cells are incubated with U0126 neurite extension is impaired compared to that in cells treated with NGF alone [16]. We, therefore, incubated PC12 cells for 5 days with 50 ng/ml NGF (NGF), with 50 ng/ml NGF and 50 μM U0126 (NGF/U0126), with 50 μM U0126 alone (U0126) or without both NGF and U0126 (control). Control and U0126- treated cells proliferated and exhibited no discernible morphological differences. Both NGF- and NGF/U0126-treated cells differentiated. However, it appeared that fewer NGF/U0126-treated cells extended neurites and that the average

Figure 2.7. The MEK inhibitor, U0126, prevents the NGF-induced increase in CT activity and CTβ2 mRNA. PC12 cells were incubated without NGF (Control), or incubated with 50 ng/ml NGF (NGF), 50 ng/ml NGF in the presence of 50 μM U0126 (NGF/U0126), or 50 μM U0126 (U0126) for 5 days. **A.** CT activity was measured in cell lysates. CT activity in control cell lysates was 2.67 fmol/min/cell. Data represent means ± SEM of three independent experiments. **B.** Total RNA was isolated, reverse transcribed and CTβ2 and cyclophilin mRNA expression were detected by RT-PCR. Results were similar in two independent experiments. * Control vs. NGF, $P < 0.01$, **NGF vs. NGF/U0126, $P < 0.05$.

Figure 2.7



neurite length at 5 days was shorter than in NGF-treated cells. These observations were not quantified. Consistent with the 9-day differentiation experiments (Fig. 2.2B), cells incubated with NGF for 5 days contained a significantly higher CT activity per cell than did control cells ($P < 0.01$, Fig. 2.7A). However, when PC12 cells were incubated with NGF in the presence of U0126, CT activity was not significantly different from that of control cells.

We used semi-quantitative RT-PCR to examine whether or not U0126 attenuated CTβ2 mRNA and CTα mRNA expression. In cells treated with NGF and U0126 together, CTβ2 mRNA abundance was appreciably less than in NGF-treated cells (Fig. 2.7B). CTα mRNA abundance was the same under all treatment conditions (data not shown). These results support our hypothesis that MAP kinase signaling mediates the NGF-dependent increase in CTβ2 expression and the subsequent increase in CT activity.

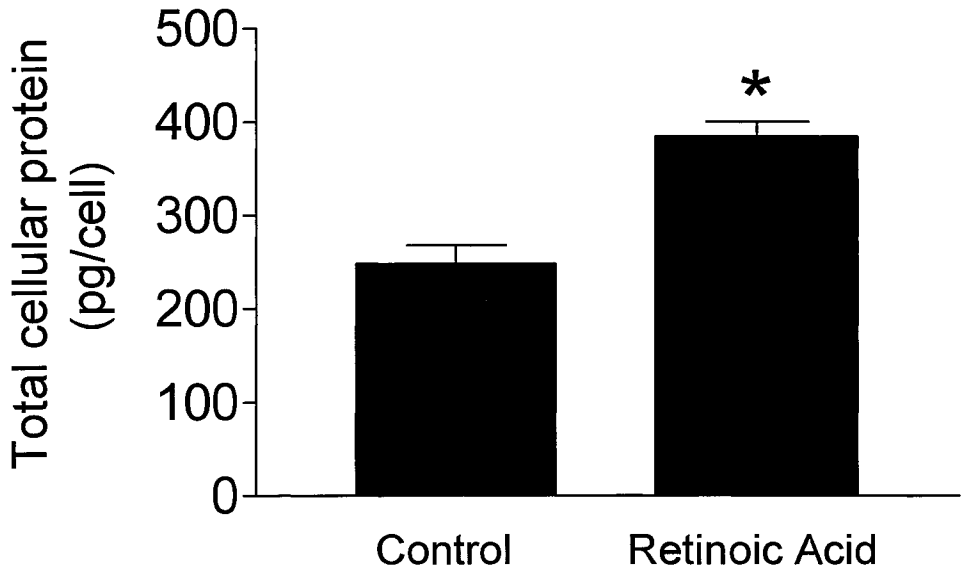
2.4.6 Retinoic Acid stimulates CT activity in Neuro2a cells.

Neurite outgrowth is characteristic of all differentiating neuronal cells. We, therefore, hypothesized that CTβ2 expression and CT activity would be increased in any cells that were actively growing neurites, rather than only in cells which differentiate upon NGF treatment. To test this hypothesis, we measured CT activity in Neuro2a cells which do not require NGF for differentiation but instead rapidly differentiate in response to treatment with retinoic acid [17]. We plated Neuro2a cells on 35 mm dishes and treated them with 20 μM retinoic acid for 48 h. Within 12 h after retinoic acid application,

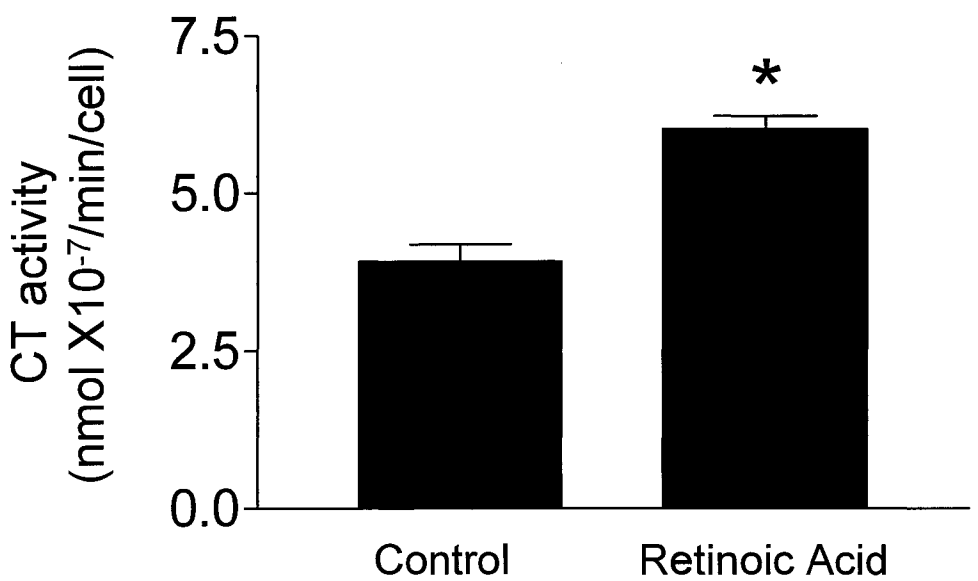
Figure 2.8. Retinoic acid increases both CT protein and activity in Neuro2a cells. Murine Neuro2a cells were incubated without retinoic acid (Control) or treated with 20 μM retinoic acid for 48 h (Retinoic acid) and then harvested as described for Fig. 2.2. **A**. Cellular protein was measured in cell lysates. **B**. CT activity was measured in cell lysates. Data represent means ± SEM of three independent experiments. * $P < 0.05$.

Figure 2.8

A.



B.



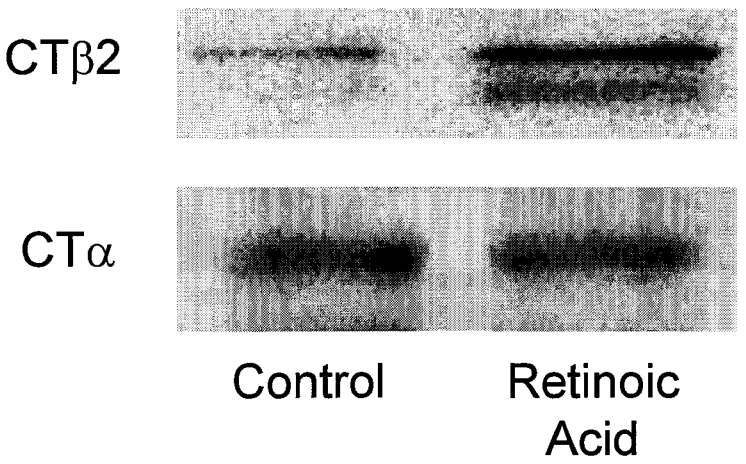
virtually all cells had stopped proliferating and had extended neurites longer than the diameter of a cell body. Retinoic acid-dependent differentiation increased cellular protein in Neuro2a cells ($P < 0.01$, Fig. 2.8A), similar to the situation in NGF-dependent differentiation of PC12 cells (Fig. 2.1A). Also similar to PC12 cells, CT activity per mg protein did not increase (not shown) whereas the activity per cell increased by 54% ($P < 0.0025$) from 3.9 fmol/min/cell in cells incubated without retinoic acid to 6.0 fmol/min/cell in cells incubated with retinoic acid (Fig. 2.8B). From these data, we conclude that in both Neuro2a and PC12 cells, which differentiate in response to retinoic acid and NGF, respectively, CT activity is significantly increased during neurite outgrowth.

2.4.7 Retinoic Acid increases the amount of CTβ2 protein in Neuro2a cells.

Since retinoic acid treatment significantly increased CT activity in Neuro2a cells, we immunoblotted Neuro2A cell lysates to determine whether or not retinoic acid increased the amount of CT protein. As shown in Fig. 2.9, the amount of CTβ2 protein was substantially higher in retinoic acid–treated cells than in control cells. The amount of CTα protein in Neuro2a cells, however, was unaffected by retinoic acid treatment. Together these results support our hypothesis that CTβ2 expression and CT activity are increased during neurite outgrowth irrespective of the method of induction of differentiation.

Figure 2.9. Retinoic acid increases the amount of CTβ2, but not CTα, protein in Neuro2A cells. Neuro2A cells were cultured as described in Fig. 2.8. Cells were harvested and cellular proteins were measured. Proteins (25 μg) from cell lysates were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS. Amounts of CTα and CTβ2 protein were assessed by immunoblotting with antibodies against CTα (**A**) or CTβ2 (**B**). Results were similar in two independent experiments.

Figure 2.9



2.5 Discussion

Neurite outgrowth places a high demand on neurons for membrane biosynthesis. This is especially true for PC biosynthesis since PC is the most abundant phospholipid in mammalian membranes. We have shown that during neurite growth of PC12 cells, NGF stimulates PC biosynthesis by specifically up-regulating the expression and activity of CTβ2. Furthermore, in Neuro2a cells, retinoic acid-induced differentiation also stimulated CTβ2 expression and increased CT activity. We conclude that in both of these neuronal cell lines, the demand for PC during neurite growth is accommodated by an elevation of CTβ2 expression, with a concomitant elevation in CT activity.

CT activity and isoform expression in NGF-treated PC12 cells.

CT is an amphitrophic enzyme, existing in a soluble form with low activity, and an active, membrane-associated form (reviewed in [18]. Translocation of CT from a soluble form to membranes is a major mechanism of regulation of CT activity [13, 14]. Accordingly, we found that in PC12 cells NGF regulated CT activity by promoting translocation of CT to membranes. Furthermore, CTβ2, but not CTα, protein increased in the membranes of NGF-treated cells (Fig. 2.6B). These observations provide the first evidence that one isoform of CT can be differentially activated over another isoform. The increase in membrane-associated CTß2 in response to NGF was not simply due to an increase in the amount of CTβ2 protein, because the cytosolic CT activity was

not increased in parallel. Previous reports have shown that the incorporation of labeled choline into PC increases dramatically when PC12 cells are exposed to NGF and that this increase in PC biosynthesis coincides with neurite outgrowth [1]. Similarly, in our experiments, PC mass per cell doubled in cells treated for 9 days with NGF. Consistent with a previous report [1], NGF treatment did not increase CT activity when measured *in vitro* per mg of protein. Nevertheless, as expected, the dramatic morphological changes of neuronal differentiation were associated with significant increases in cellular protein content (Fig. 2.1B); thus, we normalized our CT activity measurements to cell number as well as mg protein. CT activity per cell increased by 50% after 5 days of NGF treatment and by 100% after 9 days of NGF treatment (compare Fig. 2.7B to Fig. 2.2B). Although Araki and Wurtman did not report CT activity per cell, they did measure the rate of the CT-catalyzed reaction in intact cells [1]. They reported that the CT-catalyzed reaction was stimulated by ~2-fold in PC12 cells after 2 and 4 days of incubation with NGF [1]. Our data suggest that this increase in CT activity corresponds to the differential activation and expression of the CTβ2 isoform. Araki and Wurtman also reported that cholinephosphotransferase activity, which catalyzes the final step in PC biosynthesis, increased by approximately 50% after 4 days of NGF treatment [1]. Since the amount of diacylglycerol (a substrate for this enzyme) increased 4-fold after 4 days of NGF treatment, the authors speculated that the increase in cholinephosphotransferase activity

was likely due to the increase in the amount of this substrate. Thus, CPT activity may also increase to accommodate the demand for PC biosynthesis. Diacylglycerol is also a lipid activator of CT in many types of cells including human neuroblastoma [19] cells and HeLa cells [20]. In HeLa cells, diacylglycerol is increased by phorbol ester treatment and activates CT by promoting CT translocation to membranes [20]. Given that diacylglycerol levels increase 4-fold after 4 days of NGF treatment [1], it is possible that NGF activates CTβ2 by increasing diacylglycerol levels and thereby promoting the translocation of CT to membranes.

CT can also be regulated by phosphorylation [3] and NGF might regulate CTβ2 by altering its phosphorylation status. To date, the exact role of phosphorylation remains unclear in the regulation of CTα and has not been studied with CTβ2. It is known that CTα does not have to be dephosphorylated for membrane binding, but CTα activity is reduced by phosphorylation [21, 22]. Like CTα, CTβ2 protein has many potential phosphorylation sites within its carboxyl terminus [4]. However, the amino acid sequence does differ between the two isoforms. For example, unlike CTα, CTβ2 has a consensus sequence for phosphorylation by cyclin-dependent kinase 5 [4]. When a cDNA encoding CTβ2 was transfected into COS-7 cells, CTß2 became highly phosphorylated [4], but the kinases responsible for this phosphorylation have not been identified. Although it is unknown whether phosphorylation is involved in the NGF-dependent activation of CTβ2 in PC12 cells, growth factors have previously been

implicated in CT phosphorylation. In HeLa cells, that are non-neuronal cells abundantly expressing CTβ2 mRNA in addition to CTβ1 and CTα mRNAs [4], both insulin and epidermal growth factor can stimulate CT phosphorylation without altering its distribution between cytosol and membranes [23]. It should be noted that the insulin/epidermal growth factor experiments were done prior to the identification of CTβ1/2, thus it is unclear which CT isoforms were phosphorylated. In our experiments with PC12 cells, immunoblotting showed no appreciable doublet for CTß2 in membranes. However, immunoblots of proteins from cell lysates revealed the characteristic doublet (Fig. 2.6A). Perhaps the membrane-associated form of CTβ2 requires dephosphorylation or is dephosphorylated upon membrane binding. Also, when lysates from NGF-treated PC12 cells were immunoblotted, the lower molecular weight band of CTß2 predominated. In contrast, in retinoic acid-treated Neuro2a cells, the higher molecular weight band, which likely corresponds to the more-highly phosphorylated form of CTβ2 [23], was predominant.

Based on these initial observations, we speculate that phosphorylation may play a role in the regulation of CTβ2 activity during neurite outgrowth. In addition, we found that NGF increased CTβ2 mRNA levels *prior* to neurite sprouting: while 5 days of NGF treatment were necessary for 50% of PC12 cells to bear neurites, CTβ2 mRNA was up-regulated within 1 day of NGF treatment. Furthermore, CTβ2 expression was clearly dependent on NGF because when NGF was withdrawn, CTβ2 mRNA and protein levels were the same as in cells incubated without NGF. Our results are consistent with some

differential display PCR studies in rat hippocampal neurons in which CTβ2 mRNA was the most highly up-regulated transcript in response to arginine vassopressin$_{4-8}$, a peptide that facilitates memory acquisition and stimulates neurite outgrowth [6]. Arginine vasopressin$_{4-8}$ can also up-regulate NGF expression [24]. Thus, the increase in CTβ2 mRNA induced by arginine vasopression might result from an elevation in NGF expression.

To date, there is no information about the transcriptional regulation of *Pcyt1b*, the gene encoding the CTβ isoforms. Several Sp-related transcription factors regulate transcription of *Pcyt1a*, the gene encoding CTα [25]. Moreover, when Sp3 is overexpressed in murine fibroblasts, both CTα and CTβ2 mRNAs are up-regulated, suggesting that Sp3 might also regulate transcription of *Pcyt1b* [25]. The present study demonstrates that differentiating PC12 cells specifically up-regulate CTβ2 mRNA but not CTα mRNA. Thus, we speculate that a neuron-specific, or differentiation-specific, transcription factor regulates expression of the *Pcyt1b* gene during neurite outgrowth. In PC12 cells, the expression of several immediate early genes, predominantly transcription factors (e.g. MafK), is increased within 1 h of NGF exposure [26]. Unlike several other identified NGF-responsive immediate early genes, MafK, a basic region/leucine zipper transcription factor, is specifically up-regulated upon NGF exposure and is essential for neurite outgrowth in both PC12 cells and immature telencephalic neurons [27].

CTβ2 and the MAP Kinase Cascade

NGF signals through myriad signaling cascades to ensure neuronal survival and differentiation. In PC12 cells, both NGF and pituitary adenylate cyclase-activating polypeptide converge upon, and require, MAP kinase signaling for neurite outgrowth [8, 15]. Since NGF treatment up-regulated CTβ2 expression coincident with neurite outgrowth, we examined whether or not the MEK1/2 specific inhibitor, U0126, impaired CTβ2 expression. Consistent with previous studies, when PC12 cells were incubated with both NGF and U0126, fewer cells had visible neurites. Moreover, CTβ2 mRNA levels, but not CTα mRNA, decreased in the U0126/NGF-treated cells as compared to NGF treatment alone. Because CT activity was also dramatically reduced in U0126/NGF-treated cells ($P < 0.05$) so that it was no longer any different from control cells, elevations in CTβ2 expression may completely account for the elevation in CT activity during neurite outgrowth. Our laboratory recently published data that further implicate MAP kinase signaling in CTβ2 expression in oncogenic Ha-Ras-overexpressing fibroblasts [28]. Other studies, in HeLa cells, have shown that purified CT (isoform unknown) can be phosphorylated *in vitro* by p44 MAP kinase [23]; however, it is unclear which CT isoform is phosphorylated because the studies were done prior to the identification of CTβ1/2. It is possible that MAP kinase signaling governs both the elevation in CTβ2 expression via transcriptional regulation as well as phosphorylation of CTβ2 to regulate its activity.

CT Expression and Activity in Neuro2A Cells

We hypothesize that enhanced PC biosynthesis and CTβ2 expression are necessary in all neurons during neurite growth. We used Neuro2a cells as a second model of neuronal differentiation because, unlike PC12 cells, Neuro2a cells produce neurites in response to retinoic acid [17]. Similar to the differentiated PC12 cells, CT activity/cell and the amount of CTβ2 protein were significantly increased in retinoic acid-treated Neuro2a cells. Furthermore, immunoblot analyses showed that retinoic acid substantially increased the amount of CTβ2 protein. Where do retinoic acid and NGF signaling converge to up-regulate CTβ2 protein and CT activity? While little is known about how retinoic acid stimulates neurite outgrowth in Neuro2a cells, Neuro2a cell differentiation is stimulated by the ganglioside GM_2 via a c-Src-dependent sustained activation of MAP kinase [9]. In PC12 cells, NGF also induces a sustained (60 min) activation of MAP kinase which is essential for differentiation and neurite outgrowth (8). Based on these observations, we speculate that MAP kinase signaling is likely important in regulating CTβ2 expression and CT activity in both Neuro2a cells and PC12 cells.

Despite the appreciable increase in CTβ2 protein and CT activity in retinoic acid-treated Neuro2a cells, we did not detect an increase in CTβ2 mRNA. This may be explained by an increase in the stability of CTβ2 protein. Indeed, enhanced CT activity associated with neurite growth has been observed with no apparent effect on CTα or CTβ2 expression [29]. In Gaucher disease, an inherited metabolic disorder with defective lysosomal glucocerebrosidase, the

accumulation of glucosylceramide accelerated axon growth with a concomitant increase in PC biosynthesis [29]. Also, in a mouse model of Gaucher disease (*Gba*$^{-/-}$), the elevation in PC biosynthesis is directly related to activation of CT; however, neither CTα nor CTβ1/2 expression (mRNA and protein) were altered in *Gba*$^{-/-}$ mice [29]. Consequently, the authors concluded that CT was activated by post-transcriptional mechanisms [29]. Studies with cholesterol-loaded macrophages have demonstrated directly that an increase in CT activity can be elicited exclusively by post-translational mechanisms for example solely by a partial dephosphorylation of the membrane-bound pool of CT [30].

CTβ2 expression and neurite outgrowth.

As shown with *in vitro* CT activity assays of membrane and soluble fractions, PC12 cells, like all other cells, have a large reserve pool of soluble CT. An important question is: why do PC12 cells and hippocampal neurons specifically elevate CTβ2 expression during neurite outgrowth rather than mobilizing the pre-existing soluble CT pool? We speculate that CTβ2 is targeted to neurites so that PC can be synthesized *in situ* for neurite outgrowth. PC biosynthetic enzymes have been detected in rat brain synaptosomes [31], squid axoplasm [32] and distal axons of sympathetic neurons [2, 33]. CTα is unlikely to be targeted to the neurite because of the nuclear localization signal within its amino terminus. Moreover, immunofluoresecence studies have localized CTα predominantly to the

nucleus of cell [34]. In contrast, CTβ2 does not contain a nuclear localization signal and immunofluorescence studies in murine macrophages found that CTβ2 localized to the endoplasmic reticulum [4]. For these reasons, we predict that CTβ2 might be targeted to neurites.

In conclusion, we have provided evidence that enhanced CTβ2 expression and CT activity coincide with neurite outgrowth irrespective of the molecular signal for neuritogenesis. Moreover, in PC12 cells, CTβ2 expression, like neurite outgrowth, is dependent on MAP kinase signaling. We propose that CTβ2 activation accounts for the increase in CT activity that occurs during neurite outgrowth. Together, the data strongly support a role for CT activity, and specifically CTβ2 expression, in neurite outgrowth.

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Chapter 3

CTP:phosphocholine cytidylyltransferase β2 is localized to axons and required for neurite outgrowth and branch formation.

3.1 Abstract

The hallmark of neuronal differentiation is the formation and growth of neurites. Phospholipid biosynthesis increases to provide new membrane for this growth. We have shown previously that up-regulation of the expression and activity of CTP:phosphocholine cytidylyltransferase beta 2 (CTβ2), the rate-limiting enzyme in phosphatidylcholine biosynthesis, accompanies neurite outgrowth. We hypothesized that the CTβ2 isoform is specifically targeted to axons to provide a local source of phosphatidylcholine. Using immunoprecipitation techniques, we demonstrated that CTβ2 protein, but not CTα protein, is found in axons of sympathetic neurons and retinal ganglion cells. Moreover, we hypothesized that CTβ2 expression is required for neurite growth and branching. We used RNA silencing to knock down CTα and CTβ2 expression. In CTβ2-silenced PC12 cells, the numbers of primary and secondary neurites are sharply attenuated. In contrast, the length of neurites on CTβ2-silenced cells is significantly increased and the net amount of membrane does not change. Silencing CTα expression had no effect on neurite number or length. Together, these data suggest that CTβ2 is localized to the distal axons of neurons and is essential for proper neurite and branch formation. This is the first identification of a lipid biosynthetic enzyme required for neurite sprouting and branch formation.

3.2 Introduction

Since its discovery in 1976, the rat pheochromocytoma-derived PC12 cell line has become an invaluable model for studies of neuronal differentiation. In response to nerve growth factor, PC12 cells stop proliferating and differentiate into sympathetic-neuron like cells [1]. The morphological hallmark of neuronal differentiation is the sprouting and growth of neurites: long, branched extensions of the plasma membrane. In neurons, these later mature into functional axons and dendrites.

In PC12 cells, like neurons, neurite outgrowth greatly increases the surface area of the cell, thereby producing a strong demand for new membrane synthesis. Accordingly, during neurite outgrowth, biosynthesis of phosphatidylcholine (PC), the predominant phospholipid in mammalian membranes, is accelerated [2, 3]. In neurons, PC is synthesized exclusively by the Kennedy pathway (reviewed in Chapter 1). The rate-limiting reaction in the Kennedy pathway is the synthesis of CDP-choline by CTP:phosphocholine cytidylyltransferase (CT). To date, three CT isoforms, encoded by two genes, have been identified in rodents: CTα coded by the *Pcyt1a* gene and CTβ2 and CTβ3, which are transcribed from alternate start sites within the *Pcyt1b* gene [4, 5]. While CTα and CTβ2 proteins share considerable sequence identity within their catalytic and lipid-binding domains, there are key differences within their amino and carboxyl termini. In its amino terminus, CTα has a nuclear localization signal and immunofluorescence studies in multiple cell types have localized CTα to the

nucleus and endoplasmic reticulum [6]. In contrast, CTβ2 lacks a nuclear localization signal and immunofluorescent studies in murine macrophages, HeLa cells and CHO58 hamster ovary cells have localized CTβ2 to the endoplasmic reticulum and Golgi [4]. Studies on the subcellular localization of CTβ3 have not been done.

CT isoforms also have different tissue distributions: CTα is expressed in virtually all tissues, CTβ2 and CTβ3 mRNAs have the highest expression in brain [4, 5, 7]. There is a strong correlation between CTβ2 expression and growth of axons from neurons. In rat hippocampal neurons, CTβ2 mRNA is up-regulated by arginine vasopressin $_{4\text{-}8}$, a metabolite of arginine vasopressin that promotes hippocampal axon growth and facilitates memory acquisition [8]. Also, we have reported that during neurite outgrowth, PC12 and murine Neuro2a cells dramatically up-regulate CTβ2 expression and CT activity while CTα expression is unchanged [3]. We hypothesize that PC12 cells and hippocampal neurons elevate CTβ2 expression rather than mobilize the pre-existing cellular CTα pool because CTβ2 is targeted to neurites and provides PC *in situ* for growth. We used compartmented cultures of sympathetic neurons and retinal ganglion cells to examine the distribution of CTβ2 within neurons.

Until recently, conventional theory held that growing axons could not synthesize their own phospholipids to accommodate the demand for new membrane. However, the activities of PC biosynthetic enzymes have been detected in rat brain synaptosomes [9], squid axoplasm [10] and distal axons

of sympathetic neurons [11]. Metabolic labeling of the axons of sympathetic neurons showed that [^{3}H]choline is rapidly incorporated into PC and sphingomyelin within the axon [11]. However, to date, isoforms of the enzymes catalyzing PC biosynthesis within axons have not been identified.

Since CTβ2 is dramatically up-regulated during neurite outgrowth and CT activity is found in axons, we hypothesized that CTβ2 plays a role in neurite outgrowth. To determine how CTα and CTβ2 contribute to neurite formation and growth, we used RNA silencing to "knock down" their expression in differentiating PC12 cells [12] and examined the cells for any alteration in neurite formation.

Neuritogenesis includes at least three stages: neurite initiation, maintenance and elongation, and neural network formation [13]. Neurites are categorized by their location relative to the cell body. Primary neurites project directly from the cell body. Secondary neurites, or branches, project from primary neurites. The processes of primary neurite sprouting and branch formation are distinct from one another and separately regulated. We examined the number and length of both primary neurites and branches in cells with siRNA-silenced CTα or CTβ2 protein synthesis. In addition, we over-expressed a recombinant, hemagluttinin-tagged CTß2 protein in PC12 cells and assessed its effect on neurite formation.

These studies provide insight into the localization of CTβ2 within neurons and its role in neurite sprouting and branch formation.

3.3 Materials and Methods

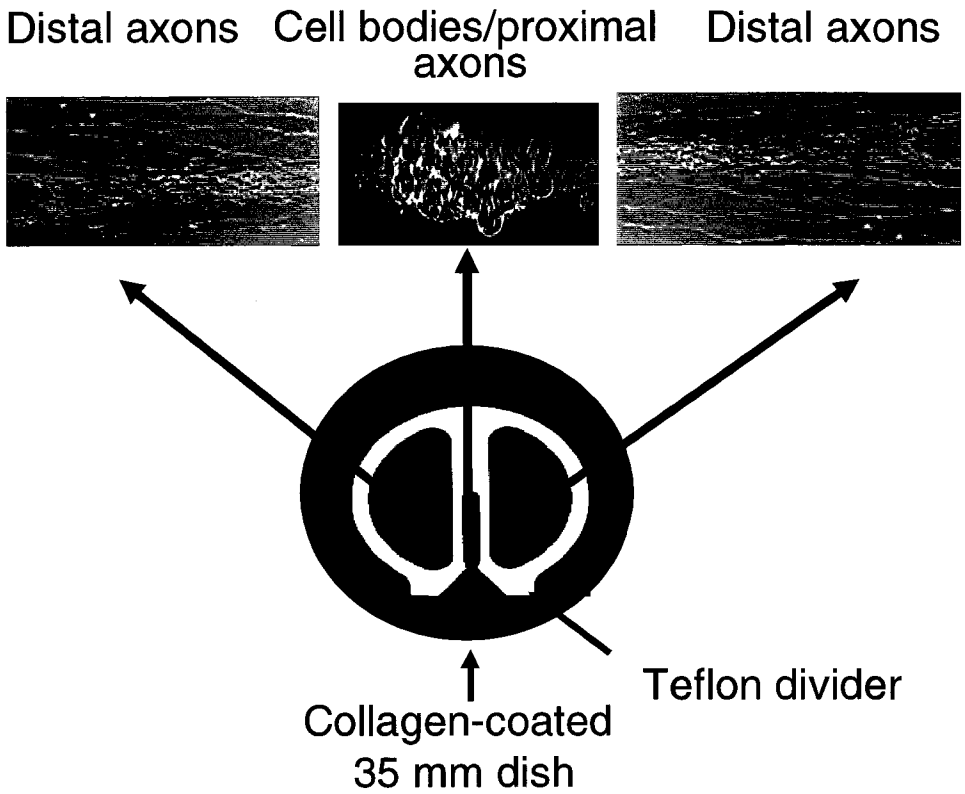
Cell culture: PC12 were obtained from American Type Cell Culture Collection. Cells were maintained in F12-K medium supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. For transfection experiments, cells were seeded on collagen-coated 35 mm dishes at a density of 2 X 10^5 cells/dish. Cells were transfected with a total of 2.5 µg of DNA per 35 mm dish. Cells were incubated overnight and then treated with 50 ng/ml 2.5S NGF (Alomone Labs). All cells were harvested on day five.

Preparation of compartmented cultures of rat sympathetic neurons and retinal ganglion cells

Sympathetic neurons from rat superior cervical ganglia were prepared as previously described [14]. Briefly, following dissection, superior cervical ganglia (SCG) from 1-day-old Sprague-Dewley rats were mechanically and enzymatically dissociated. To construct the compartmented culture dishes, culture dishes (35 mm) were coated with collagen and twenty parallel tracks were made by scratching the collagen-coated surface of the dishes. A Teflon divider (width ~0.5 mm), which creates 3 compartments, was sealed to the dish with silicone grease applied from a 20-gauge needle (Fig. 3.1). SCG were plated in the center chamber of the compartmented dishes at a density of 0.5 ganglion/dish in L15 base medium containing 2.5% rat serum (University of Alberta Laboratory Animal Services) and 1 mg/ml ascorbic acid.

Figure 3.1. Compartmented cultures of sympathetic neurons. A compartmented culture showing an entire culture and enlargement of a single track. Prior to plating the neurons, parallel scratches were made along the bottom of a collagen-coated 35 mm dish to generate tracks for axon growth. A Teflon divider was placed within the dish with a silicone grease barrier beneath it to create separate fluid environments within the center and side compartments. Dissociated neurons from rat superior cervical ganglia were plated in the center compartment. Axons extend from the center into the side compartments creating two isolated compartments: cell bodies with proximal axons and distal axons. Cell bodies/proximal axons and distal axons were harvested independently by mechanical disruption using a jet of distilled water.

Figure 3.1



Initially, all compartments contained 2.5 S nerve growth factor (Alomone labs, Israel) (100 ng/ml); however, nerve growth factor was withdrawn in the center compartment on day 7.

Retinal ganglion cells (RGC) were isolated from 1 day old rats and plated as previously described [15]. Briefly, retinal tissue was dissected from 1-day-old Sprague-Dawley rats and triturated in phosphate-buffered saline containing 0.15% trypsin inhibitor, 0.15% bovine serum albumin, and rabbit anti-macrophage antiserum (Accurate Chemical and Scientific Corp., Westbury, NY) until retinas were dissociated. Macrophages and microglia were removed by incubation of the cell suspension on a panning plate (150-mm Petri dish) coated with goat anti-rabbit IgG. RGC were re-suspended in base medium (Neurobasal medium containing glutamine (2 mM), insulin (5 μg/ml), *N*-acetylcysteine (60 μg/ml), progesterone (62 ng/ml), putrescine (16 μg/ml), sodium selenite (40 ng/ml), bovine serum albumin (0.1 mg/ml), triiodothyronine (40 ng/ml), transferrin (0.1 mg/ml), sodium pyruvate (1 mM) and 10 μM forskolin (Sigma)) to which was added 25 ng/ml brain-derived neurotrophic factor (PeproTech Inc., Rocky Hill, NJ), 50 ng/ml ciliary-derived neurotrophic factor (PeproTech Inc.), 50 ng/ml basic fibroblast growth factor (PeproTech Inc.), 2% B27 (Invitrogen), as well as 0.2% methylcellulose. Culture dishes (35 mm) were coated with poly-D-lysine (Sigma) and laminin (Sigma) and compartmentalized as described above. Isolated RGC were plated in the center chamber of the compartmented dishes at a density of 10,000–12,000 cells/dish in base medium containing the growth factors listed

above. The side compartments contained the same medium as the center compartment except that basic fibroblast growth factor was omitted and the concentration of brain-derived neurotrophic factor was increased to 75 ng/ml [15]. Proximal axons/cell bodies and distal axons of sympathetic neurons and distal axons of retinal ganglion cells were harvested after two weeks of growth.

Antibodies

Anti-M rabbit polyclonal antibodies, generated against the conserved membrane domain of rat liver CT (amino acids 256-288), were a generous gift from Dr. R. Cornell (Simon Fraser University, Vancouver, Canada). Anti-CTβ2 and anti-CTα rabbit polyclonal antibodies were generous gifts from Dr. S. Jackowski (St. Jude Children's Research Hospital, Memphis, Tennessee). The CTβ2 antibody was raised against a peptide corresponding to amino acids 347-365 of CTβ2 [4]. The CTα antibody was raised against a peptide corresponding to amino acids 1-17 of the CTα protein [4].

Immunoprecipitation and Immunoblotting:

PC12 cells, sympathetic neurons and retinal ganglion cells were rinsed with ice cold PBS and harvested into immunoprecipitation lysis buffer. Cell lysates were pre-cleared by centrifuging at 10,000 × g for 15 min at 4°C. After centrifugation, supernatants were transferred to new tubes and incubated with 3 μl of anti-M antibody on a rotating shaker at 4°C. After one h, 40 μl of a 50% slurry of protein A-Sepharose beads (pre-equilibrated with

immunoprecipitation lysis buffer) were added to the cell lysates and the lysates were incubated for an additional h at 4°C. Protein A-Sepharose bead conjugates containing immunoprecipitated CTα and CTβ2 proteins were washed three times with immunoprecipitation wash buffer (0.1% Triton X 100, 50 mM Tris-Cl, pH 7.4). CT isoforms were eluted from the protein A-Sepharose beads using SDS-PAGE sample buffer (without reducing agent) and heated at 70°C for 3 min. Equal amounts of immunoprecipitated proteins from the cell lysates were separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and transferred onto Immobilon-P transfer membranes (Millipore). Ponceau S stain was used to compare protein loading in all lanes of the gel. The membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 (TBS-T) and then incubated overnight with either anti-M (1: 1000), anti-CTα (1:1000) or anti-CTβ2 (1:1000) antibodies. The blots were washed with TBS-T for 1 h and incubated with secondary antibody, horseradish peroxidase linked to anti-rabbit IgG (1: 2500) dilution) for 1 h. After incubation, immunoreactivity was detected using Amersham Biosciences ECL reagents.

In vitro CT assays : Cells were cultured as described and collected in 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM dithiothritol, 0.1 mM phenylmethylsulfonyl fluoride and 100 μg/ml each leupeptin and aprotinin). The cells were counted and then sonicated for 20 s

at 4°C. Cell homogenates were centrifuged at 7000 X *g* for 5 min to pellet nuclei and intact cells. Aliquots of the supernatant were used for CT activity assays and immunoblotting. All protein concentrations were measured using the Bio-Rad protein assay. CT activities in the cell lysates were determined with PC:oleate vesicles by monitoring the conversion of phospho[^{3}H]choline to CDP-[^{3}H]choline as previously described [16].

Design of CTα and CTβ2-silencing RNA oligonucleotides.

Sequences specific to either CTα or CTβ2 mRNA were generated using the Ambion sequence finder algorithm (Ambion). Neither sequence showed any homology to any other sequence found in the NCBI database. Aside from their target CT isoforms, neither siCTβ2 nor siCTα-coding oligonucleotides were homologous to any other mRNAs listed in GenBank. cDNAs encoding small interfering RNAs targeted to either CTβ2 mRNA or CTa mRNA were designed using Ambion sequence finder (Ambion) and synthesized by the University of Alberta core facility. After annealing, the 50 bp cDNAs were inserted into pSILENCER 2.0 vector (siCTβ2) or pSilencer 4.1CMV (siCTα) and the plasmids were renamed pSiCTβ2 and pSiCTα. Both insertions were confirmed by sequencing.

Cloning and modification of CTβ2 cDNA

A cDNA of CTβ2, containing the entire open reading frame was cloned from a cDNA population generated from PC12 mRNA using the following primers: 5'GCCATGCCAGTAGTTACCACT (forward) and 3'GCTAAGGTTTGTGTGGGTTGTC (reverse). The amplicon was inserted into TOPO 2.1 vector and used as a template for addition of a hemagluttinin tag to the 3' end of the open reading frame. The following primer was used: 3'TCAAGCATAATCTGGAACATCATATGGATACTTCTCATCCTCATCCCCC TCACTCAT5'. After RT-PCR, the amplicon was cloned into the mammalian expression vector, pCI (Invitrogen) and named pCI-CTβ2-HA. Expression of the CTβ2-HA recombinant protein was confirmed by immunoblotting proteins of CTβ2HA-expressing cell lysates with anti-hemagluttinin and anti-CTβ2 antibodies.

Neurite Measurements

PC12 cells were cultured as described above; however, the plating density was lowered to $2X10^5$ to improve transfection efficiency. Twelve h after plating the cells, the medium was changed to F12K with no serum. Cells were co-transfected with a 3.7 kb mammalian expression vector encoding humanized recombinant GFP (phrGFP, Stratagene) and pSILENCER 2.0 (Ambion), a 3.1 kb plasmid encoding RNA-silencing oligonucleotides generated against CTβ2 or CTα. As a negative control, cells were transfected with molar equivalents of phrGFP and pSILENCER 2.0 containing a

scrambled, non-coding insert. Cells were transfected using the Lipofectamine 2000 reagent according to the manufacturer's protocol. Briefly, siRNA-encoding or control pSILENCER 2.0 vectors, along with phr-GFP were incubated with Lipofectamine 2000 in a 3:1 molar ratio for 20 min to allow DNA-reagent complexes to form and then applied to PC12 cells. After a twelve-h incubation with the Lipofectamine 2000-DNA complexes, cells were given F12K medium containing 50 ng/ml NGF to promote differentiation and neurite outgrowth. After 2, 3 and 4 days of NGF treatment, cells were viewed with fluorescence microscopy. phr-GFP and pSiCTβ2 (or pSiCTα) co-transfected cells were identified by their fluorescence at 520 nm under UV excitation. Co-transfected cells bearing at least one neurite longer than 20 pixels were considered to be differentiating cells and all of its neurites and branches were counted and measured. At each time point, at least 20 random fields of view were sampled for co-transfected cells.

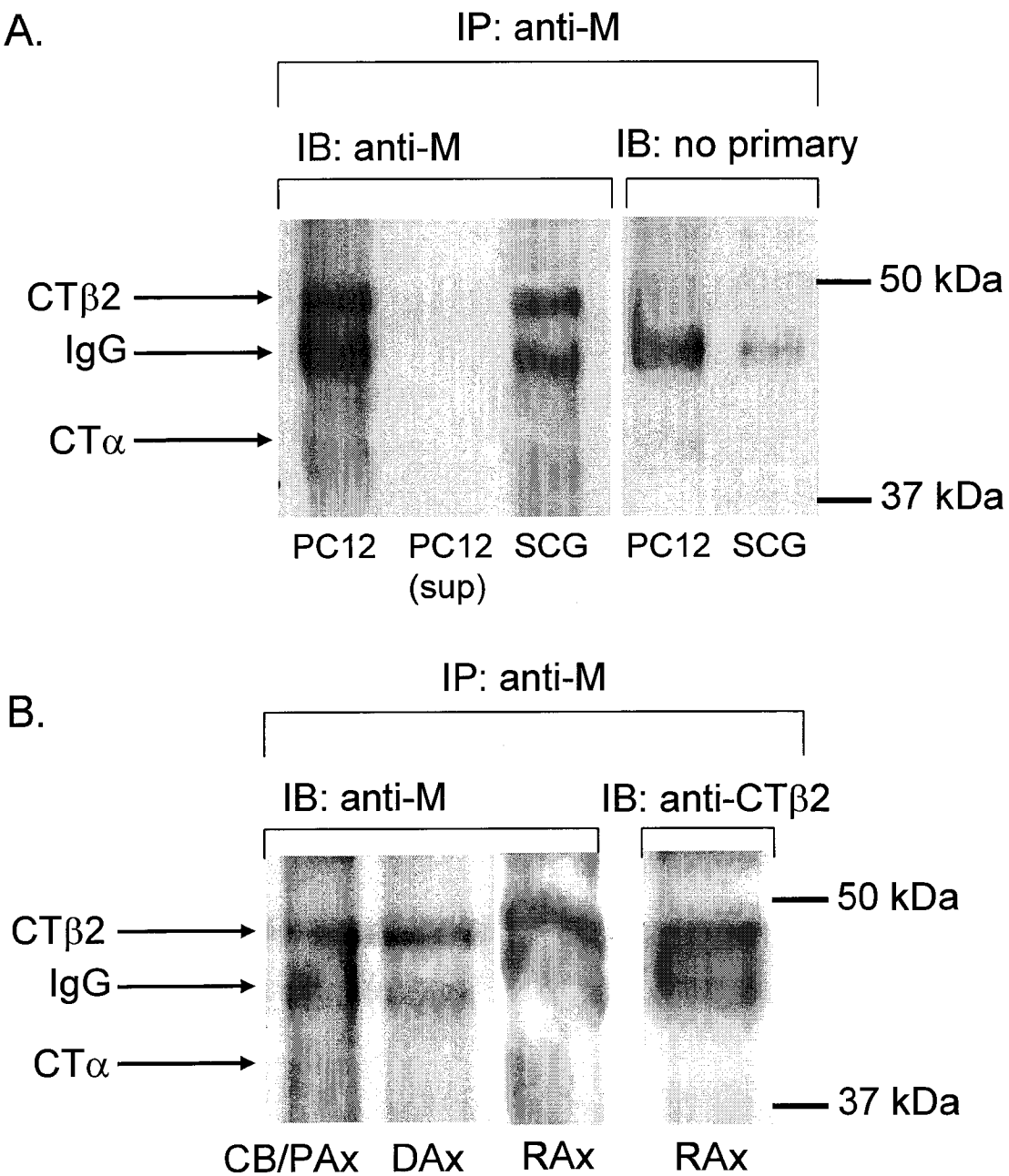
3.4 Results

3.4.1CTβ2 is localized to the axons of sympathetic neurons and retinal ganglion cells.

The subcellular localization of CT isoforms within neurons may provide insight into their relative contributions to PC biosynthesis and neurite growth. Since CTα is a predominantly nuclear isoform, we hypothesized that CTβ2 is the sole CT isoform in axons and accounts for all of the CT activity in axons. To determine whether or not this is the case, we used the anti-M antibody to immunoprecipitate CT isoforms from neurons and PC12 cells. The anti-M antibody was raised against the conserved, membrane domain of rat liver CTα, thus, it immunoprecipitates both CTα and CTβ2 proteins. To identify which anti-M immunoreactive band corresponded to which protein, we immunoblotted the anti-M immunoprecipitated proteins with specific anti-CTβ2 antibodies (data not shown) or with no primary antibody (Fig. 3.2A, Lanes 4,5). Based on these immunoblots, the upper band in Fig. 3.2A is CTβ2 protein, the middle band is the heavy chain of the anti-M antibody used in the immunoprecipitations, and the faint, lower band is CTα. Consistent with our immunoblot analyses of PC12 cells, both CTα and CTβ2 were immunoprecipitated from lysates of PC12 cells (Fig. 3.2A, Lane 1). A small amount of CTα protein was found in sympathetic neurons. (Fig. 3.2A, Lane 3). To determine whether CTβ2 is found in axons, we used compartmented cultures of sympathetic neurons and retinal ganglion cells. In compartmented cultures, the cell bodies and proximal axons are physically separated from the

Figure 3.2. CTβ2 is found in the axons of sympathetic neurons and retinal ganglion cells. A. PC12 cells and rat sympathetic neurons derived from superior cervical ganglia (SCG) were cultured as described in Materials and Methods. Lysates of PC12 cells and sympathetic neurons were immunoprecipitated (IP) with an antibody generated against the conserved membrane-binding domain of CT (anti-M). Afterward, the precipitated proteins from PC12 cells (Lanes 1, 4) and sympathetic neurons (Lanes 3, 5), and the supernatant of the precipitated proteins from PC12 cells (Lane 2), were separated by 10% acrylamide SDS-PAGE and transferred to polyvinylidene difluoride membranes. Amounts of CTα and CTβ2 protein were determined by immunoblotting (IB) with anti-M antibody (Lanes 1-3). The CTα, CTβ2-immunoreactive band and IgG heavy chain band were identified by immunoblotting with CTβ2-specific antibodies (shown in Fig. 3.2B Lane RAx) or without primary antibody (Lanes 4,5) and by their predicted molecular weight (CTα) **B.** Rat sympathetic neurons and retinal ganglion cells were cultured in compartmented cultures as described in Materials and Methods. Proteins of cell bodies/proximal axons (Lane CB/PAx) and distal axons (Lane DAx) from sympathetic neurons and distal axons of retinal ganglion cells (RAx) were immunoprecipitated (IP) with anti-M antibody, electrophoresed and immunoblotted (IB) with anti-M antibodies or anti-CTβ2 antibodies as indicated. Results were similar in two independent experiments.

Figure 3.2



distal axons by a silicone grease barrier and Teflon divider [17] (Fig. 3.1) . Thus in these cultures, distal axons can be manipulated independently from the cell bodies and proximal axons and can be isolated in pure form for biochemical analysis. As shown in Fig. 3.2B, an abundance of CTβ2 protein was immunoprecipitated from the distal axons and cell bodies of sympathetic neurons (Lanes CB/PAx and DAx) and from the distal axons of retinal ganglion cells (Lane RAx). Although a small amount of CTα could be immunoprecipitated from the cell bodies and proximal axons of sympathetic neurons, no appreciable amount of CTα was found in the distal axons of sympathetic neurons or retinal ganglion cells (Fig. 3.2B, Lanes DAx, RAx). In summary, these data provide strong evidence that CTα and CTβ2 proteins are found in the cell bodies and proximal axons of sympathetic neurons; however, CTβ2, alone, is detectable in the distal axons of both peripheral and central neurons.

3.4.2 CTβ2 expression is required for neurite outgrowth and branch formation.

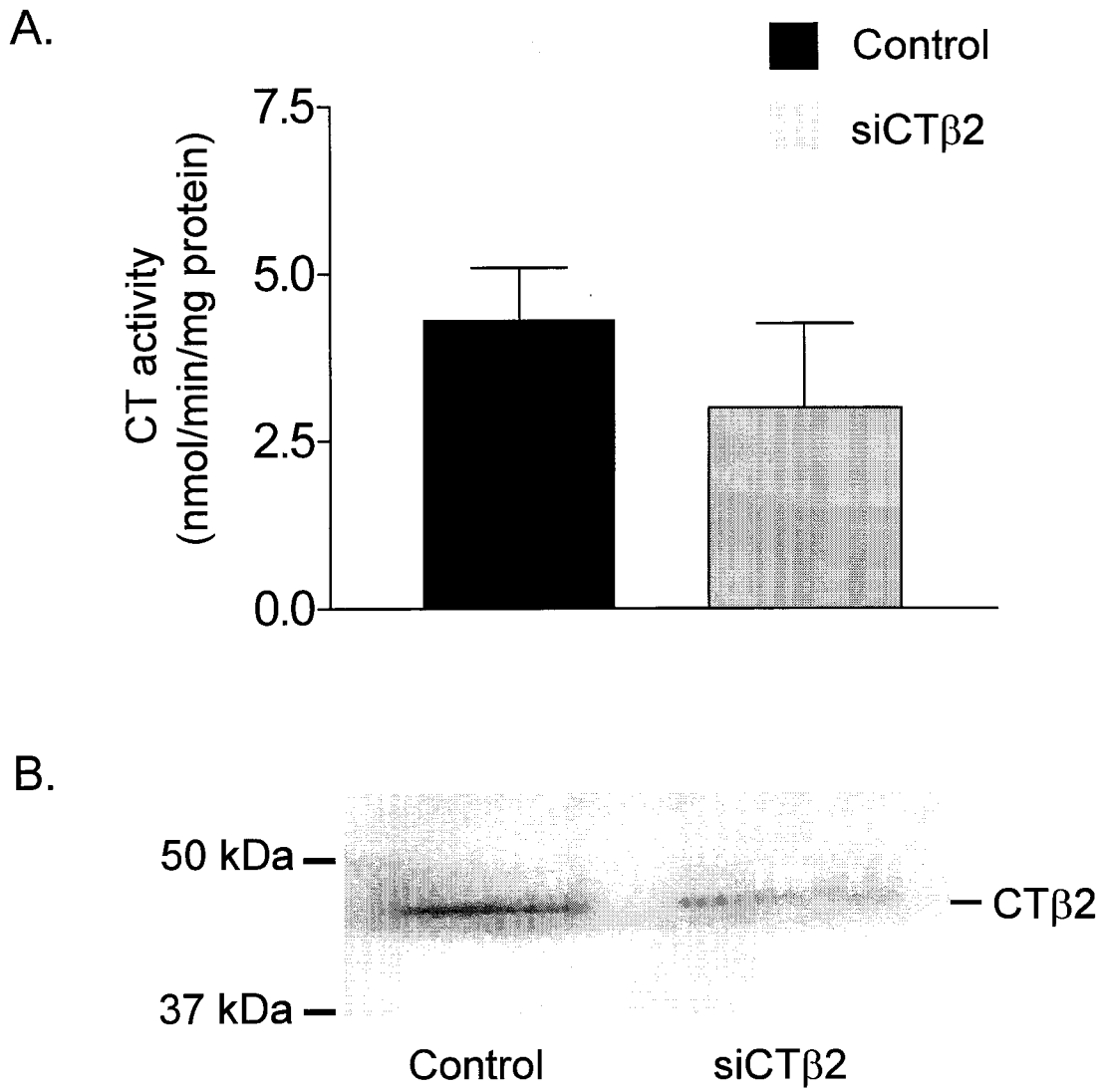
Our observations that 1) CTβ2 is found in the axons of neurons and 2) CTβ2 is dramatically up-regulated during neurite outgrowth [3] strongly suggest that CTβ2 is required for neurite growth. Thus, we hypothesized that suppression of CTβ2 expression in PC12 cells would impair neurite outgrowth while suppression of CTα expression would not affect neurite outgrowth. To test this hypothesis, we used an RNA silencing strategy to "knock down" CTα and CTβ2 expression in PC12 cells. Briefly, PC12 cells were co-transfected with

a GFP-coding plasmid and plasmids encoding small interfering RNAs targeted to CTβ2 mRNA (pSiCTβ2) or CTα (pSiCTα) or vector containing a scrambled insert (pSiCon). Because PC12 cells have a low transfection efficiency, we used Lipofectamine 2000 reagent to transfect the cells. Following transfection, the cells were treated with NGF to stimulate differentiation and neurite growth. To estimate the transfection efficiency, we identified GFP-expressing cells by fluorescence microscopy and compared the number of GFP-expressing cells to the total number of cells in several random fields of view. We approximated the transfection efficiency to be 30%.

Prior to examining pSiCTβ2-transfected cells for any discernible phenotype, we verified that the CTβ2-specific siRNA did suppress CTβ2 protein synthesis. As shown in Fig. 3.3B, cellular homogenate from pSiCTß2-transfected PC12 cells had substantially less CTβ2 protein than that of control cells. Moreover, since only ~30% of cells express the CTβ2-silencing RNA, CTβ2 protein, specifically within pSiCTβ2-transfected cells, is likely far lower than that shown in Fig. 3.3B. In addition to CTβ2 protein expression, we determined how suppression of CTβ2 protein affected CT activity *in vitro*. CT activity in cellular homogenates of pSiCTβ2-transected cells was 2.9 nmol/min/mg protein while that of control cells was 4.3 nmol/min/mg protein (Fig. 3.3A). Due to the presence of CTα and only partial RNAi-silencing of CTβ2, the reduction of CT activity is only ~30% and is not statistically significant.

Figure 3.3. SiRNA directed against CTβ2 mRNA decreases CTβ2 protein expression. PC12 cells were transfected overnight with pSiCTβ2 or vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 48 h. **A**. CT activity was measured in aliquots of homogenates from control and pSiCTβ2-transfected cells and normalized to protein. Data represent mean ± SEM of 2 independent experiments with triplicate dishes. **B.** 15 μg of proteins from the homogenates of control and pSiCTβ2-transfected cells were loaded onto 10% polyacrylamide gels containing 0.1% SDS and separated by electrophoresis. Amounts of CTβ2 protein were assessed by immunoblotting with antibodies against CTβ2. Results were similar in two independent experiments.

Figure 3.3



To assess how suppression of CTβ2 expression altered cellular morphology, phrGFP and pSiCTβ2 co-transfected cells were identified by fluorescence microscopy and compared to cells transfected with phrGFP and empty vector. Within 2 days of NGF treatment, there was a visible difference in the number of neurites per cell and after 4 days of treatment, the differences in neurite branching and neurite length is marked (Fig. 3.4, panels A and B). Specifically, the neurites of siCTβ2-expressing cells appear far longer and grow in a much more linear direction with fewer points of attachment and directional changes.

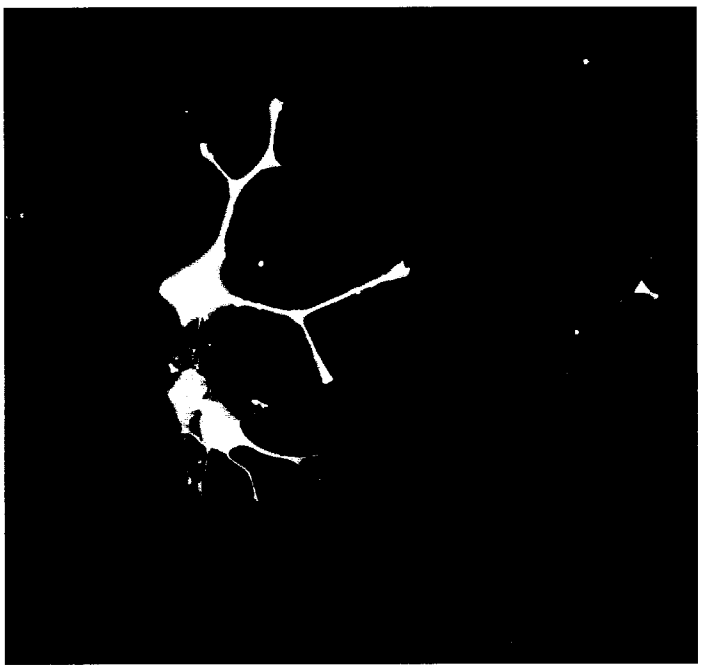
To quantitate these differences in neurite number, we counted the total number of neurites per cell (primary neurites and branches) in control and siCTβ2-expressing cells over a five day period of NGF treatment. After 2 days of NGF treatment, control cells already had 34% more neurites per cell than siCTβ2-expressing cells (3.09 ± 0 .06 vs. 2.03 ± 0.07, $p< 0.05$, Fig. 3.5A). Over the 5 days of NGF treatment, control cells steadily produced primary neurites and branches such that, by day 5, they had an average of 5.41 ± 0.35 neurites per cell. In contrast, siCTβ2-expressing cells only marginally increased their neurite number per cell from day 2 (2.03) to day 5 (2.60 neurites per cell). As a result, by day 5 of NGF treatment, siCTβ2-expressing cells had fewer than half the number of neurites of control cells (2.60 ± 0.27 vs. 5.41 ± 0.35, p = 0.003) (Fig. 3.5A).

Since primary neurites and branches are distinct neurite populations, regulated by separate signaling pathways, we analyzed how suppression of

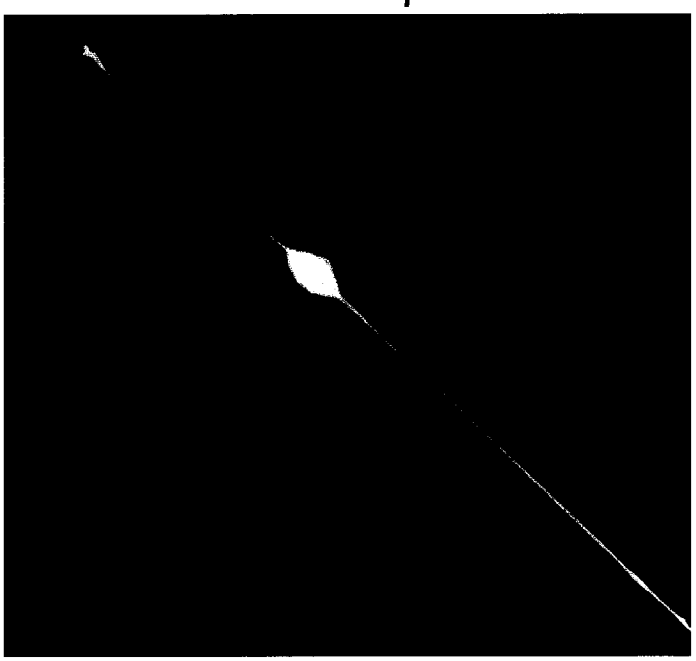
Figure 3.4. pSiCTβ2-transfected PC12 cells have decreased neurite outgrowth and branch formation. PC12 cells were co-transfected overnight with vector encoding GFP and either pSiCTβ2 (panel B) or pSILENCER vector containing a scrambled siRNA insert (Control, panel A) as described in Materials and Methods. Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 4 days. On day 4 of NGF-induced differentiation, cells were viewed under fluorescence microscopy and digital images of cells were recorded.

Figure 3.4

A. Control



B. SiCTβ2



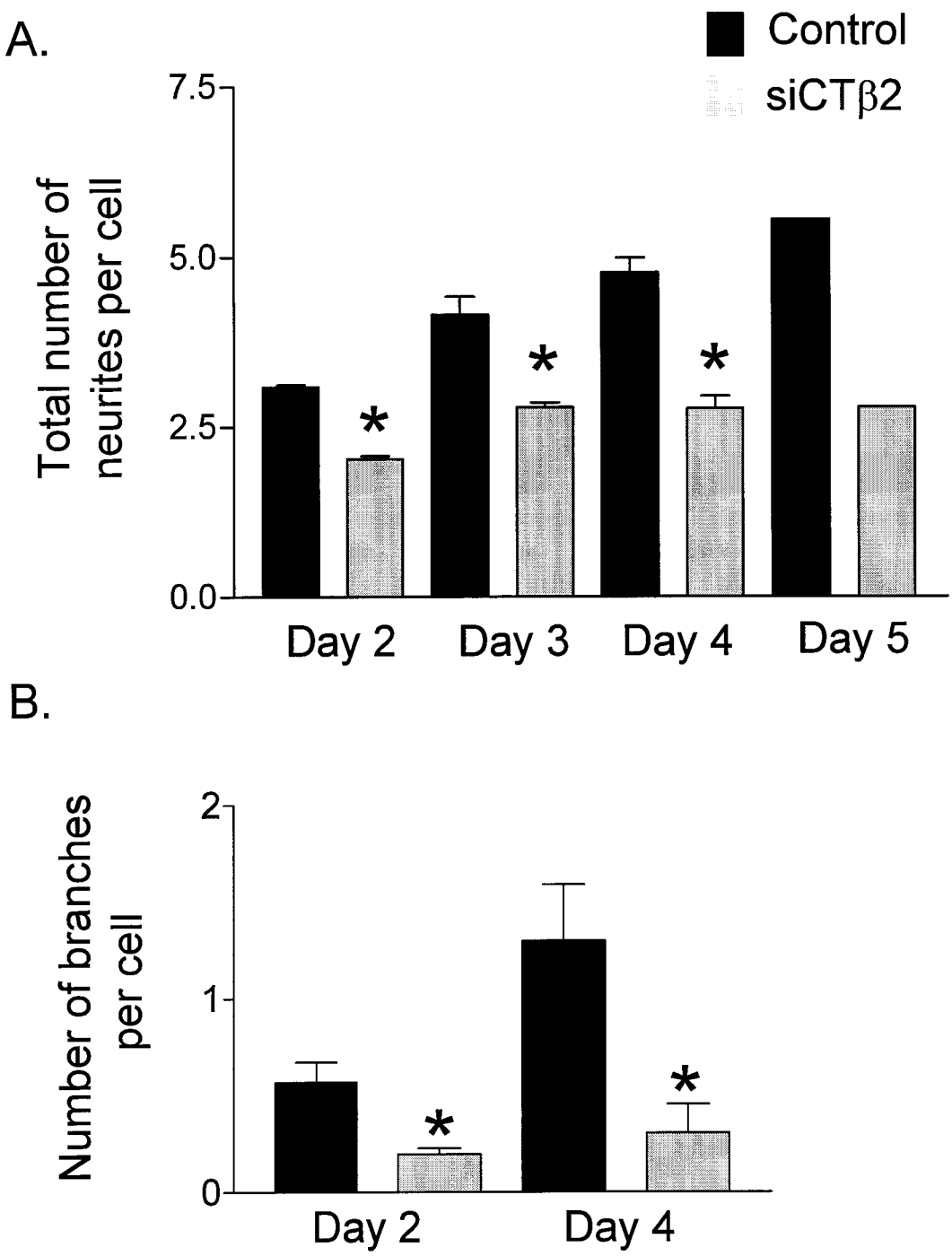
CTβ2 protein synthesis affected either primary neurite or branch populations. As shown in Fig. 3.5B, siCTβ2-expressing cells had significantly fewer branches per cell compared to that of control cells. This effect was observed after both 2 and 4 days of NGF treatment.

3.4.3 *CTβ2 is not required for neurite extension.*

Once a primary neurite has sprouted from the cell body, it continues to elongate or pauses for branch formation. Since siCTβ2-expressing PC12 cells had far fewer neurites than control cells, we measured the lengths of the primary neurites and branches to determine whether CTβ2 is also required for neurite elongation. In control cells, the average neurite length did not vary from day 2 to day 5 of NGF treatment: 84 ± 1.5 pixels (day 2) and 100 ± 37 pixels (day 5) (Fig. 3.6A). Typically, in control cells, once a neurite had reached a length of ~80 pixels, it had branched at least once thereby limiting its own elongation. In contrast, primary neurites of siCTβ2-expressing cells elongated throughout the period of NGF treatment. By day 5 of NGF treatment, neurites of siCTβ2-expressing cells were 2.5 X longer than those of control cells (245 ± 1.4 vs. 100 ± 37 pixels, $p< 0.05$). In addition to their striking length and lack of branches, neurites of pSiCTβ2-transfected cells differed morphologically as well. These neurites extended in straight lines with far fewer points of attachment or regions of localized thickening within the neurite compared to the neurites of control cells (Fig. 3.4).

Figure 3.5. CTβ2 is required for normal neurite outgrowth and branching in PC12 cells. PC12 cells were co-transfected overnight with vector encoding GFP and either pSiCTβ2 or pSILENCER vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 5 days. **A.** On days 2-5 of NGF-induced differentiation, cells were viewed under fluorescence microscopy and all neurites and branches (greater than 20 pixels) on GFP-positive cells were counted. Data represent the mean ± SEM of the total number of neurites (primary and branches) per cell. **B.** Data represent the mean ± SEM of the number of branches on GFP-positive cells. A minimum of 50 cells were scored for each timepoint. * Control vs. SiCTβ2, $P > 0.05$.

Figure 3.5



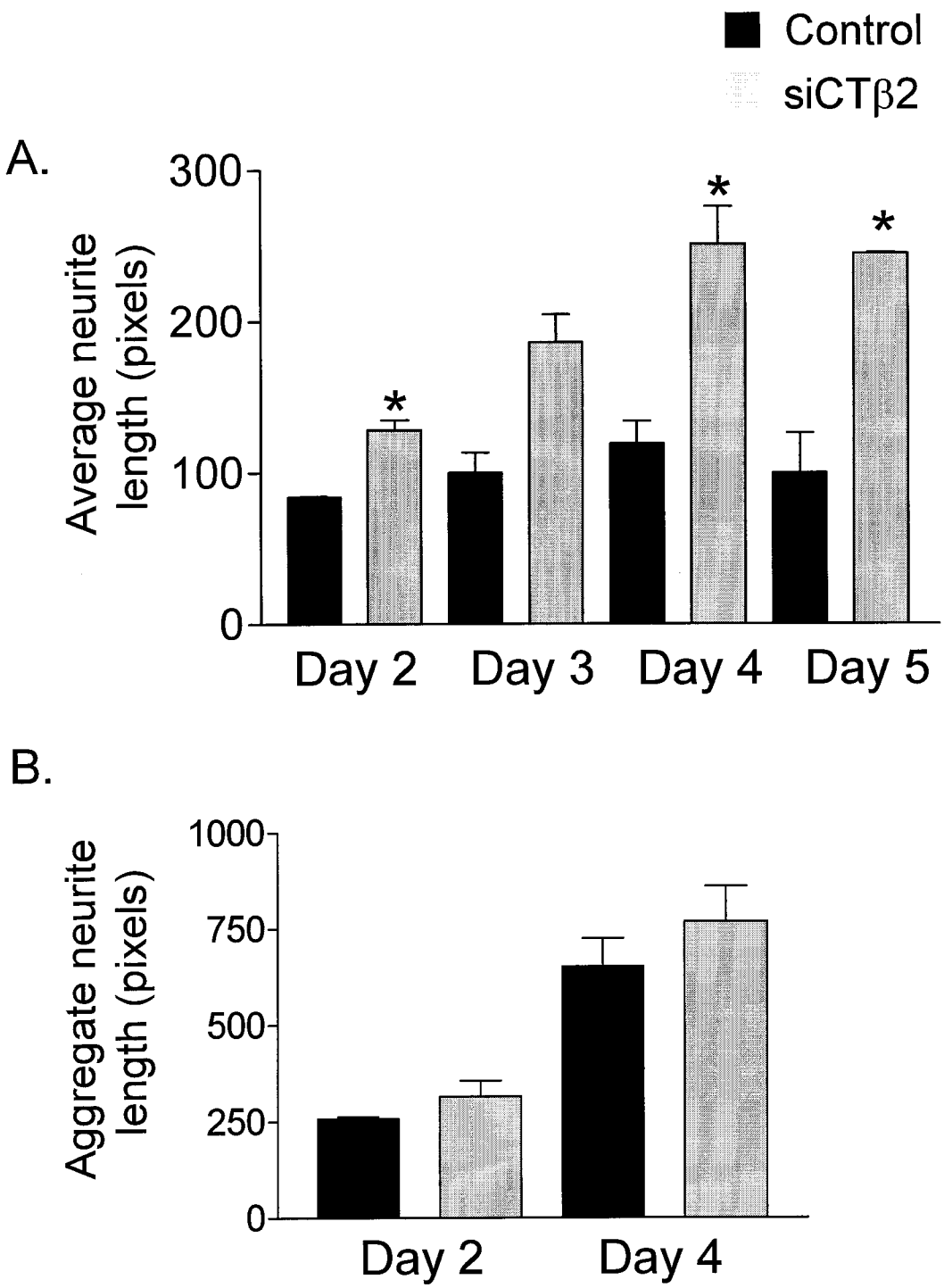
Since silencing CTβ2 expression in PC12 cells decreased CTβ2 protein, siCTβ2-expressing cells may have fewer neurites because of a general suppression of PC synthesis. To address this possibility, we calculated the aggregate length, the sum of all primary neurites and branches, extended from mock-transfected (control) and pSiCTβ2-transfected cells. After two and four days of NGF treatment, there was no significant difference in the aggregate neurite lengths between control and pSiCTβ2-transfected cells: day 2, 257 ± 5 pixels vs. 315 ± 41 pixels respectively; day 4, 580 ± 89 pixels and 713 ± 86 pixels respectively (Fig. 3.6B). In fact, the aggregate length of neurites from pSiCTβ2-transected cells was appreciably, though not significantly, higher than that of neurites from control cells. Thus, it is very unlikely that pSiCTβ2-expressing cells lack sufficient PC for neurite outgrowth and branch formation. In fact, these cells may even produce more neurite-specific membrane than control cells.

3.4.4 CTα is not required for neurite outgrowth and branch formation

Previously, we observed that differentiating PC12 cells and Neuro2a cells dramatically up-regulated CTβ2, but not CTα expression [3]. If CTα has no role in neurite formation, RNA silencing of CTα expression should not affect neurite outgrowth. We used a RNA silencing strategy to suppress CTα protein synthesis in PC12 cells [12]. Prior to examining siCTα-expressing cells for any discernible phenotype, we verified that siCTα suppressed CTα protein synthesis. As shown in Fig. 3.7B, lysates of pSiCTα-transfected cells had far

Figure 3.6. Suppression of CTβ2 protein synthesis enhances neurite elongation in PC12 cells. PC12 cells were co-transfected overnight with vector encoding GFP and either pSiCTβ2 or pSILENCER vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 5 days. **A.** On days 2-5 of NGF-dependent differentiation, cells were viewed under fluorescence microscopy and the lengths of all neurites (primary neurites and branches) on GFP-positive cells were measured. **B**. The aggregate neurite length was calculated as the summed total of all neurites (primary neurites and branches) per cell. Data represent mean ± SEM of 3 independent experiments. A minimum of 50 cells were scored for each timepoint. * Control vs. SiCTβ2, $P < 0.05$.

Figure 3.6



less CTα protein than lysates of control cells. (Fig. 3.7B). Similar to the immunoprecipitation experiments, lysates of both control and siCTα-expressing cells had much more CTβ2 protein than CTα protein, thus, the anti-M immunoreactive band corresponding to CTβ2 protein (Fig. 3.7B) was overexposed relative to the CTα band.

After 2 days of NGF treatment, we counted the total number of neurites per cell (primary neurites and branches) in control and siCTα-expressing cells. As shown in Fig. 3.7A, there was no difference in total neurites per cell between the two cell types; control cells had 3.12 ± 0.04 total neurites per cell while pSiCTα-transfected cells had 3.08 ± 0.16 total neurites per cell.

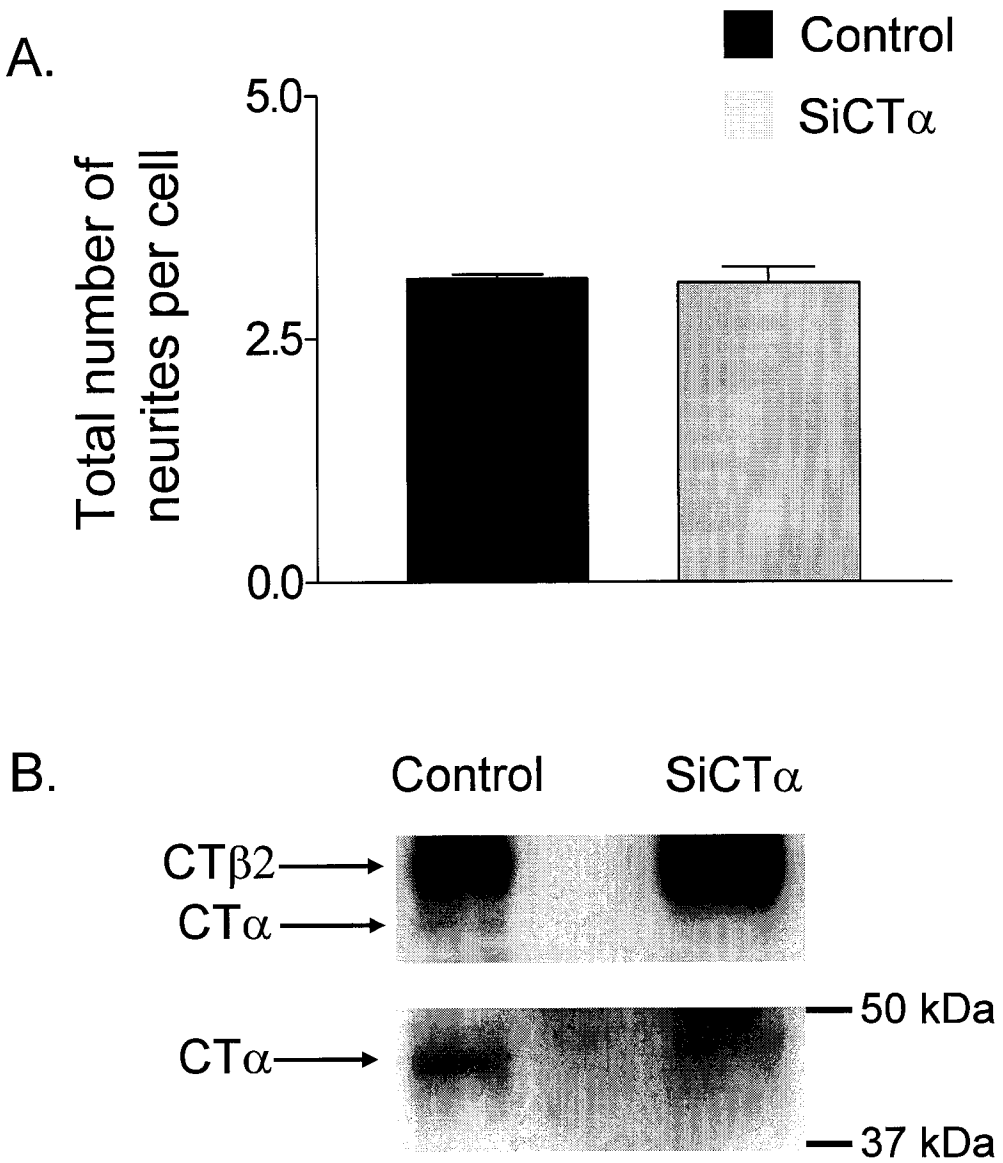
These data are consistent with our hypothesis that CTβ2 is the predominant isoform involved in neurite sprouting and branch formation. As expected, down-regulating CTα expression did appear to decrease the viability of undifferentiated, dividing PC12 cells; however, this observation was not quantified.

3.4.5 Over-expression of CTβ2 in PC12 cells stimulates neurite outgrowth and branch formation.

Since suppression of CTβ2 in PC12 cells significantly decreased neurite number, we suspected that over-expressing CTß2 would stimulate neurite sprouting and branch formation. To test this hypothesis, we generated a hemagluttinin-tagged CTβ2 recombinant protein (CTβ2-HA) encoded by the mammalian expression vector pCI. Briefly, CTβ2 cDNA was cloned from

Figure 3.7. SiRNA directed against CTα mRNA has no effect on neurite outgrowth and branch formation. PC12 cells were transfected overnight with pSiCTα or pSILENCER vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 48 hours. **A.** On day 2 of NGF treatment, cells were viewed under fluorescence microscopy and all neurites and branches (longer than 20 pixels) on GFP-positive cells were counted. Data represent the mean ± SEM of the total number of neurites (primary neurites and branches) per cell. **B.** 15 μg of proteins from homogenates of control and pSiCTα-transfected cells were separated by SDS-PAGE and transferred to PVDF membranes. Amounts of CTα protein were assessed by immunoblotting with antibodies against the membrane domain of CT (anti-M) or antiCT98. Results were similar in two independent experiments.

Figure 3.7



PC12 mRNA, modified by addition of a hemagluttinin-coding cDNA to the 3' end of the open reading frame and inserted into pCI. We verified expression of the CTβ2-HA protein by immunoblotting lysates of pCI{CTβ2HA}-transfected cells with anti-hemagluttinin antibodies. As shown in Fig. 3.9A, pCI{CTβ2-HA}-transfected cells expressed a 45 kDa, anti-HA-immunoreactive protein. This corresponds to the predicted size of the CTβ2-HA recombinant protein. Since the transfection efficiency of PC12 cells is quite low, the overall increase in CTβ2 expression was minimal in pCI{CTβ2-HA}-transfected cells (Fig. 3.9A); however, CT activity in lysates of pCI{CTβ2-HA}-transfected cells was significantly higher than that of control cells (Fig. 3.9B).

To assess the effect of CTβ2 over-expression on neurite outgrowth and branch formation, PC12 cells were co-transfected with a vector encoding GFP as well as pCI[CTβ2-HA]. After two days of NGF treatment, the cells were visualized under fluorescence microscopy to identify CTβ2-HA-expressing cells and all primary neurites and branches on these cells. As shown in Fig. 3.8A, pCI[CTβ-2HA]-transfected cells had 30 percent more total neurites than control cells: 4.9 ± 0.6 and 3.7 ± 0.4 respectively; however, this increase was not statistically significant. Moreover, while the number of both primary neurites and branches increased in pCI[CTβ-2HA]-transfected cells, neither value increased significantly (Fig. 3.8B). Primary neurites increased from 3.05 ± 0.25 neurites per cell in control cells to 3.57 ± 0.16 in pCI[CTβ-2HA]-transfected cells; branch number increased from 0.66 ± 0.31 in control cells to 1.38 ± 0.3 branches per cell in pCI[CTβ-2HA]-transfected cells.

Figure 3.8. Effect of CTβ2 over-expression on the number of neurites in differentiating PC12 cells. PC12 cells were co-transfected overnight with a vector encoding GFP along with either pCI vector with no insert (Control) or pCI-CTβ2-HA. Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for up to 5 days. After 2 days of NGF-induced differentiation, cells were viewed under fluorescence microscopy and all neurites (>20 pixels) (**A**), primary neurites (**B**) and branches per cell (**C**) of the GFP-positive cells were counted. Data represent mean ± SEM of 3 independent experiments. A minimum of 50 cells were scored for each timepoint.

Figure 3.8

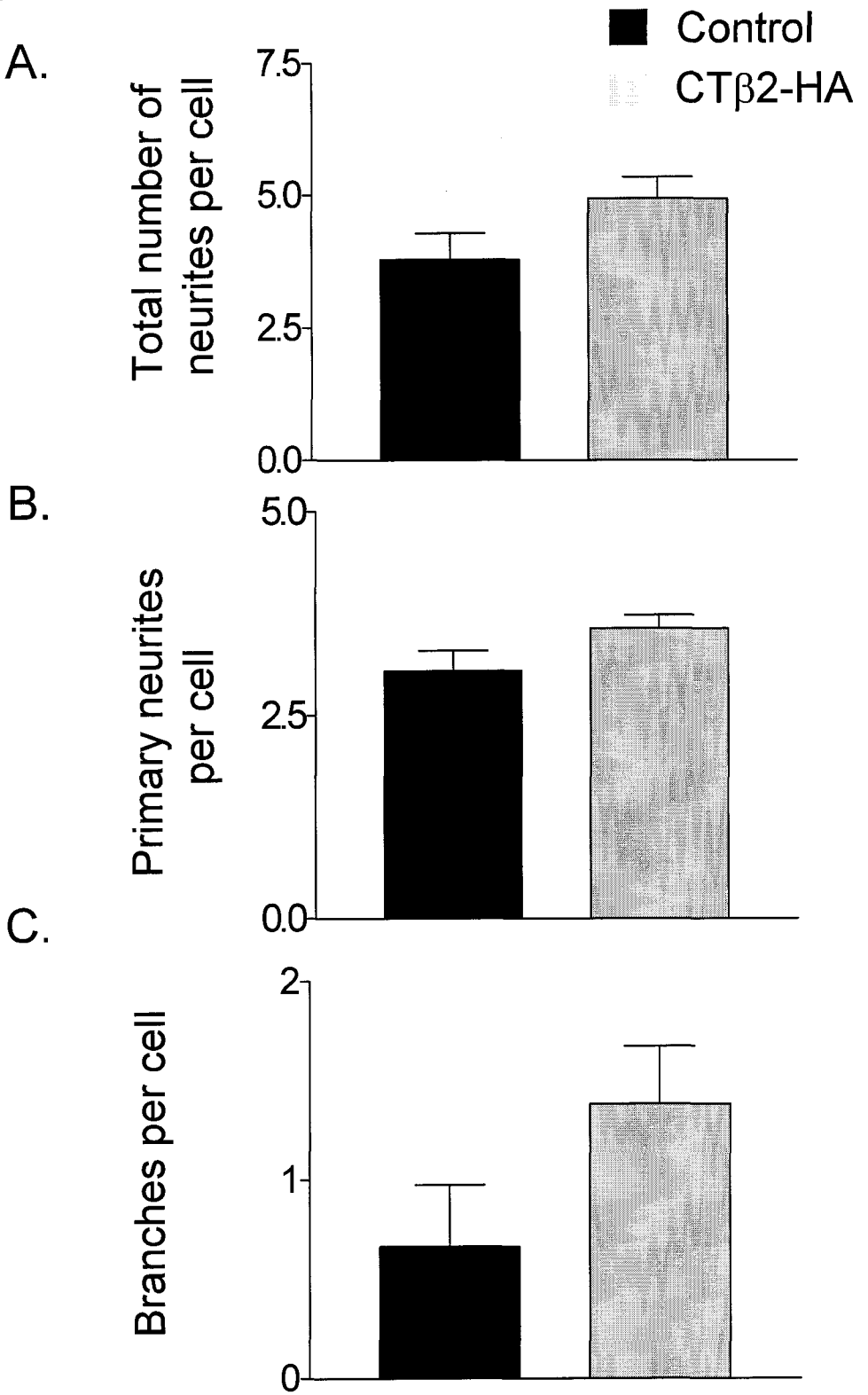
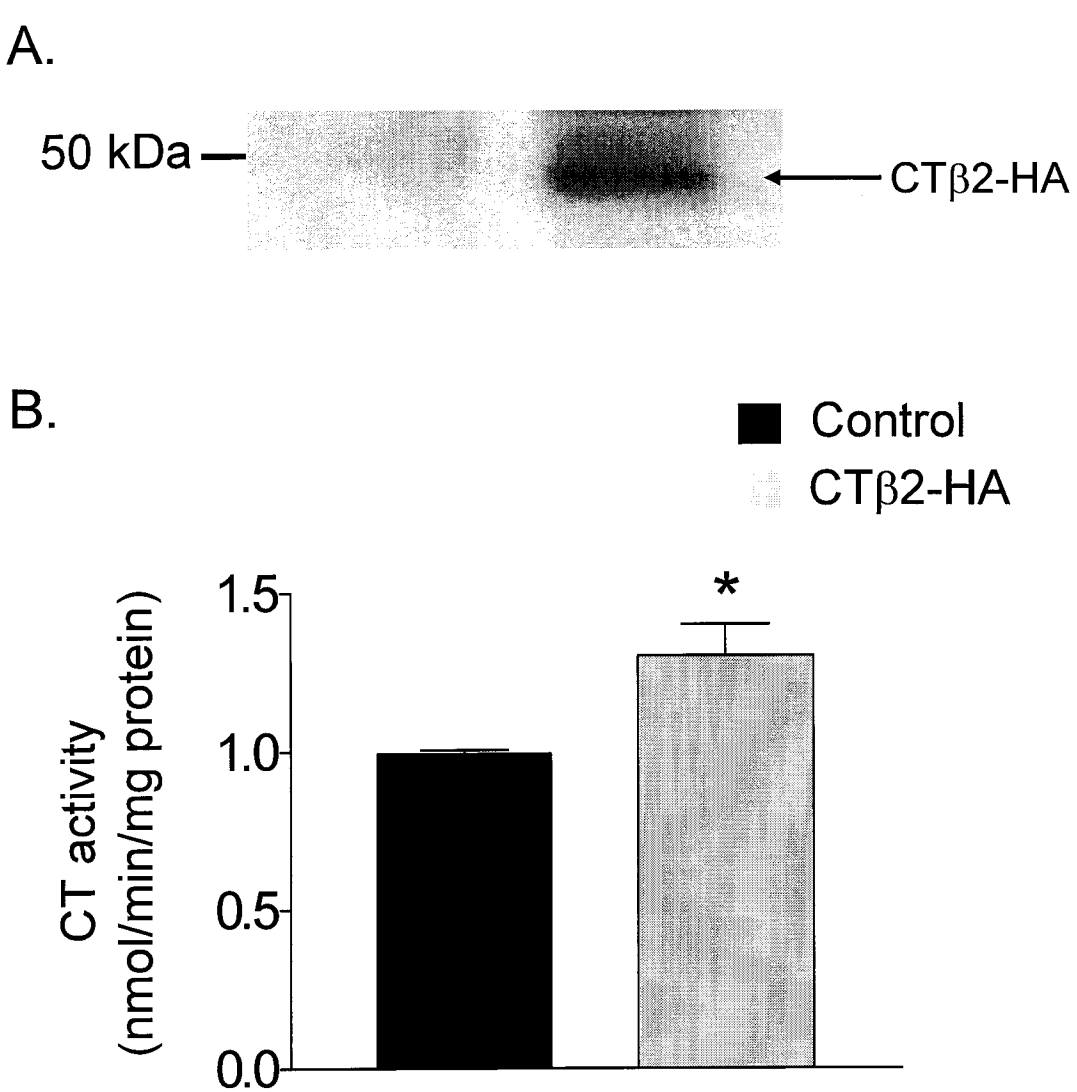


Figure 3.9. Over-expression of CTβ2 in PC12 cells. PC12 cells were co-transfected overnight with a vector encoding GFP along with either pCI vector with no insert (Control) or pCI-CTβ2-HA (CTβ2-HA) as described in Materials and Methods. Medium was changed to F12K medium containing 50 ng/ml NGF and cells were differentiated for 2 days. **A.** 15 μg of proteins from the homogenates of control and pCI-CTβ2-HA-transfected cells were loaded onto 10% polyacrylamide gels containing 0.1% SDS and separated by electrophoresis. Amounts of CTβ2-HA protein were assessed by immunoblotting with anti-hemagluttinin antibodies. Results were similar in two independent experiments. **B**. CT activity was measured in aliquots of homogenates from control (Control) and pCI-CTβ2-HA—transfected (CTβ2-HA) cells and normalized to protein. Data are calculated as means ± SEM of 2 independent experiments with triplicate dishes. Control: 1.01 ± 0.012 nmol/min/mg protein; CTβ2-HA: 1.30 ± 0.098 nmol/min/mg protein. * Control vs. CTβ2-HA, $P < 0.05$.

Figure 3.9



In conclusion, suppression of CTβ2 expression results in significant loss of both primary neurites and branches (Fig. 3.6). In contrast, while over-expression of CTβ2 did increase the numbers of both primary neurites and branches, the result was not significant due to high variation across experiments. Taken together, these data strongly suggest that CTβ2 is required for proper neurite outgrowth and specifically, branching.

3.4 Discussion

CTß2 is localized to axons.

There is considerable evidence that phospholipids are not synthesized exclusively in the neuronal cell body. Activities of the enzymes in the Kennedy pathway for PC biosynthesis have been detected in the axons of sympathetic neurons [11] and in rat brain synaptosomes [9]. In addition to PC, the biosynthesis of phosphatidylethanolamine, phosphatidylserine and sphingomyelin has been demonstrated in the axons of sympathetic neurons [11]. Accordingly, the enzymes of lipid biosynthetic pathways must be localized to axons; however such studies have not been done. One exception is GM3 synthase, a ganglioside-specific sialyltransferase, that has been found in the axons of hippocampal neurons. [18].

We have identified the axonal isoform of CTP:phosphocholine cytidylyltransferase, the rate-limiting enzyme in PC biosynthesis. CTβ2 is localized to the axons of sympathetic neurons and retinal ganglion cells (Fig. 3.2B). We were able to immunoprecipitate abundant amounts of CTβ2 protein from the distal axons of sympathetic neurons and retinal ganglion cells. We chose these neurons because sympathetic neurons are peripheral neurons while retinal ganglion cells are part of the central nervous system. Thus, CTβ2 is found in axons of neurons of both the central and peripheral nervous systems. In contrast, no appreciable amount of CTα was immunoprecipitated from the distal axons of sympathetic neurons but was immunoprecipitated, albeit in a small quantity, from cell bodies and proximal axons of sympathetic

neurons and from PC12 cells. These observations are consistent with our hypothesis that CTβ2 is localized to axons and neurites while CTα, with its nuclear localization signal, is confined to the cell body.

The differential expression of CTα and CTβ2 in the nervous system may account for the relative paucity of CTα protein within the cell bodies of sympathetic neurons. While CTβ2 mRNA levels are low in most tissues, it is the predominant isoform in the gonads, lung and brain [19] . In brain, quantitative real-time PCR of CT isoform expression has shown that CTβ2 mRNA comprised 30% of the CT transcripts [19]. In contrast, in liver, CTβ2 has a relative abundance of 0.1 compared to the abundance of CTα mRNA [19]. Despite the relative abundance of CTβ2 in brain, CTα mRNA is still the predominant transcript; however, the relative abundance of CT proteins in brain has not been determined. Also, since sympathetic neurons are part of the peripheral nervous system rather than the central nervous system, the quantitative RT-PCR analysis in brain may not be representative of the CT isoform expression profile in sympathetic neurons.

Not surprisingly, PC made in the axon is distributed to axonal membranes. At least 50% of the PC in axon membranes is derived from biosynthesis within the axon [11]. Moreover, PC synthesis in the axon is not redundant. If axons are deprived of choline or treated with inhibitors of PC biosynthesis, axonal growth is reduced by more than 50% [14, 20].

Based on its localization within axons, CTβ2, and not CTα, is likely responsible for PC biosynthesis within the axon. Thus, we hypothesized that

CTβ2 is also required for normal axonal growth. To test this hypothesis, we suppressed CTβ2 and CTα expression in differentiating PC12 cells and examined them for defects in neurite outgrowth.

CTβ2 is required for neurite sprouting and branch formation but not for neurite extension.

As Greene first predicted in 1976, PC12 cells have proven to be an invaluable model of neurite outgrowth [1]. Within 2 days of NGF treatment, PC12 cells extend visible neurites and, with continual NGF exposure, create an extensive arborization of primary neurites and branches. Silencing CTß2 protein expression in PC12 cells dramatically altered neurite morphology (Fig. 3.4) and significantly reduced neurite sprouting and branch formation (Fig. 3.5). Since CTβ2 is found both in the cell bodies and axons of neurons, we cannot conclude that it was the loss of CTβ2 expression specifically within neurites that abrogated branch formation. However, since RNA silencing of CTα expression had no effect on neurite or branch formation (Fig 3.6A), it's very likely that the localization of CTβ2 to neurites dictates its role in neurite sprouting and branch formation.

To determine whether CTβ2 is involved in neurite formation, we over-expressed hemagluttinin-tagged CTβ2 protein in differentiating PC12 cells. Consistent with our hypothesis, CTβ2-overexpressing PC12 cells had 30% more neurites than control cells; however, this result was not statistically significant (Fig. 3.8). Thus, not surprisingly, CTβ2 expression is not the sole

requirement for neurite and branch formation. Recent reports have identified several other proteins which are required for proper branching of neurites. Among them, the microtubule-binding protein, Raspotlin, and the signaling molecule, Raf, have been studied in PC12 cells [21, 22]. Like CTβ2, rapostlin is required for branch formation and in its absence, PC12 cells produce far fewer branches.

Successful neuritogenesis requires that a careful balance be struck between neurite growth and branch formation [23]. Time-lapse recording of the axons of sensory neurons showed that axons pause, for up to several hours, at the site of future branch points [24]. During the pause, microtubules become splayed and disorganized at the site of the branch point. Eventually, the microtubule network reorganizes and short microtubules project into the developing neurite (reviewed in [23]). If an axon or neurite is unable to recognize signals to pause and reorganize for branch formation, its uninterrupted growth results in abnormally long neurites. This phenomenon has been observed in PC12 cells lacking Akt, a serine/threonine kinase known to promote neurite growth and inhibit branch formation. PC12 cells lacking Akt had significantly fewer neurites than control cells; however, these neurites grew much longer than those of control cells. [25]. We observed a similar, dramatic increase in the length of neurites on pSiCTβ2-transfected cells. After 5 days of NGF treatment, the neurites of siCTβ2-expressing PC12 cells were 2.5 X longer than neurites of control cells (Fig.3.6A).

To our knowledge, however, we have identified the first lipid biosynthetic enzyme required for neurite sprouting and branch formation. Marszalek *et al.* recently reported that over-expression of the fatty acid mobilizing enzyme, acyl-CoA synthase 2, significantly increased neurite growth [26]; however, acyl-CoA synthase-2 over-expression had no effect on neurite and branch formation. The role of CTß2 in branch formation is unclear. Since it catalyzes the rate-limiting step of PC biosynthesis, we predict that CTß2 must provide PC specifically for growing neurites. Perhaps the growth cones of newly forming neurites and neurite branches, which lack an organized microtubule network, require a local source of PC for the initial phase of growth. Following injury to axons of *Aplysia* sensory neurons, the axonal membrane, rather than the cell body, provides lipid for resealing and the initial rapid growth phase after axotomy [27]. Also, Posse de Chaves *et al.* have demonstrated that treatment of distal axons of sympathetic neurons with alkylphosphocholines [20] or ceramide [28] impairs axonal PC biosynthesis and stalls growth.

In contrast, established neurites have an organized microtubule transport system and vesicles from the cell body provide membrane for neurite elongation (Chapter 1, Fig. 1.2). Since suppression of CTβ2 expression did not affect neurite elongation, either residual CTβ2 expression provided sufficient PC for growth or CTα is able to provide PC for linear neurite growth. We suspect that CTα provides PC (and vesicles) from the cell body while CTβ2 synthesizes PC within the growing neurite.

In addition to providing new membrane for neurite formation, CTβ2 may be localized to the axon to replenish PC. In a number of neuronal cell lines, phospholipase D catalyzes PC turnover [29, 30]. Also in PC12 cells, neurite outgrowth requires activation of phospholipase D [31]. Like CTβ2, phospholipase D is found in the neurites of PC12 cells, and is particularly enriched within the growth cones at the distal end of neurites. NGF-dependent activation of phospholipase D via the MAP kinase signaling cascade is an absolute requirement for neurite growth [31]. Perhaps, CTβ2 contributes to the replenishment of PC hydrolyzed by phospholipase D.

Alternatively, PC, which donates its phosphocholine group to form sphingomyelin, may be depleted during sphingomyelin biosynthesis. Sphingomyelin is synthesized in the distal axons of sympathetic neurons [11] and is essential for the formation of detergent-insoluble glycolipid complexes within the axonal membrane [32].

One additional consideration is the role of substrate availability and PC biosynthesis in neurite outgrowth. The CDP-choline pathway of PC biosynthesis is limited both by CT activity as well as the availability of substrates. For example, in PC12 cells, acceleration of PC biosynthesis is partially regulated by diacylglycerol levels and activity of choline phosphotransferase, the enzyme catalyzing the final step in the CDP-choline pathway [2, 33]. Moreover, cytidine and uridine (the product of cytidine deamination) enhance PC biosynthesis [34] in PC12 cells and rat brain slices [35], likely via stimulation of CDP-choline synthesis [36]. Interestingly,

treatment of PC12 cells with uridine, increased CDP-choline levels and enhanced neurite outgrowth and branch formation in a dose-dependent manner [37]. Enhancement of neurite outgrowth by uridine was attributed both to increases in CDP-choline as well as direct activation of pyrimidine-sensitive P2Y receptors [37]. Thus, both CTβ2 expression and substrate availability may regulate the biosynthesis of PC for neurite growth.

A more definitive evaluation of CTβ2's role in neurite outgrowth and branching requires examination of these processes in neurons with a complete deficiency of CTβ2. Recently, Jackowski *et al.* have generated mice with a disruption in *Pcyt1b*, the gene encoding CTß2. Female mice with a disruption of the CTβ2-encoding gene (*Pcyt1b*$^{-/-}$) were sterile due to ovarian dysfunction [19]. Despite the enriched expression of CTβ2 in brains of wild type mice, *Pcyt1b*$^{-/-}$ mice had no overt morphological changes in their brains. Based on our observations that CTβ2 is involved in neurite branch formation, we predict that a deficiency of CTß2 will manifest as alterations in learning and memory or motor coordination rather than as gross neuroanatomical deficits. Currently, plans are underway to evaluate these parameters in *Pcyt1b*$^{-/-}$ mice.

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Chapter 4

Studies on the regulation of CTP:phosphocholine cytidylyltransferase β2 during neurite outgrowth.

4.1 Introduction

The morphological hallmark of neuronal differentiation is the sprouting and growth of neurites: long, branched extensions of the plasma membrane which later mature into functional axons and dendrites. Neurites are categorized by their location relative to the cell body. Primary neurites project directly from the cell body. Secondary neurites, or branches, project from primary neurites (Chapter 1, Fig. 1.2). While the mechanics of branch formation are poorly understood, time-lapse recordings of sensory axon growth have elucidated that the growing tips of axons, called growth cones, pause prior to branch formation [1]. During this pause, the microtubule framework is dramatically re-organized to produce bidirectional growth [2]. The growth cone divides into two separate processes (i.e axon and branch) and the two processes grow synchronously. Consequently, branch formation stresses the axon for new membrane; therefore, axon elongation and branch formation greatly increase the demand for phospholipid biosynthesis.

The PC12 cell line has become an invaluable model for studies of neuronal differentiation and neurite outgrowth. In response to nerve growth factor (NGF), PC12 cells stop proliferating and differentiate into sympathetic-neuron like cells [3, 4]. Neurotrophins, like NGF, are essential for neuronal survival as well as growth and differentiation [5]. Neurotrophins signaling cascades share a common starting point, the trk family of neurotrophin receptors. When NGF binds to trkA, transphosphorylation of tyrosine residues results in recruitment and docking of a number of signaling proteins [5]. Recruitment of

phosphatidylinositide-3 kinase (PI3K) to the plasma membrane is achieved in this manner. PI3K has multiple downstream effectors; one of them, the serine/threonine kinase Akt, promotes motor axon and sympathetic axon growth [6] [7]. In PC12 cells, over-expression of active Akt dramatically decreased branch formation while over-expression of a dominant negative Akt protein or treatment with LY294002, a PI3K inhibitor, increased branch formation [8]. Also, in hippocampal neurons and NG108-15 cells, treatment with LY294002 promoted extensive branching of dendrites thereby implicating Akt as a negative regulator of dendritic branching [9]. In contrast, Kim *et al.* have shown that short-term LY294002 treatment impaired neurite branching in PC12 cells, suggesting that the PI3 kinase/Akt pathway is a positive regulator of neurite outgrowth. However, the dose of LY294002 used in these experiments was much higher than that associated with an enhancement of neurite branching [10]. Also, in sensory neurons lacking the apoptotic protein, Bax, over-expression of Akt enhanced axon branching [11]. In summary, PI3 kinase/Akt signaling appears to be a negative regulator of neurite branching in PC12 cells; however its role may be stimulatory in other neuronal populations.

In chapter 3, we demonstrated that CTP:phosphocholine cytidylyltransferase β2 (CTβ2) is essential for proper neurite outgrowth and branch formation. We used an RNA silencing strategy to "knock down" CTα or CTβ2 expression in PC12 cells. Silencing CTβ2 protein expression in PC12 cells dramatically altered neurite morphology (Chapter 3, Fig. 3.4) and significantly reduced

neurite sprouting and branch formation (Chapter 3, Fig. 3.5). Since CTβ2 expression is essential for proper neurite sprouting and branch formation, we determined whether inhibition of Akt stimulates neurite branching in CTβ2-deficient PC12 cells.

Like CTα, CTβ2 is heavily phosphorylated *in vivo* [12]; however, the role of phosphorylation in regulation of CTβ2 has not been studied. Within their carboxyl termini, CTα and CTβ2 share an identical, putative Akt phosphorylation motif: RSRSPS; thus we also examined whether CTβ2 is directly phosphorylated by Akt.

Another candidate kinase for CTβ2 phosphorylation is cyclin-dependent kinase 5 (cdk5), a member of the cyclin-dependent kinase family [13]. Cdks are proline-directed threonine and serine kinases which orchestrate transitions through the cell cycle [13]. Cdk5 has 60% identity to other cdks and is the sole isoform enriched in brain [14]. Cdk5 is activated by association with p35 and is required for proper neuronal migration and synaptic function. [14]. Cdk5 is also required for axon growth [15] and is activated during regeneration of facial nerve axons after injury [16].

The cdk5 consensus motif for phosphorylation is K/RSPXK/R [14]. Specific proteins phosphorylated by cdk5 include Pak1 [17], a modulator of actin dynamics and the microtubule-associated proteins (MAP) MAP1b, MAP-2 and tau [18]. Neurofilament proteins are also substrates for cdk5 phosphorylation [19] and cdk5-mediated phosphorylation of neurofilaments results in disrupted anterograde axonal transport [20].

In PC12 cells, p35, the cdk5 activator, is up-regulated by NGF treatment [21]. Interestingly, CTβ2 has a consensus site for cdk5 phosphorylation identical to that of neurofilament H [12]. Since cdk5 is essential for neurite growth, we hypothesize that cdk5 phosphorylates CTβ2 to regulate its activity in neurite sprouting and branch formation.

4.2 Materials and Methods

Cell culture. Rat pheochromocytoma cells (PC12) were obtained from American Type Cell Culture Collection. Cells were maintained in F12-K medium supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum at 37°C in a humidified atmosphere containing 5% CO_2. For transfection experiments, cells were seeded on collagen-coated 35 mm dishes at a density of 2 X 10^5 cells/dish. Cells were transfected with a total of 2.5 μg of DNA per 35 mm dish. Cells were incubated overnight and then treated with 50 ng/ml 2.5S NGF (Alomone Labs).

Cell transfection. PC12 cells were cultured as described above; however, the plating density was lowered to 2 X 10^5 to improve transfection efficiency. Twelve h after plating the cells, the medium was changed to F12K with no serum. Cells were co-transfected with plasmid encoding green fluorescent protein (GFP) (phrGFP) and pSILENCER encoding RNA-silencing oligonucleotides generated against CTβ2 or with pSILENCER containing a scrambled, non-coding insert. Cells were transfected using the Lipofectamine 2000 reagent according to the manufacturer's protocol. Briefly, siRNA-encoding or control vectors, along with phr-GFP were incubated with Lipofectamine 2000 in a 1:3 ratio for 20 min to allow DNA-reagent complexes to form and then applied to PC12 cells. After a 12 h incubation with the Lipofectamine 2000-DNA complexes, cells were given F12K medium

containing 50 ng/ml NGF and 10 μM LY294002 or an equivalent volume of vehicle.

Neurite measurements

After 2 days of NGF +/- LY294002 treatment, cells were viewed with fluorescence microscopy. pHr-GFP expressing cells were identified by their fluorescence at 520 nm under UV excitation. Co-transfected cells bearing at least one neurite longer than 20 pixels were considered to be differentiating cells and all of its neurites and branches were counted and measured. At each time point, at least 20 random fields of view were sampled.

Immunoprecipitation and Immunoblotting:

PC12 cells were rinsed with ice-cold PBS and harvested into immunoprecipitation lysis buffer. Cell lysates were pre-cleared by centrifuging at 13,000 rpm for 15 min at 4°C. After centrifugation, supernatants were transferred to new tubes and incubated with 3 μl of anti-M antibody (generated against the membrane domain of rat liver CT,), or 1 μl anti-cdk5 or 1 μl anti-A-tubulin antibodies on a rotating shaker at 4°C. After 1 h, 40 μl of a 50% slurry of protein A-Sepharose beads (pre-equilibrated with immunoprecipitation lysis buffer) were added to the cell lysates and the lysates were incubated for an additional h at 4°C. Protein A-Sepharose bead conjugates containing immunoprecipitated proteins were washed 3 times with immunoprecipitation wash buffer (0.1% Triton X 100, 50 mM Tris-HCl. pH 7.4),

eluted using SDS-PAGE sample buffer (without reducing agent) and heated at 70°C for 3 min. Equal amounts of immunoprecipitated proteins from the cell lysates were used in *in vitro* kinase assays or separated by electrophoresis on 10% polyacrylamide gels containing 0.1% SDS and transferred onto Immobilon-P transfer membranes (Millipore). Ponceau S stain was used to compare protein loading in all lanes of the gel. The membranes were blocked for 2 h with 5% skim milk in Tris-buffered saline (20 mM Tris-HCl, pH 7.5, 500 mM NaCl) containing 0.1% Tween 20 and then incubated overnight with anti-CTβ2 (1:1000) antibodies. The blots were washed with TBS-T for 1 h and incubated with secondary antibody, horseradish peroxidase linked to anti-rabbit IgG (1: 2500) dilution) for 1 h, After incubation, immunoreactivity was detected using Amersham Biosciences ECL reagents.

In vivo phosphorylation of CTβ2

PC12 cells were cultured as described above and treated with 50 ng/ml NGF for 4 days. Cells were incubated for 30 min with 50 μM roscovitine or an equivalent volume of vehicle (DMSO) in the presence of 100 μM [^{32}P] orthophosphate (Amersham). Cells were rinsed with ice cold PBS and harvested into immunoprecipitation lysis buffer. Cell lysates were pre-cleared by centrifuging at 13000 rpm for 15 min at 4°C. Following centrifugation, supernatants were transferred to new tubes and incubated with 3 μl of anti-M antibody (generated against the membrane domain of rat CTα) for 1 h on a rotating shaker at 4°C. After 1 h, 40 μl of a 50% slurry of protein A-Sepharose

beads (pre-equilibrated with PBS) were added to the cell lysates and incubated for an additional h at 4°C. Protein A-Sepharose bead conjugates containing immunoprecipitated CTβ2 were washed 3 times with immunoprecipitation wash buffer. CTβ2 was eluted from the beads using SDS-PAGE sample buffer and heated at 70°C for 3 min. Samples were separated by electrophoresis through a 10% acrylamide gel containing 1% SDS. The gel was dried overnight and then exposed to a phosphorimage screen (Kodak) for up to 3 days. Quantitation of [^{32}P]-orthophosphate-labelled CT proteins was done with a BioRad phosphoimager.

In vitro phosphorylation assays

PC12 cells were cultured as described above. Cells were rinsed with ice cold PBS and harvested into 1 ml of immunoprecipitation buffer. CTβ2 and CTα proteins were immunoprecipitated using anti-M antibody as previously described. α-tubulin was immunoprecipitated using anti-α-tubulin antibody. Cyclin-dependent kinase 5 was immunoprecipitated using 10 μl of anti-cdk5 (Santa Cruz). Following immunoprecipitation, cdk5-bound protein A-Sepharose beads were pre-warmed at 30°C for 10 min. CTβ2 protein was eluted from the anti-M antibody/protein A-Sepharose beads with 40 μl kinase buffer (50 mM Tris-HCl, pH 7.4, 10 mM $MgCl_2$ and 2 mM DTT) and boiled for 5 min. Afterward, eluted CTβ2 protein was added to cdk5-protein A-Sepharose beads in the presence of 1 mCi of [^{32}P] ATP. Samples were incubated for 15 min at 30°C. The kinase reaction was terminated by addition

of 5 X SDS-sample buffer and heating at 100°C for 3 min. Samples were separated by electrophoresis through a 10% acrylamide gel containing 1% SDS. The gel was dried overnight and then exposed to a phosphorimage screen (Kodak) for up to 3 days.

In vitro CT activity assays

Cells were cultured as described and collected in 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 2 mM EDTA, 1 mM dithiotritol, 0.1 mM phenylmethylsulfonyl fluoride and 100 μg/ml each leupeptin and aprotinin). The cells were sonicated for 20 s at 4°C. Cell homogenates were centrifuged at 7000 x g for 5 min to pellet nuclei and intact cells. For membrane and cytosol fractionation experiments, an aliquot of the cell homogenate was centrifuged at 100,000 x g for 30 min. The supernatant corresponded to the cytosol fraction; the pellet (microsomal fraction) was resuspended in homogenization buffer. All protein concentrations were measured using the Bio-Rad protein assay. CT activities in the soluble and membrane fractions were determined with PC:oleate vesicles by monitoring the conversion of phospho[^{3}H]choline to CDP-[^{3}H]choline as previously described [22].

4.3 Results

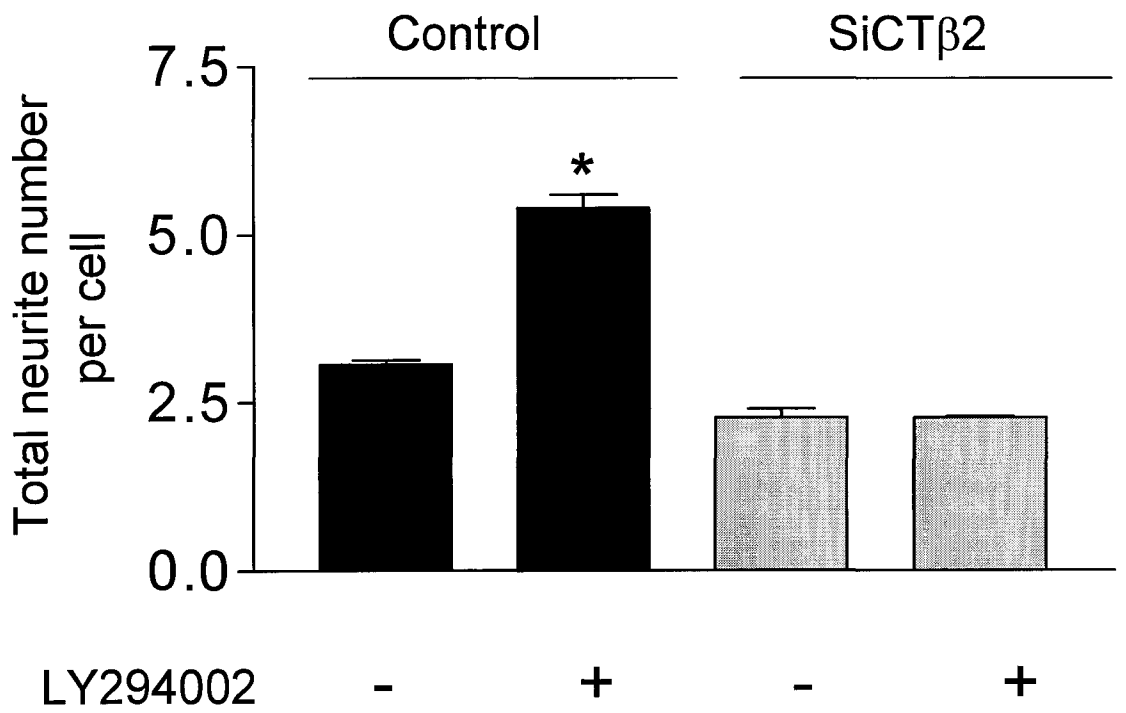
4.3.1 CTβ2 is required for LY294002-dependent stimulation of neurite branching.

Neurite outgrowth and branching is one of the morphological hallmarks of neuronal differentiation. In PC12 cells, Akt is a negative regulator of neurite branching; treatment with LY294002, an inhibitor of PI3K, abolished Akt activation and promoted neurite branching [8]. Since we have established a role for CTβ2 in neurite outgrowth and branch formation, we hypothesized that CTβ2 is a downstream effector of the PI3K/Akt signaling cascade. To test this hypothesis, we compared the effect of LY294002 on neurite outgrowth and branch formation in control and CTβ2-deficient PC12 cells. Prior to assessing the effect of LY294002 on neurite outgrowth, immunoblotting with anti-phospho Akt antibodies was done to confirm that LY294002 inhibited Akt phosphorylation. Treatment with 10 μM LY294002 significantly decreased the amount of phospho-Akt immunoreactive proteins compared to that of untreated cells. Similar to Higuchi *et al.* [8], we observed that cells treated with LY294002 had significantly more neurites than untreated cells: 5.4 ± 0.2 vs 3.1 ± 0.06 neurites per cell respectively, $p<0.01$) (Fig. 4.1). In sharp contrast, LY294002 had no effect on neurite number in CTβ2-deficient cells; untreated siCTβ2-transfected cells had 2.05 ± 0.16 neurites per cell and LY294002 treated siCTβ2-transfected cells had 1.95 ± 0.02 neurites per cell.

In addition to total neurite number, we counted primary neurites and branches, individually, to determine whether LY294002 specifically affected

Figure 4.1. CTβ2 is required for LY294002-dependent neurite outgrowth and branching in PC12 cells. PC12 cells were co-transfected overnight with vector encoding GFP and either pSiCTβ2 (SiCTβ2) or vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and 10 μM LY294002 (Control/Ly and SiCTβ2/Ly) or an equivalent volume of vehicle (Control and SiCTβ2) and cells were differentiated for 2 days. On day 2 of NGF-induced differentiation, cells were viewed under fluorescence microscopy and all neurites and branches (greater than 20 pixels) on GFP-positive cells were counted. Data represent the mean ± SEM of the total number of neurites (primary and branches) on GFP-positive cells. A minimum of 50 cells were scored for each timepoint. * Control vs. Control/Ly294002, $P < 0.05$.

Figure 4.1



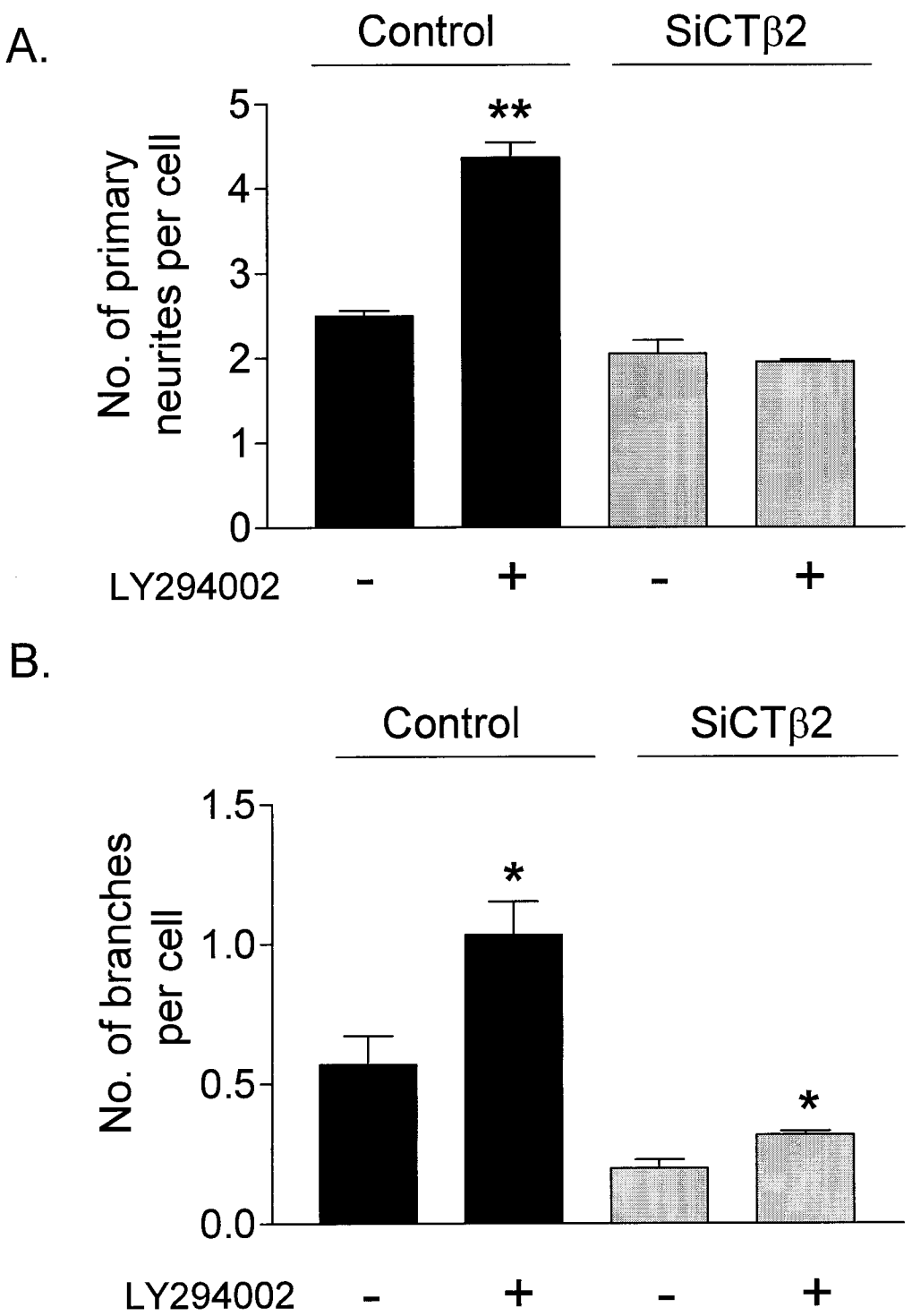
either of these neurite subpopulations. In untreated cells, treatment with LY294002 significantly increased both the number of primary neurites as well as the number of branches (Fig. 4.2). In si-CTβ2 transfected cells, however, LY294002 treatment had no effect on the number of primary neurites. Also, treatment with LY294002 nearly doubled the number of branches per cell in control cells: 0.57 ± 0.10 to 1.03 ± 0.12 per cell $p < 0.05$ (Fig. 4.2B). In contrast, CTβ2-deficient cells had far fewer branches than control cells (0.19 ± 0.03 branches per cell, Fig. 4.2B) and LY294002 only increased the number of branches by ~ 50% to 0.31 ± 0.01 branches per cell. Thus, while LY294002 did increase branch number slightly in CTβ2-deficient cells, they still had 70% fewer branches than control cells treated with LY294002. Based on these experiments, we can conclude that CTβ2 is involved in LY294002-stimulated primary neurite outgrowth and branch formation in PC12 cells.

Since treatment with a PI-3 kinase inhibitor, LY294002, did not stimulate neurite branching in CTβ2-deficient cells, we speculated that CTβ2 may be a downstream target of the PI-3 kinase/Akt signaling pathway. CTβ2 is heavily phosphorylated *in vivo* [12] and we identified a putative Akt phosphorylation motif within the carboxyl termini of both CTα and CTβ2.

To test if CTß2 were a substrate for phosphorylation, we did phosphorylation studies on CTβ2 in PC12 cells with long-term exposure to LY294002. Differentiated PC12 cells were incubated with 10 μM LY294002 or an equivalent volume of vehicle for 48 h. Then, cells were incubated with [^{32}P]orthophosphate for 1 h and CTβ2 proteins were immunoprecipitated and

Figure 4.2. LY294002-stimulated primary neurite and branch formation requires CTβ2 expression. PC12 cells were co-transfected overnight with vector encoding GFP and either pSiCTβ2 (SiCTβ2) or vector containing a scrambled siRNA insert (Control). Medium was changed to F12K medium containing 50 ng/ml NGF and 10 μM LY294002 (Control/Ly and SiCTβ2/Ly) or an equivalent volume of vehicle (Control and SiCTβ2) and cells were differentiated for 2 days. On day 2 of NGF-induced differentiation, cells were viewed under fluorescence microscopy and all primary neurites (A) and branches (B) (greater than 20 pixels) on GFP-positive cells were counted. Data represent the mean ± SEM of the number of primary neurites or branches per GFP-positive cell. A minimum of 50 cells were scored for each time point. ** (Control vs. Control/LY, $P < 0.01$). * (Control vs. Control/LY, $P < 0.05$; SiCTβ2 vs. SiCTβ2/LY, $P < 0.05$).

Figure 4.2



separated by SDS-PAGE. [^{32}P]-labelled CTβ2 proteins were identified by phosphoimage analysis. Treatment with LY294002 had no appreciable effect on [^{32}P]-labeling of CTβ2 proteins and the incorporation of [^{32}P] orthophosphate was not quantitated (Fig. 4.3). Thus, in NGF-differentiated PC12 cells, we conclude that CTβ2 is not a substrate for PI-3 kinase/Akt phosphorylation.

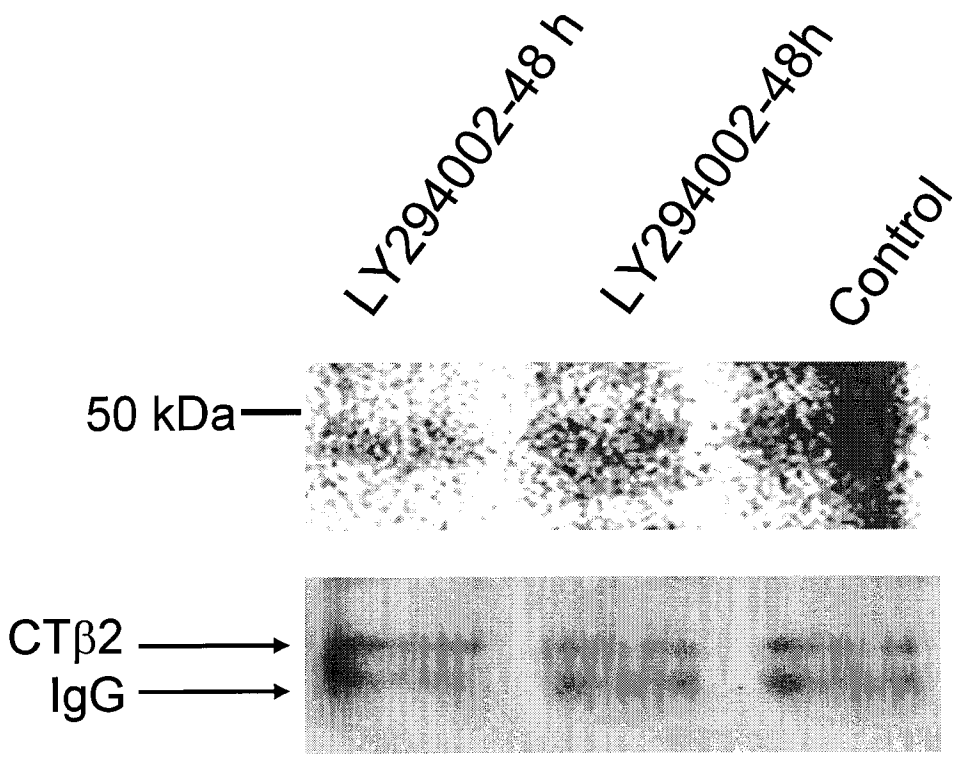
4.3.2 Inhibition of cyclin-dependent kinases decreases neurite outgrowth

In human neuroblastoma SK-N-BE(2)C cells, both roscovitine and over-expression of dominant negative cdk5 protein dramatically decreased neurite outgrowth in response to retinoic acid [23]. Roscovitine is an ATP-directed non-specific inhibitor of cyclin-dependent kinases [24, 25] and has been used to inhibit cdk5 within neurons [26]. We induced differentiation of PC12 cells with nerve growth factor for 24 h and then continued the differentiation in the presence or absence of 50 μM roscovitine for an additional 2 days. Roscovitine-treated PC12 cells had 2.0 neurites per cell and vehicle-treated cells had 3.5 neurites per cell. Since we measured only 30 cells in each treatment group further experiments are required to confirm that roscovitine treatment significantly decreases neurite outgrowth in PC12 cells.

Figure 4.3. CTβ2 is not phosphorylated by Akt

PC12 cells were treated with 50 ng/ml NGF overnight and then treated for 48 h with 50 ng/ml NGF and 10 μM LY294002 (LY294002-48h), or an equivalent volume of vehicle (Control). All treatment groups were labeled with 100 mM [^{32}P] orthophosphate for 1 h. CTβ2 proteins were immunoprecipitated from cell lysates as described in Methods, separated by SDS-PAGE and exposed to a phosphoimage cassette for 3 days. Upper panel: phosphoimage analysis of [^{32}P]-labelled CTβ2 proteins. Lower panel: anti-CTβ2 immunoblot of CTβ2-immunoprecipitated proteins. CTβ2 and anti-M IgG heavy chain bands were identified as described in Chapter 3. Results are representative of two independent experiments.

Figure 4.3



4.3.3 Cdk5 phosphorylates CTβ2 in vitro *and roscovitine alters CTβ2 phosphorylation* in vivo

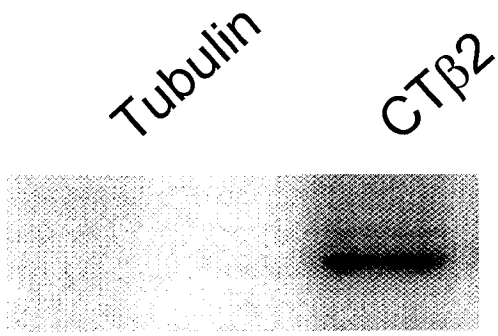
CTβ2 has a putative cdk5 phosphorylation consensus motif within its carboxyl terminus and we hypothesized that cdk 5 may directly phosphorylate CTβ2. To test this hypothesis, CTβ2 and tubulin proteins were immunoprecipitated from PC12 cell homogenates and used as substrates for cdk5 phosphorylation in an *in vitro* kinase assay. We used tubulin as a negative control because it lacks a cdk5 phosphorylation motif and is not known to be phosphorylated by cdk5. As shown in Fig. 4.4A, CTβ2 is readily phosphorylated *in vitro* by cdk5 (Lane 2); however, tubulin is not phosphorylated by cdk5 (Lane 1). Immunoblotting verified that equivalent amounts of tubulin and CTβ2 proteins were used in the assays.

Since cdk5 phosphorylates CTβ2 *in vitro*, we determined whether cdk5 phosphorylates CTβ2 *in vivo*. If CTβ2 were phosphorylated by cdk5 *in vivo*, treatment with roscovitine, a cdk inhibitor, should diminish cdk5-dependent labeling of CTβ2 protein with [^{32}P] orthophosphate. To test this hypothesis, PC12 cells were treated with roscovitine for either 1 h or 48 h prior to metabolic labeling of the cells with [^{32}P] orthophosphate. Afterward, CT proteins were immunoprecipitated from the PC12 cell lysates and separated by SDS-PAGE as described in the Methods. The [^{32}P] orthophosphate-derived radioactivity associated with CTβ2 was assessed from two separate gels in two independent experiments. As shown in Fig. 4.4B, CTβ2 proteins from cells treated for 1 h with roscovitine incorporated far less (66 ± 6 %)[^{32}P]

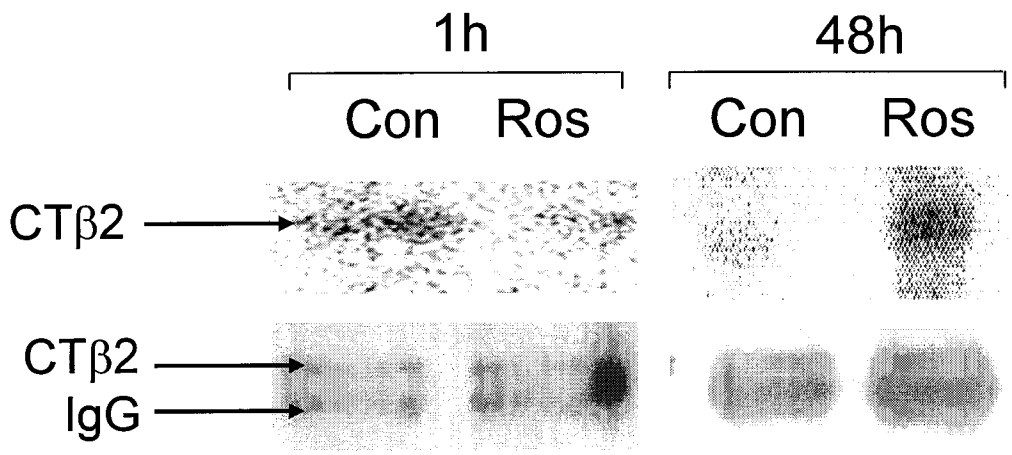
Figure 4.4. CTβ2 is phosphorylated *in vitro* by cdk5 and its phosphorylation is altered *in vivo* by roscovitine. A. *In vitro* cdk5 kinase assay. CTβ2, α-tubulin and cdk5 proteins were immunoprecipitated from lysates of PC12 cells with anti-M, anti-tubulin and anti-cdk5 antibodies respectively. Immunoprecipitated cdk5 was incubated with either tubulin or CTβ2 and [^{32}P] ATP in *in vitro* kinase buffer for 15 min and the proteins were separated by SDS-PAGE and exposed to a phosphoimage screen for 3 days. Amounts of [^{32}P]-labelled CTβ2 and α-tubulin proteins were determined by phosphoimage analysis. **B**. *In vivo* phosphorylation of CTβ2. PC12 cells were treated with 50 ng/ml NGF overnight and then treated for 48 h with 50 ng/ml NGF and 25 μM roscovitine or an equivalent volume of vehicle (Control). Cells were switched to phosphate-free DMEM containing 100 mM [^{32}P] orthophosphate for 1 h. CTβ2 proteins were immunoprecipitated from cell lysates, separated by SDS-PAGE and exposed to phosphoimage cassette for 3 days. Upper panel: phosphoimage analysis of [^{32}P]-labelled CTβ2 proteins. The apparent difference in [^{32}P] orthophosphate CTβ2-labelling in control cells from the 1 and 48h time points is an artifact of phosphoimaging enhancement. Lower panel: anti-CTβ2 immunoblot of CTβ2-immunoprecipitated proteins. CTβ2 and anti-M IgG heavy chain bands were identified as described in Chapter 3. Results are representative of two independent experiments.

Figure 4.4

A.



B.



orthophosphate than CTβ2 immunoprecipitated from untreated cells. This observation strongly suggests that CTβ2 is a substrate for cdk5. In striking contrast, long-term treatment with roscovitine had the opposite effect: CTβ2 proteins from cells treated with roscovitine were labeled with far more (50 ± 15%) [^{32}P] orthophosphate than CTβ2 proteins from untreated cells (Fig. 4.4B, upper panel). Equivalent volumes of immunoprecipitated CTβ2 proteins were used for immunoblotting (Fig. 4.4B lower panel) to confirm that protein loading was equivalent in all lanes of the gel. These results were similar in three independent experiments.

We have previously reported that CTβ2 is localized to the axons of sympathetic neurons and retinal ganglion cells (Chapter 3, Fig. 3.2). Thus, we examined the effect of roscovitine treatment on CTβ2 phosphorylation within distal axons. The distal axons of compartmented cultures of sympathetic neurons were treated with 50 μM roscovitine for 12 h and then incubated in phosphate-free medium supplemented with [^{32}P] orthophosphate for 1 h. Immunoprecipitated CTβ2 proteins were separated by SDS-PAGE and equivalent CTβ2 proteins were detected by immunoblotting with anti-CTβ2 antibodies; however, subsequent phosphoimage analysis showed no detectable incorporation of [^{32}P] orthophosphate into either control or roscovitine-treated axons (data not shown). We suspect that there was insufficient immunoprecipitation of [^{32}P] orthophosphate-labelled CTβ2 protein to generate detectable radioactivity.

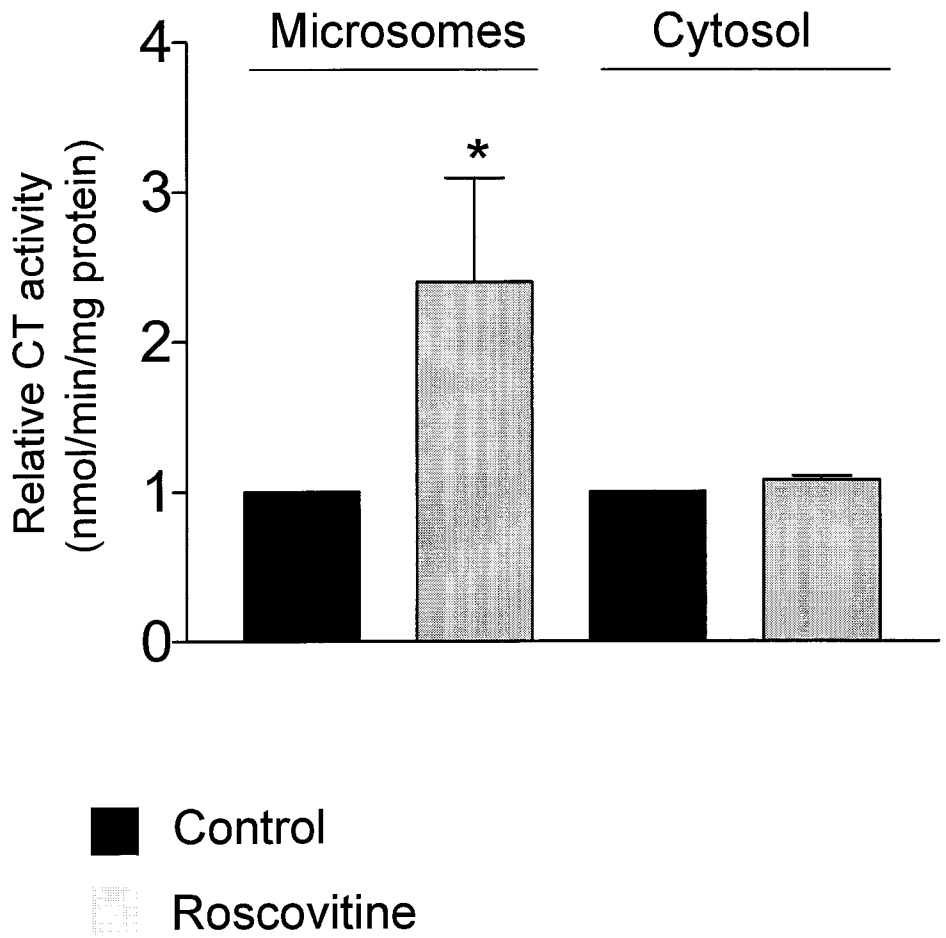
4.3.4 *Roscovitine increases microsomal CT activity in PC12 cells*

Since phosphorylation of CTβ2 by cdk5 is altered by roscovitine treatment, we hypothesized that roscovitine may also affect CTβ2 activity. CT, an amphitrophic enzyme is activated by translocation from the cytosol to membranes. [27]. Previously, we reported that NGF promoted the translocation of CTβ2, but not CTα, from cytosol and membranes [28] thus, we assayed soluble and membrane fractions to determine if roscovitine altered the NGF-stimulated translocation of CTβ2 to membranes. To test this hypothesis, we induced differentiation of PC12 cells with 50ng/ml NGF overnight and then switched the medium to 50ng/ml NGF with either 25 μM roscovitine or an equivalent volume of DMSO. Forty-eight h later, cells were harvested and microsomal membranes and cytosol fractions were prepared as described in the Methods. CT activity was measured in the aliquots by incorporation of [^{3}H]phosphocholine to [^{3}H]CDP-choline as previously described. As shown in Fig. 4.5, the relative CT activity in microsomal membranes from roscovitine-treated cells was 2.4 times higher than that of control cells. In contrast, there was no difference in cytosolic CT activity between control and roscovitine-treated cells.

CTα does not have a cdk5 phosphorylation consensus motif nor is it known to be phosphorylated *in vivo* by any cyclin-dependent kinase; however, since roscovitine inhibits all cyclin-dependent kinases, we cannot dismiss the possibility that the effect of roscovitine on microsomal CT activity is not

Figure 4.5. Roscovitine increases microsomal CT activity but not soluble CT activity in PC12 cells. PC12 cells were treated with 50 ng/ml NGF overnight and then treated for 48 h with 50 ng/ml NGF and 25 μM roscovitine, or an equivalent volume of vehicle (Control). Cell homogenates were centrifuged at 100,000 X g for 30 min. CT activity was measured in the resolubilized pellet (membranes) and the supernatant (cytosol). CT activity was 0.52 nmol/min/mg in the membrane fraction of control cells and 1.27 nmol/min/mg protein in the soluble fraction of control cells.

Figure 4.5



exclusive to an inhibitory action on CTβ2. Interestingly, roscovitine did not affect cytosolic CT activity, thus translocation of CTβ2 from cytosol to membranes is unlikely to account for the increase in microsomal CT activity. Also, although we suspect that the increased microsomal CT activity is due to CTβ2, it is possible that long-term treatment with roscovitine alters PC metabolism throughout the cell, resulting in altered distribution of CTα protein. Examination of the distribution of CT proteins to microsomal or cytosolic subcellular fractions would address this possibility.

4.4 Discussion

CTβ2 is required for LY294002-stimulated neurite branching

Previously, we reported that CTβ2 is required for neurite outgrowth and branch formation. In addition, we now report that CTβ2 is required for LY294002-stimulated neurite branching. LY294002, through inhibition of PI3K prevents Akt activation [8]. Akt is a serine/threonine kinase and phosphorylates proteins with the following consensus motif: RXRXXS/T [29]. Within their carboxyl termini, CTα and CTβ2 share an identical, putative Akt phosphorylation motif: RSRSPS. Since treatment with LY294002 did not stimulate neurite branching in CTβ2-deficient cells and CTβ2 has an Akt phosphorylation motif, we examined whether Akt phosphorylated CTβ2 *in vivo*. LY294002 treatment of PC12 cells did not alter [^{32}P]-labeling of CTβ2 protein (Fig. 4.3). Thus, CTβ2 is unlikely to be directly regulated by Akt.

These data are consistent with a recent report describing a role for Akt in dendritic branching of hippocampal neurons. Like branching of neurites in PC12 cells, branching of dendrites is characterized by extension with subsequent retraction. Leemhuis *et al.* found that inhibition of Akt activity with LY294002 treatment specifically impaired dendritic branch retraction [9]. That is, the dendrites of LY294002-treated neurons sprouted the same number of branches but lost far fewer of them than did untreated cells. CTβ2 provides PC, and thus membrane, for neurite and branch formation therefore, CTβ2 likely participates in neurite sprouting rather than neurite retraction. If CTβ2 contributes to neurite formation and LY294002 treatment impairs neurite

retraction, a lack of CTβ2 protein would impair the ability of Ly294002 treatment to prevent neurite retraction. Also, since the LY294002-dependent stimulation of primary neurite formation was completely abolished in CTβ2-deficient cells whereas branch formation was impaired by approximately twenty percent, perhaps CTβ2 may play a stronger role in primary neurite formation than in branch formation.

Role of cdk5-mediated CTβ2 phosphorylation.

CTβ2 is heavily phosphorylated *in vivo*; however, studies of CTβ2 phosphorylation have not been reported [30]. In HeLa cells, both insulin and epidermal growth factor (EGF) can stimulate CT phosphorylation [31]. Recently, Lykidis *et al.* reported that HeLa cells abundantly express both CTα and CTβ isoform mRNAs [12], thus, it is possible that CTβ2 is phosphorylated in response to growth factors.

Nerve growth factor signaling activates a vast number of intracellular signaling molecules [5]. Cyclin-dependent kinase 5 and its co-activator, p35, are among the NGF-activated proteins [21] and have been strongly implicated in axon growth and recovery after injury [15, 16] . Since CTβ2 has a cdk5 phosphorylation consensus motif within its carboxyl terminus, we hypothesized that cdk 5 may directly phosphorylate CTβ2. Indeed, cdk5 heavily phosphorylated CTβ2 *in vitro* (Fig. 4.4A). In addition, short-term treatment of PC12 cells with roscovitine sharply decreased CTβ2 phosphorylation. Thus, we predicted that cdk5 directly regulates CTβ2 activity

via phosphorylation. However, long-term treatment with roscovitine resulted in a dramatic increase in CTβ2 phosphorylation suggesting that chronic inhibition of cdk5 enhances CTβ2 phosphorylation.

Since long-term treatment with roscovitine enhanced phosphorylation of CTβ2, we tested whether roscovitine affected CTβ2 activity *in vitro*. Interestingly, the relative CT activity in microsomal membranes from roscovitine-treated cells was 2.4 times higher than that of control cells; however, cytosolic CT activity was unaffected (Fig. 4.5). Since long-term roscovitine treatment enhanced CTβ2 phosphorylation and had no effect on cytosolic CT activity, it is unlikely that cdk5-dependent phosphorylation promotes CTβ2 translocation from cytosol to membranes. One alternative hypothesis is that cdk5 phosphorylation of CTβ2 alters CTβ2's susceptibility to proteolytic degradation. Wieprecht *et al.* demonstrated that oloumoucine, another inhibitor of cyclin-dependent kinases, increased proteolytic degradation of CT in human keratinocytes [32]. Also, in neurons, Cdk5 can phosphorylate target proteins to alter their susceptibility to proteolysis. Over-expression of cdk5 and its co-activator, p35, in neurons and CHO cells increased the phosphorylation and protein stability of presenilin 1 protein [33] and tyrosine hydroxylase. [34]. Paradoxically, long-term treatment with roscovitine increased microsomal CT activity and CTβ2 phosphorylation; however, neurite outgrowth was reduced. We suspect that other substrates of cdk5 act up-stream of CTβ2 to re-organize the growth cone prior to branch formation.

One additional point needs to be addressed. In differentiation of L6 myoblasts, PI3 kinase and Akt are up-stream modulators of cdk5 activity [35]. However, studies in $cdk5^{-/-}$ mice suggest that cdk5 is an upstream modulator of Akt [26]. Clearly, PI3 kinase and cdk5 signaling have an element of "cross talk" that complicates interpretation of the use of LY294002 and roscovitine, inhibitors of Akt and cdk5 respectively.

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Chapter 5

Conclusions and future directions

Phosphatidylcholine is the principal constituent of eukaryotic membranes. Chapter 1 illustrates the importance of PC in cellular homeostasis and describes the CDP-choline pathway of PC biosynthesis. Since CTP:phosphocholine cytidylyltransferase catalyzes the rate-limiting reaction of this pathway, CT is the focus of this discussion. There are multiple isoforms of CT in rodents: CTα, CTβ2 and CTβ3 [1-3]. Structure and organization of *Pcyt1a* and *Pcyt1b*, the two genes encoding the three CT isoforms, are reviewed as is the functional organization of the CT proteins. Also, the unique sub-cellular localization and tissue distribution of CT isoforms is used to illustrate the non-redundant roles of CT within cellular PC metabolism.

In particular, Chapter 1 elaborates on PC metabolism and the expression of CT isoforms and regulation of CT activity within the nervous system. During development, the rapid growth of neurites produces a strong demand for PC. The overall objective of this work is to evaluate how CT isoforms and PC biosynthesis contribute to neurite outgrowth and branching.

In chapter 2, evidence for the specific up-regulation of CTβ2 expression during neuronal differentiation is presented. Using two neuronal cell culture systems, PC12 and murine neuroblastoma cells, CT activity *in vitro* and PC biosynthesis were measured. In addition, CT α and CTβ2 mRNA and protein levels were quantitated during neurite outgrowth. In PC12 cells, nerve growth factor (NGF) enhances CTβ2 expression and elevates PC levels coincident with neurite outgrowth. Since NGF signals through the MAP kinase cascade, specific inhibitors of MAP kinase signaling (U0126) were used to determine

how NGF regulates CTβ2 expression. U0126 treatment inhibited neurite outgrowth and abolished the NGF-dependent up-regulation of CTβ2 expression.

Recently, the CTβ2-specific promoter of the *Pcyt1b* gene was cloned from murine neuroblastoma cells (C. Banchio, personal communication). Future studies will include identification of *cis*-acting transcriptional elements and the cognate transcription factors involved in regulation of CTβ2 expression during neuronal differentiation. In chapter 2, we have shown NGF up-regulates CTβ2 mRNA in neurons; however the signaling cascade of this up-regulation is unclear past the point of MAP kinase activation [4]. Several transcription factors, including MafK [5], Nex1 [6], Sox21 [7]and activator protein-1 [8], have been implicated in NGF-dependent neurite outgrowth, The CTβ2 promoter will be examined for putative transcriptional elements which bind any of these transcription factors.

Since CTα and CTβ2 are both expressed in neurons, experiments presented in chapter 3 examined the specific role that CTβ2 fulfills during neurite outgrowth There is strong evidence that phospholipids, including PC, are synthesized both in axons as well as in the cell bodies of neurons [9-12]. We demonstrated that CTβ2 is targeted to the distal axons of both sympathetic neurons and retinal ganglion cells. In contrast, CTα is localized to the cell bodies and proximal axons but is not found in the distal axons of sympathetic neurons.

Protein palmitoylation, the covalent attachment of palmitate to cysteine residues, is an established method of targeting neuronal proteins to axons [13]. For example, growth associated protein 43 (GAP-43) is targeted to axonal growth cones by palmitoylation [14]. Husseini *et al.* have also demonstrated that palmitoylated GAP-43 localizes to detergent-insoluble glycolipid (DIG) domains; these DIG domains are the proposed transport mechanism for delivery of GAP-43 to the growth cone [15].

We hypothesize that CTβ2 is specifically targeted to axons by post-translational protein modification. Interestingly, within the amino terminus of CTβ2, we've identified several cysteine residues arranged in a putative palmitoylation motif (amino acids 46-51: SSCQCQ, Figure 1.5). CTα does not contain these cysteine residues. Future experimentation includes radiolabelling of neurons with [^{125}I]-iodopalmitate to determine whether CTβ2 protein is palmitoylated. Preliminary studies have been attempted; however insufficient [^{125}I]-iodopalmitate was incorporated into CTβ2 or a control protein (known to be palmitoylated) to determine whether CTβ2 is palmitoylated *in vivo*.

Chapter 3 also examined the roles of individual CT isoforms in neurite outgrowth. RNA silencing and recombinant cDNA strategies were used to either "knockdown" or over-express CTα or CTβ2 in differentiating PC12 cells. We provide strong evidence that CTβ2 is required for neurite sprouting and branch formation but not for neurite extension. To our knowledge, this is the first identification of a phospholipid biosynthetic enzyme required for neurite

outgrowth and branching. We hypothesize that CTβ2, which is localized to neurites, provides a local source of PC during the dramatic cytoskeletal re-organization that occurs with new neurite or branch formation

A more definitive evaluation of CTβ2's role in neurite outgrowth and branching requires examination of these processes in neurons with a complete deficiency of CTβ2 (CTβ2$^{-/-}$). Recently, Jackowski *et al.* generated mice with a disruption in *Pcyt1b*, the gene encoding CTß2 [16]. Based on our observations that CTβ2 is involved in neurite branch formation, we predict that a deficiency of CTβ2 will manifest as alterations in learning or motor coordination rather than as gross neuropathological changes. In addition, we plan to examine the susceptibility of neurons from CTβ2$^{-/-}$ mice to ischemic insults and neurotoxins. As discussed earlier, CDP-choline, the product of the CT reaction, is neuroprotective against both chronic neurodegenerative disorders and acute ischemic insults [17]. Currently, plans are underway to evaluate these parameters in *Pcyt1b*$^{-/-}$ mice.

One important caveat to these proposed experiments concerns the CTβ expression profile in mice. Mice express both CTβ2 and CTβ3 protein [2] whereas rats (and rat-derived PC12 cells) express CTβ2 but not CTβ3 protein [4].

In Chapter 4, the role and regulation of CTβ2 in neurite growth was further investigated. In PC12 cells, both the PI3 kinase/Akt pathway [18, 19] and cyclin-dependent kinase 5 [20] have been implicated in neurite outgrowth and branch formation. Specifically, Akt is a negative regulator of neurite branching

and treatment with LY294002, an Akt inhibitor, stimulates neurite outgrowth and branch formation [19]. Interestingly, CTβ2 contains putative consensus sequences for both kinases within its phosphorylation domain (Chapter 1, Fig. 5). Using LY294002, an Akt inhibitor, we determined that LY294002-dependent stimulation of neurite outgrowth, particularly the primary neurite population, required CTβ2 expression; however, CTβ2 itself was not phosphorylated by Akt.

In contrast, CTβ2 was phosphorylated by cdk5 *in vitro* and its phosphorylation was impaired with short-term roscovitine treatment (a cdk5 inhibitor) *in vivo.* Long-term treatment with roscovitine impaired neurite outgrowth and enhanced CTβ2 phosphorylation, consistent with the proposed negative regulatory effect of phosphorylation of CT activity. However, surprisingly, long-term (48 hour) treatment with roscovitine increased, rather than decreased, microsomal activity of CT *in vitro*. Since *in vitro* CT activity assays measure all CT isoforms, we cannot conclude whether the increase in microsomal CT activity is due to CTα, CTβ2 or both isoforms. Examination of the microsomal and cytosolic distribution of CTα and CTβ2 proteins during roscovitine treatment will clarify which CT isoform is involved. Alternatively, roscovitine may not alter the sub-cellular distribution of CT proteins at all. Future studies are required to clarify how cdk5 regulates CTβ2 in neurons.

Our data demonstrate a unique role for CTβ2 in neurite outgrowth and branch formation. Moreover, to our knowledge, we have identified the first

phospholipid biosynthetic enzyme to be targeted to axons and propose that protein palmitoylation of CTβ2 specifically targets it to the neurite.

We provide strong evidence that CTβ2 is phosphorylated by cdk5 *in vitro* and *in vivo*, thereby identifying the first kinase to play a role in regulation of CTb2 activity. Taken together, these studies provide important insight into the role of CT isoforms and PC biosynthesis within neurons. The wide-ranging therapeutic uses of CDP-choline in acute ischemia and chronic neurodegenerative diseases underscore the importance of delineating how CT isoforms contribute to neuronal function and recovery after injury.

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