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NOVEL MODULATION OF A LIPID SECOND MESSENGER AS A FUNCTION OF NEUROTROPHIN RECEPTORS

by

TERRI KAGAN

A dissertation submitted to the Graduate Faculty in Biology in partial fulfillment of the requirements for the degree of Doctor of Philosophy
The City University of New York

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Abstract

NOVEL MODULATION OF A LIPID SECOND MESSENGER AS A FUNCTION OF NEUROTROPHIN RECEPTORS

by

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Adviser: Dr. Zahra Zakeri

Ceramide is a lipid second messenger that is involved in the propagation of diverse signaling pathways that ultimately lead to either differentiation or apoptosis in a cell specific manner. Since two distinct pathways (SM hydrolysis or neosynthesis) can generate endogenous ceramide, its origination may be vital to its downstream signaling activity. In this study we have investigated the role of ceramide in the induction of differentiation and cell death in the rat pheochromocytoma (PC12) cell line, with specific reference to its origin.

Since the PC12 cell line responds to nerve growth factor (NGF) by expressing a neuronal phenotype, we first asked if ceramide mediates differentiation in response to NGF and whether the cell's expression of neurotrophin receptors affects its ability to generate ceramide. We established that NGF-induced PC12 cell differentiation activates both ceramide pathways but requires only SM hydrolysis, and that overexpression of the high affinity $p140^{trkA}$ NGF receptor results in a cell that requires both ceramide pathways to generate less ceramide in response to NGF. We further established that $p140^{trkA}$ overexpressing cells (TrkA) under-express the low affinity $p75^{NTR}$ receptor. We next determined that apoptosis in neuronal PC12 cells induced by NGF depletion is mediated

by ceramide synthesis, while both pathways mediate ethanol-induced apoptosis. Similarly, we demonstrated that TrkA cells require both pathways but generate smaller amounts of ceramide in response to either NGF depletion or ethanol. We next looked to the mitochondria as a possible downstream target of ceramide. We found that differentiation always accompanied mitochondrial hyperpolarization while apoptosis involved loss of either mitochondrial integrity or enzyme activity. We also established that TrkA cells exhibit mitochondrial hyperpolarization and/or protection against loss of mitochondrial integrity and function in response to inducers of cell death. Finally we examined the ability of the mitochondrial coenzyme CoQ_{10} to interact with the apoptotic machinery. We found that similar to TrkA overexpression, exogenous CoQ_{10} both inhibits ceramide synthesis and prevents loss of mitochondrial integrity and function. Finally, we present a model which suggests that neurotrophin receptors may interact with the apoptotic machinery upstream of the mitochondria, blocking apoptosis through modulation of downstream sphingolipid generation and mitochondrial function.

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I. INTRODUCTION

Prologue

Neurons are specialized post-mitotic cells, which are not easily replaced when they die. During normal embryonic development an overabundance of neurons is produced and "pruned" so that the final number is matched to the size of the target tissue to be innervated. The selective death of these neurons occurs by apoptosis, in a physiological, regulated and controlled manner (Kerr '65; '71; Kerr et al. '72; '91; Bursch et al. '00; Wylie et al. '80). Both differentiation and selective apoptosis of neuronal cells is mediated by growth factors and when development is complete, neuronal cell loss is not normally a major factor in either organismal homeostasis or aging. However, since neurons are terminally differentiated cells, once neurons are lost to disease, the damage is irreversible and inevitable. Death of neurons is implicated in a variety of disorders including Alzheimer's disease (AD), Parkinson's disease (PD), Huntington disease (HD), ethanol poisoning (Fetal Alcohol Syndrome) and cerebral ischemia (see review by Ariga et al. '98; West and Goodlet, '90, Cotman and Anderson '95, Johnson et al. '95). However, despite considerable progress in identifying the extra- and intra-cellular factors, gene products and proteases involved in committing a cell to either specialization or self-destruction, we do not yet fully understand how a cell interprets these signals thereby committing itself to its fate.

Ceramide is one such signal that has recently emerged as an important second messenger and reports have shown that it regulates cell proliferation (Boucher et al. '95; Okazaki et al. '90, Dobrowsky et al. '94), differentiation (Fishbein et al. '93), and growth arrest (Dbaibo et al. '95). In addition, both neuronal differentiation mediated by

specific growth factor receptors (Dobrowsky et al. '94) and neuronal apoptosis induced by growth factor withdrawal are also mediated by ceramide (Venkatarana and Futerman '00). The ability of ceramide to mediate such diverse processes may result from triggering different downstream targets, such as caspases, mitogen activated protein kinases, and stress activated protein kinases (Hannun '96). More recently, reports suggest that caspases may not be required for apoptosis (Hakem et al '98), that caspase precursors have a mitochondrial distribution (Mancini et al '98) and that mitochondrial function determines the specific mode of cell death (Ankarcrona et al '95). Caspases have also been identified as upstream of mitochondrial events (Genestier et al '98). On the other hand, ceramide generation is reported to activate caspases and to be inhibited by the mitochondrial anti-apoptotic Bcl-2 protein (Yoshimura et al. '98). Interestingly, the low affinity nerve growth factor (NGF) receptor is reported to signal through a ceramide-caspase dependent pathway that is differentially regulated by the Bcl-2 family (Lievremont et al. '99; Coulson et al '99).

This study will focus on the ceramide signal transduction pathway and its relationships with mitochondrial activation and growth factor receptor expression in differentiating and dying neurons. To do so, we will: a) investigate the mechanisms by which ceramide levels are altered with respect to neurotrophin receptor expression in both differentiating and dying neuronal cells, b) examine the ability of neurotrophin receptor expression to delay activation of cell death, c) determine whether apoptosis induced by a variety of agents use the same ceramide signaling pathways as does growth factor withdrawal, d) establish whether ceramide-mediated differentiation or apoptosis alters mitochondrial integrity or function, and e) investigate the ability of the

mitochondrial coenzyme ubiquinone to alter either mitochondrial function or ceramide generation as well as to prevent apoptosis.

1. Concepts of Cell Death and Differentiation

Cell division, differentiation, and death all play important roles in the organizing of cells to form tissues and organs during embryonic development as well as during adult homeostasis and regulation. Nevertheless, the relationship among these diverse processes is not fully understood.

The decision for a cell to continue to proliferate or to exit the cell cycle by differentiation, quiescence, or death is determined by a wide variety of cellular and extracellular factors. These factors include substrates and soluble signaling molecules as well as interactions with neighboring cells, cell lineage, genotype, developmental stage, stage of cell cycle, metabolic second messengers and many more. This study examines the significance of specific signals in cell differentiation and death in order to better understand these relationships and provide insight to specific disease states.

A. Importance of differentiation

Differentiation is an aspect of patterning, development, morphogenesis, and teratogenesis. Differentiation occurs not only in embryonic development and morphogenesis but also in the homeostasis of adult organisms. Differentiating cells in adult organisms include keratinocytes, epithelial cells, chondrocytes, osteocytes, myocytes, hepatocytes, lymphocytes, and erythrocytes. Differentiation also allows specific tissues in mature animals to respond to varying functional demands. For example, in mature female mammals the epithelial cell population of the mammary gland is minor while the bulk of the gland is composed of adipose stromal tissue. Pregnancy results in selective differentiation of the mammary secretory epithelium and loss of

adipose cell volume. Similar functional alternations and selective differentiation are evident in the immune system as well as in other types of secretory and non-secretory tissue (Medina et al. '00).

As with other mechanisms, differentiation is sometimes maladaptive. These defective differentiation signals often play a major role in tumor and cancer growth. The activation by estrogen of the BRCA1 gene, which induces normal differentiation in the mammary cells of younger women, instead stimulates transformed cells, resulting in breast cancer in older women (see review by Hilakivi-Clarke '00). In other examples, the maladaptive neuroendocrine-like differentiation of pancreatic cells has been implicated in pancreatic carcinoma (Tezel et al. '00), and the loss of differentiative ability in leukemic cells has been implicated in the uncontrolled growth characteristic of leukemogenesis. In fact, reversal of a putative "differentiative block" has become an essential element in therapeutic treatment of certain cancers (see review by Randolph '00).

B. Importance of cell death

Cell death is also a fundamental part of embryonic development, morphogenesis, and teratogenesis, as well as to aging of the central nervous system, tumor growth, and disease. Cell death patterns new structures, deletes unneeded ones, controls cell number or eliminates abnormal, misplaced, nonfunctional or harmful cells (see review by Vaux and Korsmeyer '99; Saunders '66; Williams and Bell, '91; Zakeri and Ahuja, '94; Coucouvanis and Martin '95; Raff '92; Hakem et al. '96; Cohen '91; Killeen and Littman '96).

Inappropriate activation or inhibition of the correct spatial and temporal sequence of cell death results in a variety of diseases. In adult or embryo, repression of normally occurring cell death can result in tumor development or malignant cancer (Reed et al. '90; Carson and Ribiero '93; Mountz and Gause '93; Williams '91; King et al. '96; Lu et al. '96). Excessive cell death may also result from post-ischemic tissue reperfusion (Benitez-Bribiesca '00), persistent infections such as influenza or HIV (Mori and Kimura '00; Losa and Graber '00), autoimmune disease such as Systemic Lupus Erythematosis (SLE, Navratil and Ahearn '00), or neurodegenerative diseases such as Parkinson (PD) and Alzheimer's disease (AD, see review by Ariga et al. '98).

a. Necrosis

Cell death can be identified by a variety of biochemical and morphological hallmarks. Historically, necrosis is considered to be both a non-physiologic and violent form of cell death (Kerr et al. '72; Schweichel and Merker '73; Wyllie '74; Arends et al. '90). Necrotic cells typically rupture following toxic insult and osmotic lysis of cell content is followed by an inflammatory response. Necrosis does not require the input of energy or the synthesis of either proteins or nucleic acids. Necrosis is also associated with dramatic changes in mitochondrial ultrastructure and rapid ATP depletion (Leist et al. '97). Furthermore, Nicotera et al. ('99) and others have recently shown that intracellular energy levels and mitochondrial function are rapidly compromised in necrotic cell death (Arora '98) and that pre-emptying cells of ATP can switch the type of death caused by toxic agents to necrosis.

In contrast, physiologic cell death (PCD) is characterized as an active, inherently programmed and tightly regulated fragmentation of a cell followed by phagocytosis of the fragments without inflammation. PCD is an evolutionarily conserved and genetically regulated mechanism and therefore requires energy input, the synthesis of macromolecules, or *de novo* gene transcription. Nevertheless, although PCD and necrosis are considered conceptually distinct forms of cell death, there is increasing evidence that they represent only the extreme ends of a wide range of possible morphological and biochemical deaths (Schweichel and Merker '73; Clarke '90; Schwarz et al. '93; Zakeri et al. '95; Bursch et al. '96; Bursch et al. '00).

b. Apoptosis

In classical "apoptosis" or Type I cell death as defined by Clarke ('90), a characteristic condensed nuclear morphology is accompanied by fragmentation of the DNA into microsomal ladders before other morphological changes are evident. Both cytoplasmic condensations and nuclear condensations precede membrane blebbing and cellular fragmentation into apoptotic bodies. Organelles are still intact in condensed cells and cell fragments, and cell membranes are preserved so that lysis does not occur; however, cytoskeletal elements disintegrate during early stages. Apoptotic cells are eventually phagocytosed and degraded *in vivo* with minimal lysosomal involvement or toxic lysis (Kerr et al. '72; Wyllie et al. '80; Ahuja and Zakeri '94; Bursch et al. '00). More recently it has become clear that mitochondria exhibit significant changes in their structure and function during apoptosis and in fact play a major role in the process of

apoptosis (Susin et al. '98; Skulachev '98; Kroemer et al. '98; Green and Reed '98; Brenner and Kroemer '00).

c. Autophagy

One of the major parameters of PCD in secretory cells, or in cells possessing relatively large cytoplasms, appears to be activation of lysosomal enzymes (Lockshin and Williams '64; Halaby et al. '94; Bursch et al. '96, '00). This type of death is called "autophagy" or Type II cell death and is characterized by the early formation of cytoplasmic autophagic vacuoles and prominent lysosomal activity. In autophagic cells, DNA fragmentation appears to be a late event and is evident only in the final stages (Zakeri et al. '95; Bursch '96). Autophagic cell death includes degradation of Golgi apparatus, polyribosomes and endoplasmic reticulum while the integrity of cytoskeletal elements like intermediate and microfilaments may or may not be preserved (Jochová et al. '97; Bursch et al. '00). In vivo, autophagic cells are eventually phagocytosed by neighboring cells much like apoptotic cells (Schweichel and Merker '73).

d. Other

Although most dying cells fall into one of the previous two categories, PCD comprises subtypes that do not all follow the same morphology or biochemistry. In his review of cell death, Clarke ('90) described Type III cell death as cell fragmentation without either condensation or lysosomal involvement. Type III cells die by regulated mechanisms that do not involve either nuclease activation or autophagy. Even more recently, Roach and Clarke ('00) have described non-apoptotic dead chondrocytes *in*

vivo. They term these cells 'dark chondrocytes' because of their convoluted nuclei, patchy chromatin condensations, and dark cytoplasm full of excessive endoplasmic reticulum. Immature chondrocytes were also found that exhibited convoluted nuclei and patchy chromatin condensations, but with an expanded endoplasmic reticular lumen and both cytoplasm and organelles reduced to dark, worm-like inclusions. These cells are termed 'paralyzed' and Roach and Clarke suggest that both they and 'dark' chondrocytes in essence are apoptotic cells that could not be phagocytosed due to their confinement within their lacunae *in vivo*.

Thus, although in many instances the ordered execution of a cell results in characteristic morphological and biochemical changes that can be specifically termed "apoptosis" or "autophagy", subroutines of the degradation program may not be active in all cases of cell death, which results in differences in the morphology and biochemistry of dying cells. Furthermore, since all forms of cell death overlap to some extent, the same insult may give rise to one form or another or even an intermediate. Moreover, it is often the intensity of the initial insult and the histology of the cell that decides the prevalence of a specific type of death and the morphology varies according to the system under examination.

C. Signals regulating differentiation and death

Cells control differentiation and death through activation of internally regulated programs that initiate specific signal transduction pathways. Signaling includes three stages: signal induction, propagation via signaling cascade, and execution. Although the signal induction stage varies with the stimulus, the process eventually converges on

common propagation and execution stages. Some signal inductions begin with specific ligands engaging cell surface receptors that mediate the external signaling events by modulating downstream activity in the form of second messengers and biochemical pathways. There is however multiplicity of receptors and crosstalk between signals, receptors and pathways, with the end result dependent on the relative contribution of each. In fact, recent studies have revealed that switching between the different gene programs that produce proliferation, differentiation and death is controlled through a complex interplay between the exterior soluble factors and insoluble extracellular matrix molecules, available cell surface receptors, static mechanical forces that produce changes in cell shape, and internal signal transduction pathways (see review by Vaux and Korsmeyer '99).

a. External cues

Many different types of signals can induce cellular differentiation or death and the same external signals can be translated differently internally depending on the type and status of cell studied. There are so many agents that can function as signals for differentiation or death, that it is inadvisable to list them all here and I will confine this discussion to a small subset of signals relevant to my research.

Chemicals released from one set of cells can act upon the secretory cells, cells in the immediate surrounding, or even cells at a great distance in an autocrine, paracrine, or endocrine fashion. The most common signaling molecules include retinoids, steroid and peptide hormones, and growth factors. Retinoids induce apoptosis in keratinocytes and interdigital cells (Zakeri and Ahuja '94), and direct the differentiation of neuroblastoma,

leukemic, mammary and pancreatic cells (Reynolds '00; Moriya et al. '00). Ecdysone and juvenile hormone also regulate both differentiation and apoptosis during the development of Drosophila (see review by Baehrecke '00) or other insects (Halaby and Zakeri '97), and estrogen regulates cell growth, differentiation, and death in mammary epithelial cells, mammary duct tissue (Russo et al. '00) and ovarian follicles (Schedin et al. '00). Estrogen can also promote mammary tumor development largely due to its ability to induce differentiation (Yoshidome et al. '00).

The epidermal growth factor (EGF) family and the transforming growth factor-beta (TGF-beta) family play central roles in the regulation of growth as in for example keratinocyte expansion (see review by Hashimoto '00). Other growth factors, like insulin-like growth factors (IGFI,IGFII) regulate muscle differentiation (Florini et al. '91; Ewton et al. '94; Montarras et al. '96), while fibroblast growth factors (FGF, bFGF), nerve growth factor (NGF), and brain-derived neurotrophic factor (BDNF) all improve survival and promote differentiation of neurons, and exert a neuroprotective effect as well when neurons are challenged with metabolic stress or cytotoxins (Nakagami et al. '97; Hossain et al. '97; Casaccia-Bonnefil et al. '99; Avola et al. '00; Reuss and Unsicker '00; Hardcastle et al. '00). Furthermore, the activity of these growth factors is not confined to neuronal tissue, as FGF also regulates epithelial morphogenesis (Kettunen et al. '00) and NGF is implicated in myogenesis (Rende et al. '00) and in hepatic cell pathophysiology (Cassiman et al. '01).

Other factors that can either induce proliferation and promote cellular survival, and/or cause growth arrest and apoptosis include oncogenes (reviewed in Blagosklonny '99), ionizing radiation (Herskind and Rodemann '00; Itoh and Horio '01), inhibitors of

protein synthesis like cycloheximide (Higami et al. '00) and nucleus damaging agents such as the topoisomerase inhibitor, camptothecin (CAM) (Aller et al. '92; Zhang et al. '00; Sanchez-Alcazar et al. '00).

b. Cell surface receptors

As indicated, many stimuli can trigger either cell death or differentiation. Thus an absolute requirement in the initiation of signaling is the presence of appropriate receptor proteins on the exterior plasma membrane of the target cell. Cell adhesion molecules like selectins and integrins are only two of many plasma membrane proteins that function in such signal-transducing roles (reviewed in Gonzalez-Amaro et al. '99). Decreases in the level of integrin expression and degree of cell adhesion are among the first signs of both differentiation and death (Davidowitz et al. '01) and may be part of a negative feedback system. Interactions of integrins with extracellular matrix proteins normally activate focal adhesion kinase (FAK), and suppress apoptosis in healthy epithelial and endothelial cells (Frisch et al. '96; Crouch et al. '96).

Specific cells possess cell surface receptors called death receptors. These receptors detect the presence of specific extracellular death signals and trigger some form of rapid cellular destruction (Nagata '97; Ashkenazi and Dixit '98; reviewed in Wehrli et al. '00). Expression and signaling by death receptors is essential for key physiologic functions in various systems including the functional integrity of skin (Leithauser et al. '93; Matsue et al. '95; Viard et al. '98) and the immune response (Zheng et al. '95; Brunner et al. '95; Ashkenazi and Dixit '98).

Death receptors are a growing family of Type I transmembrane proteins that belong to the TNFR family of receptors and contain a cytoplasmic sequence named a death domain (DD). At least six members of this subfamily have been identified in humans including Fas (APO-1/CD95), TNFR1, TRAMP, TRAIL, TRAIL-R1, TRAIL-R2. The DD couples the receptor to cysteine protease (caspase) cascades, which trigger apoptosis following proteolytic degradation of specific substrates such as poly-(ADP-ribose)-polymerase (PARP). Furthermore, although all death receptors can transmit such death inducing signals, only TNFR1 and TRAIL can also induce specific expression of specific genes such as NF-κB (see review by Wehrli et al. '00). It should be pointed out that although NF-κB is often induced in response to apoptotic stimuli, it is not specifically a pro-apoptotic transcription factor. In fact NF-κB activation can be required to suppress apoptosis such as that induced by oncogenic *ras* (Mayo et al. '97) and inhibition of NF-κB can cause apoptosis (Taglialatela et al. '97).

c. Specific Signaling Pathways

Although many different types of stimuli or ligand/receptor interactions can induce either cell death or differentiation, once the decision has been made, the execution of either program seems to involve a relatively limited number of intracellular cascades or pathways. Ligands bind to receptors and recruit adaptor molecules such as TRADD, FADD/MORT-1, RAIDD, MADD, TRAF-1, -2, and FLIP, which in turn recruit enzymatic proteins such as RIP, NIK or caspases –8 and –10 (See review by Salvesen and Dixit '97). The interactions among these proteins is mediated by the DD on the

receptor, the death effector domain (DED) on the adaptor molecule and the caspase recruiting domain (CARD) on the recruited proteins.

i. Fas (Apo-1/CD95)

One key to triggering PCD appears to be the protein receptor Fas (Apo1/CD95), found on the surface of many different types of cells (see review by Raoul et al. '00). Fas/Fas ligand (FasL) interactions are involved in both the proliferative (Freiberg et al. '97) and cell death response induced in a variety of cell types by a variety of stimuli as well as in death induced by trophic deprivation in PC12 cells (Le Niculescu et al. '99), cerebellar granule cells (Brunet et al. '99) and spinal motoneurons (Raoul et al. '99). Thus one of the best studied signaling pathway is that induced by binding of FasL to Fas (Figure 1, pp. 114-5).

FasL is expressed mainly on the immune system's activated T lymphocytes, which also express Fas. The purpose of FasL is apparently to allow T cells to kill target cells which express Fas, by triggering the caspase cascade and prompting apoptosis, as well as to regulate T cell activity by triggering apoptosis in other T cells (Kagi et al. '94; Lowin et al. '94; Hahne et al. '96; Braun et al. '96). Recent evidence indicates that other cells may also co-express Fas and FasL thus causing their own suicide (Kohji and Matsumoto '00) and FasL expression is upregulated in neurons dying from injury or stress (Herdegen et al. '98; Martin-Villalba et al. '99; Raoul et al. '99). There are at least two potential mechanisms that could be involved. One involves Akt (protein kinase B/PKB) kinase, which is a serine threonine kinase. Akt kinase is believed to be constitutively activated in the presence of trophic factors and to sequester in the

cytoplasm a phosphorylated form of a specific transcription factor (forkhead-like protein FKHRL1). This prevents the transcription of the FKHRL1 binding site that contains a FasL gene and thereby promotes survival over apoptosis (Brunet et al. '99). Alternatively, signal induced activation of Fas in FasL expressing cells could explain neuronal loss *in vivo* (Cheema et al. '99).

There are several separate downstream pathways possible following binding of FasL to Fas. After ligation, Fas multimerizes and recruits the adaptor molecule FADD (Fas associated death domain). Together they form a death inducing signaling complex (DISC), which in turn causes autocatalytic activation of caspase-8 and initiation of apoptosis by cleavage and activation of downstream effector caspases such as caspase –3, -6 and –7, leading to irreversible cleavage of proteins necessary for maintaining cell structure, DNA synthesis and repair (Kischkel et al. '95).

In another downstream pathway, Fas recruits the adaptor protein DAXX, which links the receptor to the Jun N-terminal kinase (JNK) pathway described below in Figure 2 (Chang et al. '99). In yet another pathway, Fas signals are activated by death associated protein (DAP) kinase, which is localized to the cytoskeleton and the function of which is upstream of caspase-8 (Cohen et al. '99). Fas may also signal through mitogen activated kinase kinases (MKKs) or by the phosphatase and tensin homolog (PTEN), which dephosphorylates phosphoinositol (3,4,5)-triphosphate (PIP-3) (Toyoshima et al. '97; Stambolic et al. '98). Fas can also be directly activated at the plasma membrane independently of its ligand by UV light, which thereby also induces apoptosis (Aragane et al. '98). Ceramide, has been both implicated in (Cifone et al. '93; Tepper et al. '95; Cifone et al. '95) and ruled out of (Watts et al. '97) the mediation of

Fas induced apoptosis and this contradiction may result from the different mechanisms by which ceramide may be derived.

ii. JNK/stress activated protein kinase (SAPK) and mitogen activated protein kinase (MAPK) pathways

Among the pathways that activate or are activated by Fas or TNFR signaling are the: stress-activated protein kinase-1 (SAPK1/JNK), SAPK-2/p38, and mitogen activated protein kinase (MAPK/ERK) pathways. These pathways are important central elements used by mammalian cells to orchestrate the signals received by a cell from its environment (Waskiewicz and Cooper '95). Nevertheless, although they are all involved in the modulation of apoptosis and survival, they are structurally related, and there is crosstalk between the various kinase cascades, these pathways are activated by very different stimuli. The ERKs are activated by growth factors whereas-depending on the cell line and context of activation-the SAPK/JNKs are activated either upstream or downstream of caspase activity and downstream of environmental stress (Mosser et al. '97; Cardone et al. '97; Harada and Sugimoto '99). In addition, activation of MAPK pathways may inhibit SAPK/JNK activation-induced apoptosis (Ariga et al. '98).

The JNK/SAPK1 pathway is initiated by activation of MAP/ERK kinase kinase (MEKK)-1(Figure 2, pp. 116-7). MEKK-1 can be activated by any of a variety of signaling pathways including: TNF/TNFR interactions via TRAF; activation of ceramide activated protein kinase (CAPK); and mediation of reactive oxygen species (ROS) which include free radicals, peroxides and superoxides. MEKK-1 activation in turn leads to activation of SEK1(also called c-jun kinase kinase/JNKK or MKK4), which then

activates JNK. JNK subsequently phosphorylates c-jun, increases transcription of c-jun protein, and activates transcription of AP-1 (Xia et al. '95; Virdee et al '97; Anderson and Tolkovsky '99). Activation of JNK and phosphorylation of c-Jun are required for the induction of apoptosis in a variety of situations (Xia et al. '95, Eilers et al. '98; Luo et al. '98). The JNK pathway also modulates the activity of activating protein (AP)-1, and activated phospho-c-Jun, with the participation of AP-1 and NF-κB, can induce transcription of FasL (Kasibhatla et al. '98; Le-Niculescu et al. '99).

The SAPK2/p38 pathway involves activation of MKK3, a protein kinase related to MKK4, and subsequent activation of p38 (Raingeaud et al. '95; Derijard et al. '95). This appears to be the mechanism by which early membrane blebbing during stress-induced apoptosis is regulated via F-actin reorganization (Huot et al. '98), in addition to being a requirement of neurite outgrowth during differentiation of PC12 cells (Morooka and Nishida '98).

The MAPK pathway (Figure 3, pp. 118-9) is also called the survival pathway and involves sequential activation of $p21^{ras}$ (Ras), the kinase Raf and B-Raf (Raf-1), the phosphorylation and activation of MEK (MEK-1), MKK 1/2, extracellular signal regulated protein kinase –1 or –2 (ERK 1/2), mitogen activated protein kinase (MAPK) and finally suppression of JNK activity.

D. The Role of Mitochondria in Differentiation and Death

Mitochondria also play a central role in apoptosis induced by receptor ligand-mediated signaling (see reviews by Kroemer and Reed '00 and Desagher and Martinou '00). Mitochondria are comprised of two distinct compartments that are separated from

each other by the inner mitochondrial membrane. This membrane contains the protein complexes vital to cellular respiration and is almost totally impermeable in physiological conditions, which allows the electron transport chain to create an electrochemical gradient ($\Delta\Psi_m$) necessary for driving ATP synthesis.

Studies indicate that in most cases of apoptosis, disruption of the inner transmembrane potential ($\Delta\Psi_m$) precedes changes in cellular morphology, nuclear fragmentation or exposure of phosphatidylserine (PS) (Liu et al. '96; Yang et al. '97; Kim et al. '97). The permeabilization of inner and/or outer mitochondrial membranes constitutes part of the decision/effector phase of apoptosis but is possibly only one of several pathways leading to caspase activation. Mitochondrial permeabilization is required in some cases of apoptosis (Liu et al. '96; Xiang et al. '96; Kluck etal '97; Green and Reed '98) and dispensable for others (Nicholson and Thornberry '97; Scaffidi et al. '98; Ashkenazi and Dixit '98), and there exist multiple bi-directional links between mitochondria and caspases (Green and Kroemer '98).

Mitochondrial membrane permeabilization (MMP) occurs in three stages: initiation, decision, and degradation. Depending on the system being studied and the stimuli used to activate the cytoplasmic cell death machinery, a variety of effector molecules such as cytochrome c (*cyt c*), "apoptosis inducing factor" (AIF), and certain pro-caspases, accumulate during the initiation phase (see review by Kroemer and Reed '00). In most instances, disruption of the inner membrane and leakage of *cyt c* from the intermembrane space precedes changes in cellular morphology or nuclear fragmentation (Liu et al. '96; Yang et al. '97; Kim et al. '97). In fact, neutralizing *cyt c* by injecting

antibodies into the cytosol is often sufficient to prevent NGF withdrawal-induced apoptosis in neuronal cells (Neame et al. '98).

During the second stage, the mitochondrial membrane becomes permeable and finally, in the last stage, proteins normally confined to the mitochondria leak out, resulting in irreversible loss of mitochondrial functions and activation of cellular degradation by caspases and nucleases. Permeabilization occurs through the activation of the mitochondrial permeability transition pore (MPTP, PTP), which is also called the PTP complex (PTPC), megachannel, or megapore (reviewed in Jacotot et al. '00). This pore is a very large conductance channel in the inner mitochondrial membrane and its opening is associated with an abrupt increase in permeability of the inner mitochondrial membrane to solutes smaller than 1.5 kD; this change is known as the permeability transition (PT; reviewed in Bernardi '99). The PT causes rapid depolarization of the mitochondria and dissipation of $\Delta\Psi_m$, uncoupling of oxidative phosphorylation and mitochondrial swelling (Zoratti and Szabo '95; reviewed in Bernardi et al. '99). The PT cannot be directly visualized but can be detected by confocal microscopy, which measures the loss of fluorescent cations escaping from the mitochondria resulting from $\Delta\Psi_m$ (Nieminen et al. '95).

The release of pro-apoptotic proteins such as *cyt c*, or the production of a reactive oxygen species (ROS) like nitric oxide (NO) can activate caspases (Figure 4, pp. 120-1; reviewed in Desagher et al. '00; Yanase et al. '00; Sanchez-Alcazar et al. '00; Scorrano et al. '00; Han et al. '01). *Cyt c* in particular can activate caspase-3 and is involved, in concert with apoptotic protease activating factor-1 (Apaf-1), in the formation of a complex called the apoptosome. In the presence of ATP this complex recruits and

activates procaspase-9, which in turn activates the rest of the caspase cascade (Liu et al. '96; Green and Kroemer '98). More recently, a 50 kD caspase-independent death effector called AIF has been identified in mammals. AIF is normally localized in the mitochondrial intermembrane space and, following induction of apoptosis, it translocates to the nucleus where it directly causes chromatin condensation and DNA fragmentation (see review by Lorenzo et al. '99; Susin et al. '99).

One integral mitochondrial membrane protein is Bcl-2. It is a 26 kD anti-apoptotic protein and the product of the *bcl-2* oncogene (de Jong et al. '94; Allsopp et al. '93). Although the mode of action of Bcl-2 is unknown, most researchers believe that it either prevents $\Delta\Psi_m$ depolarization through its interactions with the MPTP, inhibits release of *cyt c*, or functions as an antioxidant (Marchetti et al. '96; Kluck et al. '97; Armstrong et al. '01).

The mitochondrial electron transport chain has been implicated as the main locale for ROS generation mediated by apoptosis induced by ceramide, staurosporine, dopamine, and other cytotoxic drugs (Quillet-Mary et al. '97; Heerdt et al. '00; Jones et al. '00; Davis et al. '01). Nonetheless, an alternative apoptotic cascade has recently been identified; it is induced by staurosporine and associated with an early transient elevation of $\Delta\Psi_m$ in colon carcinoma cells but is independent of growth arrest, dissipation of $\Delta\Psi_m$, ROS production, or neosynthesis of either RNA or protein (Heerdt et al. '00).

ROS or free radicals can directly cause DNA strand breaks and base modifications that result in cell death (Ha et al. '98). DNA can similarly be damaged by lipid peroxyl and alkoxyl radicals (Hruszkewycz and Bertold '90; Zhang, and Sevanian '91; Park and Floyd '92). ROS may escape through their mishandling in the

mitochondria (Kroemer et al. '01). Lipid peroxidation can also damage proteins, especially those associated with membranes (Zwizinski and Schmid '92). C*yt c* is also released from the mitochondria after disruption of $\Delta\Psi_m$ caused by increased ROS induced by cytotoxic stimuli (Kroemer et al. '01).

ROS release resulting from mitochondrial dysfunction are believed to be involved many instances of neurodegeneration including NGF- or serum-deprivation induced apoptosis (Greenlund et al. '95; Satoh et al. '96), oxidative stress-induced death (Satoh et al. '97), Parkinson's disease (PD, Jenner '92; Cassarino et al. '97; Sheehan et al. '97), Alzheimer's disease (AD, Behl et al. '94; Cassarino et al. '98; Guo et al. '99; Passer et al. '99), Friedreich ataxia (FRDA, Koutnikova et al. '97), Huntington disease (HD, Cooper and Schapira '97; Sawa et al. '99), and amyotrophic lateral sclerosis (ALS, Carri et al. '97; Kostic et al. '97; Matthews et al. '98; Klivenyi et al. '99). Oxidative products have also been demonstrated to activate the JNK/SAPK apoptotic pathway (Verheij et al. '96); this activation can be inhibited by antioxidants (Luo et al. '98).

Nitric oxide (NO) is just one example of a short-lived ROS that is produced in the mitochondria. It has been suggested that JNK activation mediates the formation of NO (Chung et al. '99). NO itself can either stimulate (Troy et al. '96) or inhibit (Lipton et al. '93; Farinelli et al. '96; De Nadai et al. '00) apoptosis depending on the specifics of the cell system in question. Furthermore, high activity of nitric oxide synthase (NOS) appears to protect against apoptosis (Lievremont et al. '99). Targets of NO include NMDA receptor, tyrosine residues, $p21^{ras}$, and reduced glutathione (GSH) (reviewed in Lander et al. '96). GSH regulates the cellular redox state and controls both signal transduction and gene expression (Sies '99). GSH also inhibits nSMase activity induced

by TNFα in Molt4 leukemia and MCF7 mammary carcinoma cells. Moreover, although GSH does not inhibit ceramide-induced apoptosis (Liu and Hannun '97; Liu et al. '98), its depletion has been observed in response to many inducers of apoptosis including TNFα, Fas, viral infections, and glutamate (Ishii et al. '92; Kato et al. '92; Zhong et al. '93; Shoji et al. '95; Phelps et al. '95; Chiba et al. '96; Ciriolo et al. '97; Froissard et al. '97).

a. CoQ_{10}: A Significant Mitochondrial Enzyme

The reports on mitochondrial activation during cell death suggest the possibility of involvement of mitochondrial components such as ubiquinone (coenzyme Q_{10}, CoQ_{10}). CoQ_{10} is found in many cell types and is likely present in all cells. It is synthesized in the endoplasmic reticulum and Golgi apparatus from where it is translocated to other cytosolic compartments (Rawn '89). CoQ_{10} is well established as a vital component of the respiratory chain localized in the inner mitochondrial membrane, where it serves as a highly mobile carrier of electrons and protons between the flavoproteins and the cytochromes. As a carrier, one molecule of CoQ_{10} can transfer two electrons and two protons. The reduced form of CoQ_{10} is known as ubiquinon; in this form it picks up a proton and changes to its free radical form, known as ubisemiquinone. This intermediate product can pick up another proton to give ubiquinol, which is the oxidized form of CoQ_{10}.

Although its molecular and cellular mode of action is not understood, CoQ_{10} has been used clinically for several decades to treat patients suffering from many illnesses (Densnuelle et al. '89; Folkers et al. '88; Langsjoen et al. '88; Topi et al. '89; Kato et al.

'90; Bianchi et al.'99; Park et al.'99; Barbieri et al.'99; Kieburtz '99; Eriksson et al.'99; Khatta et al.'00). CoQ_{10} has been implicated as an important factor in cell death, and apoptosis may be induced via inhibition of isoprenoid production, which is a major step in the biosynthesis of CoQ_{10} (Iimura et al. '97). CoQ_{10} stimulates cell proliferation in serum free conditions *in vitro* (Sun et al '92; Crane et al. '94), and thus it may also play a role in cell growth. CoQ_{10} may also be pro-apoptotic (Kagan et al. '99; Teranishi et al. '99; Fernandez-Ayala et al. '00; Brancato et al. '00), or pro-tumorigenic, as the marked fatality of several cancers has been linked with exceptionally low levels of CoQ_{10} (Bliznakov '73; Folkers et al. '93; Jolliet et al. '98).

E. Second messengers in differentiation and dearth

Signaling cascades are based in part on second messengers whose generation unleashes the downstream cellular responses. The criteria used to evaluate whether a specific molecule is a second messenger include identification of transient changes in levels detected in response to extracellular signals. Examples of second messengers include *cyt c*, free oxygen radicals and ROS generated in the mitochondria as described above as well as cyclic AMP (cAMP), calcium ions (Ca^{2+}), and lipid signal transduction molecules such as phosphoinositol-3 (PI-3), protein kinase C (PKC) and the sphingolipid ceramide.

cAMP is an important second messenger with immunomodulatory properties. It simultaneously triggers both apoptosis and differentiation in the same culture (Seite et al. '00), and like other second messengers, the messages relayed by cAMP are cell specific and vary among cell types and physiological states. As an example, increased

intracellular cAMP inhibits cytokine production in effector Th1 cells, but stimulates cytokine production and activation of p38 MAPK in Th2 cells (Zhang et al. '97; Chen et al. '00). cAMP analogs also increase MAP kinase activity in pheochromocytoma (PC12) cells and potentiate both NGF-activation of MAP kinase and NGF-induced neurite outgrowth (Frodin et al. '94, Young et al. '94).

Calcium (Ca^{2+}) is another important intracellular signal that is critical for normal development and survival of neurons (Mills and Kater '90). Increased intracellular Ca^{2+} has also been associated *in vivo* with neurodegenerative changes occurring after trauma or ischaemia in normal aging and in various neuropathologies although downstream events of Ca^{2+} influx are not understood (Choi '90, Kirischuk et al. '92). It might be that Ca^{2+} influx activates key catabolic enzymes that comprise the apoptotic machinery, or that emptying Ca^{2+} stores disrupts intracellular architecture and allows key effectors of apoptosis access to their substrates (see review by McConkey and Orrenius '96). Nevertheless, increases in intracellular Ca^{2+} increase the production of ROS, and that oxidative stress in turn, results in increased intracellular Ca^{2+} influx (Oyama et al. '96).

Activated phospholipid-dependent kinases such as the protein kinase C (PKC) family are also important regulators of growth and differentiation (Nishizuka '92; Hug and Sarre '93; Michie et al. '01). PKCs comprise a family of Ca^{2+} activated, phospholipid-dependent serine/threonine protein kinases that play a critical role in signaling cell proliferation, activation of cellular function, differentiation, and apoptosis (reviewed in Musashi et al. '00). Most cells express multiple isoforms of PKC, and each isoform has a specific function and cellular localization (Mochly-Rosen '95). PKCs may be activated by other 2^{nd} messengers and in turn activate other downstream signals

apparatus from ceramide synthesized in the endoplasmic reticulum and redistributed in a non vesicle-mediated (Kok et al. '98), ATP-dependent manner (Fukasawa et al. '99). It is composed of a sphingoid base backbone, which is predominantly sphingosine with a fatty acid in amide linkage at the second position of the sphingoid base, and a phosphocholine head group. SM is synthesized by the transfer of phosphocholine from phosphatidyl choline to ceramide at the lumenal surface of the Golgi apparatus (Barenholz and Thomson '80; Merrill and Wang '92; Futerman et al. '90).

Many reviews have been written on the metabolism of SM and the role in apoptosis and stress of its hydrolytic product, ceramide (Hannun '94; Pushkareva et al. '95; Kolesnick and Fuks '95; Hannun '96; Ballou et al. '96; Haimovitz-Friedman et al. '97; Alessenko '00). SM can be hydrolyzed by the action of sphingomyelin phosphodiesterases (SMases) (Okazaki et al. '89; Okazaki et al. '94; Wiegmann et al. '94; Heller and Kronke '94; Santana et al. '96). So far, five types of SMases have been identified as occurring naturally and their structures, regulation, and roles are reviewed in Liu et al. ('97).

Two specific types SMases are involved in determining the route of SM degradation that yields ceramide and phosphocholine during cell differentiation and death. They are the lysosomal acidic SMase (aSMase) (Brady et al. '65; Barenholz et al. '66) and the plasma membrane bound neutral Mg^{2+}-dependent SMase (nSMase) (Hostetler and Yazaki '79), whose catalytic surface is located on the intracellular surface (Tomiuk et al. '98).

NSMase exhibits the greatest amount of enzymatic activity in brain cells as compared to all other cell types (Tomiuk et al. '98). NSMase is also concentrated in

(Wang et al. '99). Cycloheximide (CHX)-induced apoptosis may even be mediated by PKC activation of sphingosine (Alessenko et al. '97).

2. The Generation And Function of Ceramide

Lipids are major structural components of intracellular vesicles and membranes as well as important cellular second messengers. Sphingolipids (SLs) in particular are a large class of membrane lipids that all contain either sphingosine or a related sphingoid base, with amide linked fatty acyl groups or other various head groups at the carbon 1 (C1) position. Many reviews have been written on the structure and function of SLs (Hailman et al. '94; Hakomori et al. '98; Barenholz and Thompson '99). They are specifically enriched in neuronal membranes where they are implicated as mediators of various regulatory events.

A. Sphingolipid biosynthesis and the sphingomyelin cycle

SL synthesis begins in the endoplasmic reticulum and continues in the plasma membrane, lysosomes, secretory vesicles, endosome or other intracellular sites (Merrill and Wang '92; Jeckel and Wieland '93) where SLs are recycled and remodeled by hydrolases (Hassler and Bell '93) and subsequently reduced and further processed to form a variety of complex phospho- and glyco-sphingolipids (Figure 5, pp. 122-3).

Sphingomyelin (SM; N-acylsphingosine-1-phosphocholine) is a major structural component of the mammalian myelin sheath and a prominent component of plasma membranes, plasma lipoproteins, and cell membranes of mammalian cells (White '73; Barenholz and Thomson '80; Merrill and Jones '90). SM is found in the outer leaflet of the lipid bilayer (Slotte et al. '90). *De novo* or neosynthesis of SM occurs in the Golgi

neural tissues and cells of neural origin (Gatt '76; Spence et al. '82) and following induction of differentiation by retinoic acid, its activity increases in parallel with neuronal maturation in both rat brain (Spence and Burgess '78) and neuro2a neuroblastoma cells (RA) (Riboni et al. '95). Recently nuclear specific nSMases have also been identified and their action might result in localized nuclear accumulation of ceramide, which could be involved in specific signaling events or apoptosis (Tsugane et al. '99).

Natural ceramide (N-acyl sphingosine) exists primarily embedded within the membrane lipid bilayer (Venkataraman and Futerman '01). It is a sphingolipid with a sphingoid base and a hydroxyl group at C-1 and an amide linked fatty acyl chain varying in length from 16 to 24 carbons (Ballou et al. '96). Ceramide is synthesized by the acylation of sphinganine at the cytosolic surface of endoplasmic reticulum followed by dehydrogenation of dihydroceramide to ceramide (Hirschberg et al. '93). Ceramide can then be metabolized to glucosylceramide (GlcCer) at the cytosolic surface of the pre- or early Golgi apparatus compartment (Futerman and Pagano '91; Trinchera et al. '91a, b; Jeckel et al. '92) or to SM at the lumenal surface (Futerman et al. '90; Jeckel et al. '90) of the cis or medial Golgi apparatus compartment. Ceramide apparently can also be synthesized at the surface of other endomembranes as ceramide synthase has more recently been isolated from fractions of both mitochondrial and nuclear membranes (Shimeno et al. '98).

Ceramide is a key intermediate in SM metabolism; newly synthesized ceramide can be used as a precursor for SM synthesis, and transference of a phosphocholine from phosphatidylcholine to ceramide forms SM and diacylglycerol and completes the SM

cycle (Hannun '94). Newly synthesized ceramide can also be used as a precursor in the generation of a host of other mainly structural sphingolipids and glycosphingolipids. Ceramide can be modified to form a variety of non-structural SLs as well, and the quantitative levels of ceramide metabolites in the cell are important in determining the dynamic balance among apoptotic, differentiative and proliferative signals.

B. The Physiological Importance of SLs

SLs play many roles in regulating the differentiation of neurons, and the quantitative levels of sphingomyelin metabolites in the cell are equally important in determining the dynamic balance between apoptotic, differentiative and proliferative signals. In fact it is quite possible that SM hydrolysis to ceramide during apoptosis alters cell surface morphology by phospholipid scrambling (Tepper et al. '00) and that membrane blebbing during apoptosis results from differential packing density of SM vs. ceramide in the lipid bilayer (Venkataraman and Futerman '01).

We can use the ceramide metabolite sphingosine (Sph) to illustrate the way modified SLs can produce multiple effects. Sph is formed by the hydrolytic action of ceramidase on ceramide and, since it is an endogenous inhibitor of PKC, it inhibits many cell functions. Sph is increased in cells as a result of action of inducers of apoptosis as well as inducing apoptosis on its own. Sph can also activate MAP kinase and thereby release Ca^{2+} from intracellular stores, thus regulating cell proliferation (Carpio et al. '99). Sph and its methylated derivative N,N,-dimethyl-sphingosine (DMS) also induce apoptosis in several human cancer lines (Sweeney et al. '96).

By contrast, the phosphorylated derivative of Sph, sphingosine 1-phosphate (Sph1P or SPP) is mitogenic in a variety of cell types (Van Brocklyn et al. '98; Pyne et al. '96; Bornfeldt et al. '95; Gomez-Munoz et al. '95; Zhang et al. '91). Both Sph and SPP are regulated by the cell cycle and they in turn determine the type of signaling induced by factors like PDGF (Fatatis and Miler '99). SPP mediates a proliferative signal through Ca^{2+} mobilization and therefore plays a cytoprotective role (reviewed in Alessenko '00). SPP also suppresses ceramide- (Edsall et al. '97) and serum deprivation-induced apoptosis in HL60 cells (Cuvillier et al. '96), PC12 cells (Van Brocklyn et al. '98),and Jurkat T cells (Olivera et al. '99) as well as Fas-induced apoptosis in Jurkat T cells (Cuvillier et al. '96; Olivera et al. '99). SPP formation is activated by growth and survival factors like PDGF (Olivera and Spiegel '93; Bornfeldt et al. '95;Olivera et al. '99), NGF (Edsall et al. '97), and PKC activation (Mazurek et al. '94; Buehrer et al. '96). In fact it has been suggested that SPP formation suppresses apoptosis by stimulating ERK and thus counteracting the ceramide induced activation of SAPK/JNK in 3T3 fibroblasts and U937 cells (Cuvillier et al. '96).

Mitochondria- and nucleus- specific ceramidases have recently been identified, suggesting the existence of a specific mitochondrial or nuclear pools of ceramide (El Bawab et al. '00) that can be hydrolyzed to form mitochondria-specific pools of either Sph or SPP and that may be involved in apoptosis (Tsugane et al. '99). Moreover, neutral and alkaline ceramidases are also secreted into culture media by murine endothelial cells, macrophages, and human fibroblasts and may participate in extracellular SM metabolism (Romiti et al. '00).

C. The Physiological Importance Of Ceramide As A 2nd Messenger

As already stated, ceramide is an important signaling molecule responsible for multiple effects including proliferation, differentiation, inhibition of neurite outgrowth, and cell death (Okazaki et al. '90; Kim et al. '91; Olivera and Spiegel '93). Ceramide interacts with target proteins such as kinase suppressor of Ras (CAPK), protein kinase c-Raf, protein kinase Cξ (van Blitterswijk '98), CAPP (Galadari et al. '98), and cathepsin D (Heinrich et al. '99). Ceramide appears to facilitate TNF-R1 activated signal transduction by increasing TRADD recruitment to the DISC complex and activating caspase 8 (De Nadai et al. '00). Ceramide also mediates apoptosis induced by environmental stresses through the activation of SAPK/JNK cascades (Verheij et al. '96; Szabo et al. '96; Cuvillier et al. '96). Meanwhile, other research shows that ceramide induced cell death in specific systems is independent of the Fas ligand/caspase pathway (Bras et al. '00).

a. Ceramide and Growth Arrest

Changes in *de novo* synthesis of ceramide may function in the endogenous regulation of cell cycle progression (Lee et al. '98). Moreover, growth arrest of γδT cells can be induced by *de novo* synthesis of ceramide mediated by ligation of the WC1 cell surface receptor (Kirkham et al. '00).

b. Ceramide and Differentiation

Biosynthesis of SL in the form of GlcCer and dihydroceramide is required for axon elongation and neurite outgrowth in cultured hippocampal neurons (Harel and

Futerman '93; Merrill et al. '96). Alone at low concentrations, ceramide can also cause increases in mean axon length (Schwarz et al. '95). More specifically, ceramide functions as a modulator of the formation of minor processes from lamellipodia during the initial stages of neuronal development, while GlcCer modulates axonal growth (Schwarz and Futerman '97; Futerman et al. '98). In addition, while ceramide is required for dendritic growth in cerebellar Purkinje cells (Furuya et al. '95), GlcCer is required for neurite outgrowth in neuroblastoma cells (Uemura et al. '91). In addition, the development of neuronal polarity appears to be based at least in part on SL synthesis and localization during axonogenesis and axon elongation (Hirschberg et al. '96).

Although GlcCer is not necessary for initial minor process formation, ceramide must be metabolized to GlcCer to sustain axon growth, (Schwarz and Futerman '97). All SLs enhance the formation of minor neuronal processes from lamellipodia in primary cultures of hippocampal neurons, but only GlcCer is required for both normal and accelerated axon growth and inhibition of such SL synthesis inhibits axonal growth and branching (Schwarz et al. '95; Futerman et al. '96). Furthermore, the ability of specific factors like bFGF to stimulate neuronal growth appears to be dependent on GlcCer synthesis, since GlcCer is an essential membrane-lipid building block (Boldin and Futerman '97).

Long term inhibition of ceramide synthase by a specific inhibitor, fumonisin B_1 (FB_1), interferes with cytokinesis, microvillus formation, formation of long processes, and formation of actin cytoskeleton in 3T3 fibroblasts. Inhibition of ceramide synthesis also reversibly blocks cell proliferation and DNA synthesis and upregulates GlcCer without altering SM synthesis (Meivar-Levy et al. '97; Meivar-Levy and Futerman '99).

Exogenous glycosphingolipids (GSLs), and retinoic acid (RA) can also induce differentiation of neurons in primary cultures and both Neuro2a and SH-SY5Y neuroblastoma cells *in vitro* (review in Tettamanti and Riboni '94; Tettamanti et al. '96).

c. Ceramide and Cell Death

The mechanism of ceramide-mediated apoptosis is highly conserved. Induction of REAPER (RPR) expression in *Drosophila* results in apoptosis associated with increased ceramide production (Pronk et al. '96). Virus entry into cells can also trigger apoptosis mediated by ceramide generated from SMase activity (Jan et al. '00).

Defective ceramide signaling has been implicated in the genesis of resistance to apoptosis induced by UV and ionizing radiation, TNF-α, FasL, IgM, and glucocorticoids (Michael et al. '97; Metz et al. '96; Quintans et al. '94; Cifone et al. '93; Obeid et al. '93). Furthermore, the product of the CLN3 gene enhances growth and protects against apoptosis induced by vincristine, staurosporine, and etoposide by blunting or attenuating ceramide generation (Puranam et al. '00).

Apoptosis induced by environmental stresses is initiated through ceramide mediated cascades (Szabo et al. '96). Ceramide levels increase sharply in response to cytokines such as TNF-α, FasL (Cifone et al. '93; Tepper et al. '95; Gulbins et al. '94), and vitamin D_3 (Kim et al. '91; Okazaki et al. '90; Obeid et al. '93:' Verheij et al. '96). Ceramide levels also increase in cells exposed to ionizing radiation (Haimovitz-Friedman et al. '94), withdrawal of serum (Jayadev et al. '95), UV-C radiation, heat shock (Verheij et al. '96), and chemotherapeutic agents (Bose et al. '95). The generation of ceramide has been viewed as a causative factor and not merely correlative largely because

exogenously added synthetic ceramide analogs mimic some of the biological effects of TNF-α and vitamin D3 by inducing apoptosis (Okazaki et al. '90; Obeid et al. '93).

Ceramide signaling pathways are also implicated in non-apoptotic cell death. Exogenous ceramide induces hepatocyte necrosis following disruption of mitochondrial function via induction of the mitochondrial membrane permeability transition (MMPT) (Arora et al. '97).

d. The Localization of Ceramide Synthesis

The presence of SM in different sub-cellular compartments implies that the location of ceramide generation can mediate different biological effects (Linardic and Hannun '94; Andriew et al. '95; Bettaieb et al. '96). Although short acyl chain ceramides, which have only recently been found in nature, can spontaneously transfer among and within lipid bilayers (Bai and Pagano '97; Karasawa et al. '99), most naturally occurring ceramides, which possess long acyl chains (eg. C_{18}-ceramide), cannot spontaneously transfer between lipid bilayers (Simon et al. '99) and ceramide generated in lysosomes cannot escape (Chatelut et al. '98). Thus downstream signaling targets must translocate to the membrane where ceramide is generated and we may speculate that this may be the mechanism by which different subsets of downstream targets can interact with ceramide generated by specific intracellular membranes or subcellular compartments.

Differentiation of neuro2a neuroblastoma cells with RA requires both extralysosomal nSMase-dependent SM hydrolysis and newly synthesized ceramide synthesis by N-acylation of Sph (Riboni et al. '95). Increased N-acylation of

neosynthesized Sph to form ceramide is also reported in RA-induced differentiation of GH_4C_1 cells (Kalen et al. '92). Furthermore, Riboni et al. ('95) suggest that ceramide is used in a bioregulatory role and not simply as a trigger. Ceramide may act as a negative regulator in sympathetic neurons, since its localized elevation in distal neurites inhibits their growth (de Chaves et al. '97).

Generation of ceramide by SMase activity is the traditional pathway that has been described for mediation of apoptosis. Following induction of apoptosis by either TNF-α or IL-1β, ceramide is generated by nSMase activity at the inner leaflet of the plasma membrane (Linardic and Hannun '94; Andrieu et al. '95; Adam-Klages '96; Andrieu et al. '96; Bettaieb et al. '96), while Fas- and TNFα-induced SM hydrolysis occurs at endolysosomal compartments and is mediated by aSMase (Schutze et al. '92; Cifone et al. '93; Wiegmann et al. '94). TNF-α induces nSMase generated ceramide-mediated apoptosis in U937 and HL60 cells (Jarvis et al. '94). TNF-α also induces both ceramide- and Sph-mediated apoptosis in cardiac myocytes (Krown et al. '96). TNFα-TNFR ligation activates both nSMase and aSMase (Wiegmann et al. '94) and the ceramide derived from aSMase hydrolysis is believed to underlie TNFα-induced cell death (Adam et al. '96) as well as PT dependent apoptosis induced by GD-3 ganglioside (De Maria et al. '97; Scorrano et al. '99).

NSMase activity has also been implicated in pro-inflammatory response and ERK1 cascade (Zhang et al. '97), although U937 cells overexpressing cloned mammalian nSMase show only moderately elevated concentrations of ceramide and no activation of ERK1 following TNF-α stimulation (Tomiuk et al. '98). Both etoposide, a topoisomerase II inhibitor, and ionizing radiation also induce apoptosis through SMase-

modulated ceramide increase (Chmura et al. '97; Sawada et al. '00; Chmura et al. '00). Similarly, ceramide from aSMase has been implicated as a mediator of killing by IL-4 and interferon-γ or following treatment with ionizing or ultraviolet radiation, heat shock, oxidative stress, or serum or NGF withdrawal (see review by Sawai and Hannun '99).

Neosynthesis of ceramide is an equally important pathway for ceramide generation during induction of cell death. CPT- and TPA-induced apoptosis appear to be mediated by *de novo* ceramide synthesis (Suzuki et al. '97; Garzotto et al. '98), with TPA-induction of apoptosis mediated by activation of the PKC pathway by newly synthesized ceramide. Furthermore, newly synthesized ceramide generated by ceramide synthase is implicated in cell killing by anthracycline antibiotics such as doxorubicin and daunorubicin, which are used as chemotherapeutic agents in the treatment of acute leukemias, malignant lymphomas and solid tumors (Bose et al. '95; Lucci et al. '99). Nevertheless, killing by anthracyclines is not mediated solely by ceramide neosynthesis, as blocking such activity with FB_1 blocks ceramide accumulation without preventing apoptosis (Di Bartolomeo et al. '00). Doxorubicin-induced apoptosis activates aSMase and causes increases in intracellular ceramide (Andrieu-Abadie et al. '99). In contrast, daunorubicin-induced apoptosis stimulates both SM hydrolysis (Jaffrezou et al. '96) and *de novo* ceramide synthesis in hen granulosa as well as both P388 and U937 cells (Bose et al. '95; Witty et al. '96). Furthermore anthracycline induced apoptosis can also be mediated by ceramide induced activation of Fas signaling (Herr et al. '97).

3. The Role of NGF in Neuronal Differentiation and Death

Mature neurons are among the most long-lived differentiated cell types in mammals. Nevertheless, a significant number of neurons die during embryogenesis and

development of the nervous system. Moreover, neuronal death is a significant feature of neurodegenerative diseases such as Alzheimer's, such as Parkinson's.

To a large extent, neuronal survival depends on trophic support and the survival of developing neurons is directly related to the availability of their innervating targets. Furthermore, only those neurons that succeed in establishing the right synaptic connections obtain sufficient trophic support to enable survival (see review by Levi-Montalcini '96). This support is provided in the form of neurotrophins such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and fibroblast growth factors (FGFs).

A. NGF receptors

NGF receptors are neurotrophin specific and are expressed in a lineage-restricted manner enabling distinctions among cell types that express the various receptors (Wang et al. '98). There are two main subfamilies of neurotrophin receptors. The first subfamily consists of the p75 neurotrophin ($p75^{NTR}$) receptor, which is a 75 kD glycosylated type I integral membrane protein. Based on structural homologies in the extracellular domains, $p75^{NTR}$ has been classified as a member of the Fas/TNFR family (Chao et al. '95). However $p75^{NTR}$ does not contain an intracellular death domain similar to that of other TNFR family members (Liepinsh et al. '97) and therefore its signal transduction mechanisms may be very different from other members of the family. The $p75^{NTR}$ receptor is activated weakly by all neurotrophins and robustly by BDNF. When co-expressed with other neurotrophin receptors, $p75^{NTR}$ enhances responses to preferred high affinity ligands. $p75^{NTR}$ also associates with TNFR-associated factor 6 (TRAF6),

which may function as its signal transducer for NGF (Khursigara et al. '99). Several downstream signaling pathways have been implicated for $p75^{NTR}$ including induction of both NF-κB and JNK activity, which will be discussed below (Carter et al. '96; Kuner and Hertel '98; Bhakar et al. '99; see review by Coulson '99).

The second subfamily of neurotrophin receptors is the Trk kinase family of receptors, which consists of TrkA, TrkB and TrkC tyrosine kinases. These receptors are members of a larger family of receptor tyrosine kinases that includes the EGF, FGF, and PDGF receptors (reviewed in Schlessinger et al. '92). The Trk family of tyrosine kinases acts as high affinity receptors for NGF, BDNF and NT-3 respectively, although TrkA can also bind NT 4/5 and NT-3 but not at all BDNF (reviewed in Korsching '93, Wright and Snider '95, Birge '98; Bamji et al. '98).

Each neurotrophin/receptor pair has a distinctive role in the developing mammalian nervous system. Mice carrying targeted mutations in TrkB receptors have significant increases in apoptotic cell death in hippocampus, striatum, and thalamus during early postnatal life (Alcantara et al. '97) while mice lacking TrkC exhibit defects in muscle afferents (Tessarollo '94). Disruptions of TrkA receptors result in apoptosis of trigeminal, sympathetic and dorsal root ganglia neurons, and cholinergic basal forebrain projections to hippocampus and cortex (Smeyne et al. '94).

Trk receptors are also expressed in adults where they may modulate neuronal function (McMahon et al. '94). Beginning at early stages, TrkA expressing cholinergic neurons are reduced in Alzheimer's disease (AD) patients (Hefti and Mash '89; Salehi et al. '96; Dubus '00; Mufson '00; Savaskan '00). There is also enhanced TrkA and NGF expression in both neural and non-neural tissue in Crohn's disease and Ulcerative Colitis

(di Mola et al. '00) as well as in ductal pancreatic carcinomas (Miknyoczki et al. '96), which suggests activation of this pathway in the chronic inflammation and phenotype associated with these conditions.

The TrkA NGF receptor (p^{140}TrkA) is a 140 kD integral membrane protein, the role of which is quite complex and is the subject of our research as described here. Following NGF binding to the TrkA receptor, receptor auto-phosphorylation initiates a signal transduction cascade (Figure 6, pp. 124-5) and subsequent activation of diverse intracellular signaling pathways including both $p21^{ras}$ (Ras) and PI 3-kinase (reviewed in Birge et al. '98). One of the first events following NGF ligation is docking of the adaptor proteins Shc or Shb to Trk receptors (Stephens et al. '94; Karlsson et al. '98), which triggers the activation of the small GTP-binding protein Ras, and then subsequently activation of Raf-1 and the downstream MAP kinase cascade, and suppression of JNK activity (Boulton et al. '91; Robbins et al. '92; Vaillancourt et al. '94; see review by Yuan and Yankner '00). Activated Raf-1 also binds Bcl-2 on the mitochondrial membrane; this binding inhibits the mitochondrial death promoter bax. In fact, NGF induces activation of PI-3 kinase, which in turn produces phosphotidylinositol 4,5 biphosphate (PIP2), which activates Akt (PKB), which inhibits apoptosis (Virdee et al. '99).

Reciprocal interactions between Trk and $p75^{NTR}$ signaling pathways can dictate cellular responses to particular signals (Kaplan and Miller '97). Recently it has even been suggested that cross talk between these pathways is regulated by caveolin (Bilderback et al. '99). Caveolin is a key structural protein in the morphogenesis of caveolae (Parton '96; Okamoto et al. '98), and a putative transformation suppressor protein. Caveolae are considered to be potential localized centers for signal transduction

through tyrosine kinase (Mineo et al. '96; Liu et al. '96, '97), SL (Liu and Anderson '95; Bilderback et al. '97, '99), and phosphoinositide 3-kinase (PI(3)K) signaling pathways (Pike and Casey '96; Hope and Pike '96).

B. Evidence that NGF Receptors play a role in differentiation

NGF receptors and NGF are critical during differentiation and development of the sympathetic nervous system. NGF promotes enhanced speed of differentiation, increased process formation, and increased size of responsive neurons (Levi-Montalcini '66). NGF receptors and NGF are equally critical in the growth and development of tumors. Rat T9 and SHSY-5Y glioblastoma cells, as well as PC12 and other transformed cells, also undergo differentiation in response to neurotrophin/receptor interactions (Scott et al. '86; Marushige et al. '87; Yaeger et al. '92; Gallo et al. '00). Differentiation in T9 cells is mediated by activation of SM hydrolysis, with a resulting 2 fold increase in ceramide, and regulated through the $p75^{NTR}$. NGF induced-differentiation of cultured hippocampal neurons is also mediated by N-SMase generated ceramide signaling downstream of $p75^{NTR}$ (Brann et al. '99). These result suggests that SM hydrolysis may be a prominent step in cells that primarily express p75 such as cells forming gliomas, schwannomas (Kahle and Hertel '92), melanomas (Rabizadeh et al. '93), or brain cells of AD patients (Yaar et al. '97).

Many of the classic trophic signals elicited by the neurotrophins require the presence of a Trk family member. NGF induces a 4 fold increase in SM accumulation in PC12 cells that is suppressed by inhibition of the TrkA receptor (Piccinotti et al. '00). Moreover, extended C_2-ceramide exposure increases TrkA receptor homodimer

formation and increases NGF-induced TrkA activation, as well as activation of both ERK1/2 and PI3-kinase (MacPhee and Barker '99).

Neurite outgrowth during NGF induced differentiation of PC12 cells has been attributed to activation of the MAP kinase pathway by TrkA (Kaplan et al. '91a, b). Furthermore NGF responsiveness in both PC12 and neuroblastoma cells is mediated by TrkA receptor (Greene '77; Sonnenfeld and Ishii '85; Green et al. '86). Overexpression of TrkA in PC12 cells accelerates NGF-induced differentiation, possibly through enhanced autophosphorylation of the Trk receptor (Hempstead et al. '92), while expression of p75 in sympathoadrenal cells leads to enhanced differentiation following NGF stimulation (Verdi et al. '94). Thus, NGF-induced differentiation in PC12 cells involves a TrkA-mediated downstream ceramide pathway which may or may not require SM hydrolysis or ceramide generation.

C. Evidence that NGF Receptors play a role in death

As we described above, exogenously administered NGF can prevent naturally occurring neuronal death during development (Levi-Montalcini and Angeletti '68, Hamburger et al. '81). Withdrawal of NGF results in activation of the JNK/p38 signaling pathway by MKK-3 or –4 signaling (Xia et al. '95). Furthermore, death induced by withdrawal of growth factors requires the participation of caspases, and apoptotic protease-activating factor 1 (Apaf-1), in addition to Trk and $p75^{NTR}$ receptors, and the p53 tumor suppressor protein also appears to be an essential component of the TrkA/$p75^{NTR}$ apoptotic signaling cascade, most likely as a convergent downstream target of the MEKK/JNK pathway (Aloyz et al. '98).

The death inducing activity of $p75^{NTR}$ has been documented in neuronal cells. However, the predominant physiologic role of $p75^{NTR}$ is complex and dependent on cell context and $p75^{NTR}$ can act either autonomously or in concert with Trk receptors to enhance or reduce responses to Trk ligands (reviewed in Barker '98 and Casaccia-Bonnefil et al. '98). Binding of ligand to $p75^{NTR}$ can activate NF-κB (Casaccia-Bonnefil et al. '98). However activation of death pathways depends on the presence or absence of TrkA as well as on the physiological status of the neuron at the time of activation. Furthermore, death-inducing activities may actually reflect the inhibition of the other neurotrophin receptors rather than the direct activation of a death pathway downstream of $p75^{NTR}$ (Wiese et al. '99).

During the first two postnatal weeks of life, sympathetic neurons compete for limited amounts of target-derived NGF, which mediates neuronal survival by activating TrkA receptors on neuronal terminals (Campenot '82; Kaplan et al. '91; Senger et al. '97). In the absence of TrkA receptors, $p75^{NTR}$ generates a death signal (rev in Casaccia-Bonnefil et al. '98); likewise ligand mediated activation of $p75^{NTR}$ is sufficient to cause sympathetic neuron apoptosis (Bamji et al. '98). During appropriate developmental sympathetic neuronal death, $p75^{NTR}$ is activated coincident with suboptimal NGF/TrkA survival signals (reviewed in Miller and Kaplan '98). Furthermore, SK-N-BE neuroblastoma cell clones transfected with $p75^{NTR}$ but lacking Trk receptors undergo extensive spontaneous apoptosis (Bunone et al. '97).

NGF also activates the sphingomyelin cycle in T9 and NIH 3T3 cells through $p75^{NTR}$-mediated SM hydrolysis (Dobrowsky et al. '94) suggesting that activation of the sphingomyelin cycle through $p75^{NTR}$ may mediate NGF-induced differentiation. The role

of $p75^{NTR}$ in mediating neuronal death may explain its localization in rat Purkinje cells following traumatic injury (Martinez-Murillo et al. '98). Crosstalk pathways between $p75^{NTR}$ and $p140^{trkA}$ exist and co-expression of $p140^{trkA}$ with $p75^{NTR}$ abolishes $p75^{NTR}$-dependent SM hydrolysis and apoptosis in both oligodendrocytes and PC12 cells (Dobrowsky et al. '95; Yoon et al. '98). Moreover, in oligodendrocytes this rescue is furthermore correlated with activation of the MAP kinase cascade and suppression of c-jun kinase activity, without affecting induction of NF-κB (Yoon et al. '98).

The PI(3)K-Akt pathway is also central to neuronal survival (see review by Yuan and Yankner '00). PI(3)K enzymes can be activated by recruitment to an activated Trk receptor or through activated Ras. Once activated, they in turn catalyze the formation of lipid 3'-phosphorylated phosphoinositides which regulate localization of Akt. As mentioned above, active Akt protein supports the survival of neurons and its absence results in transcription of FasL and death.

Both SMase and ceramide neosynthesis are implicated in the apoptosis of neuronal cells. Increased ceramide is implicated in choline deficiency-induced apoptosis in differentiated PC12 cells (Yen et al. '99). A ceramide activated protein kinase (CAPK) that mediates apoptosis through the SM pathway has been identified as kinase suppressor of Ras (Zhang et al. '97). Exogenous phosphatidylcholine can suppress apoptosis induced by C_2-ceramide and aSMase in primary cultures of cerebellar granule neurons (Ramos et al. '00). Binding of NGF to $p75^{NTR}$ also activates aSMase and releases ceramide in neuronal cells (Dobrowsky et al. '94; '95). Similarly ceramide produced by exogenously added bacterial aSMase overcomes NGF deprivation (Itoh and Horigoe '95). Furthermore, ceramide neosynthesis has been implicated in cell death

induced by TNF-α/ cycloheximide in cerebral endothelial cells (Xu et al. '98). Nevertheless, although SK-N-BE neuroblastoma cells that possess only $p75^{NTR}$ receptors exhibit increased levels of ceramide and caspase activity when they undergo spontaneous apoptosis (Bunone et al. '97), resistant clones also show high levels of ceramide without spontaneous apoptosis (Lievremont et al. '99).

4. The Model System

We used PC12 cells and the Trk 6-24 PC12 cell line to investigate the mechanisms of neuronal differentiation as well as neuronal degeneration due to apoptosis. PC12 cells have been used extensively by others to examine neuronal cell differentiation and the mechanisms of NGF action. Trk 6-24 PC12 is a clonal cell line that has been stably transfected with the ^{p140}TrkA NGF receptor.

A. PC12 Cells

PC12 cells are derived from rat adrenal pheochromocytoma cells and were developed to mimic neuronal differentiation in vitro (Greene and Tischler '78). PC12 cells are adherent, normally proliferative, chromaffin cells that are induced by transient exposure to NGF to enter G0 arrest and differentiate into postmitotic cells that exhibit neuronal properties (Greene and Tischler '76; Burstein and Greene '78). Notable markers of differentiation in these cells include: increases in glucose oxidation and lipid biosynthesis, increases in contractile fibrillar proteins in cell perikarya and axons, polymerization of tubulin to microtubules, synthesis of noradrenaline, and exhibition of electrical excitability.

PC12 cells are also used as an *in vitro* model to study neuronal cell death. Like sympathetic neurons, neuronal PC12 cells are NGF-dependent and undergo apoptotic degeneration in its absence (Greene et al. '86; Mesner et al. '92; Hartfield et al. '97). Both naïve and neuronal PC12 cells are also sensitive to cytotoxic agents such as ceramide, CAM, retinoic acid and staurosporine (Mills et al. '96; Park et al. '97; France-Lanord '97), and our laboratory, in conjunction with Dr. Karen Newell's laboratory at the University of Vermont, has found that PC12 cells express FasL during cell death induced by NGF depletion.

NGF potentiates neurite outgrowth in PC12 cells via its receptors $p140^{trkA}$ and $p75^{NTR}$, which can activate a variety of downstream intracellular signaling molecules (Kaplan et al. '91a,b; Klein et al. '91; Carter and Downes '92; Wood et al. '92, Jaiswal et al. '94, Lange-Carter and Johnson '94; Robbins et al. '92, Boulton et al. '91, Vaillancourt et al. '94). The high affinity binding sites of NGF in PC12 cells are $p140^{trkA}$ dependent although a fraction of these sites requires both receptors (Ryden et al. '97). Expression of these different NGF receptor subtypes varies at the surface of PC12 cells in a cell cycle specific manner, with the high affinity $p140^{trkA}$ mainly expressed in M and early G1 phases, and the low affinity $p75^{NTR}$ expressed in late G1, S, and G2 phases (Urdiales et al. '98).

B. TrkA Cells

We also used the Trk 6-24 PC12 cell line, which we call TrkA throughout this paper. TrkA cells express 20 fold more $p140^{trkA}$ receptor as a result of which they differentiate 2-3 times faster than PC12 cells and do not go into G0 arrest even after

achieving a differentiated morphology (Hempstead et al. '92). TrkA cells are independent of growth factor and do not undergo apoptosis when NGF is withdrawn (Glassman et al. '97) and we have found that they do not express FasL following NGF depletion.

II. RESULTS AND DISCUSSION:

Aims:

Although compilation of this data suggests that ceramide is an important regulator of both neuronal differentiation and death, the mechanism of ceramide generation during either differentiation or death of PC12 cells remains unresolved. Furthermore, the involvement of ceramide and the mechanism by which it is generated in TrkA cells is also unresolved. In addition, the involvement of the mitochondria and specifically the mitochondrial respiratory enzyme ubiquinone (CoQ_{10}) in the initiation of either differentiation or death in both PC12 and TrkA cells has not been substantiated. To address these questions, we examined: a) the mechanisms by which ceramide levels are altered by the induction of differentiation by NGF with respect to TrkA receptor expression; b) the ability of TrkA receptor expression to delay activation of cell death by a variety of cell death inducing agents; c) the mechanism by which ceramide is generated following activation of cell death by NGF withdrawal, ethanol, or camptothecin with respect to TrkA receptor expression; d) the effect of TrkA receptor expression on the disruption of mitochondrial function following activation of cell death by NGF withdrawal, exogenous ceramide and ethanol; e) the ability of CoQ_{10} to prevent apoptosis and its correlation to alterations in both mitochondrial function and ceramide generation.

Chapter I

The Role of Ceramide in the Differentiation of PC12 and TrkA Cells

A. Objective

The proliferation, differentiation, and survival of neurons in the central nervous system are regulated by interactions between NGF and $p75^{NTR}$ and $p140^{trk}$ (TrkA), its respective low, and high affinity receptors. These interactions activate diverse intracellular signaling pathways that are further regulated and mediated by mechanisms not fully understood. Activation of the TrkA receptor inhibits $p75^{NTR}$-mediated activity such as SM hydrolysis (Bose et al. '95). In response to NGF stimulation, TrkA overexpressing cells produce a neuronal phenotype faster than control cells. Furthermore, although different pathways can be used to generate ceramide, it is not known if either synthetic pathway is involved in differentiation, nor is it known what role TrkA plays. We have therefore used TrkA cells, a PC12 cell line that exhibits a 20-fold overexpression of the constitutively active TrkA receptor, to examine the role of the two ceramide pathways in the differentiation of neuronal cells.

B. Materials and Methods

Cell Culture and Treatment

Rat adrenal pheochromocytoma (PC12) cells were obtained from the American Type Culture Collection (ATCC). Trk 6-24 PC12 cells (TrkA) were a gift of Dr. Raymond Birge (Rockefeller University, NY). PC12 cells were maintained in RPMI-

1640 supplemented with 5% FBS, 10% horse serum, 50 U/ml penicillin and 100 μg/ml streptomycin. TrkA cells were maintained in DMEM with 2% FBS, 1% horse serum, 50 U/ml penicillin, 100 μg/ml streptomycin and 100 μg/ml G418. All cell culture media were obtained from Gibco BRL. The cultures were maintained in a humidified atmosphere in 95% air and 5% CO_2 at 37°C.

Naïve cells were induced to differentiate and maintained by transfer to DMEM supplemented with 2% FBS, 1% horse serum, 0.05ng/ml nerve growth factor (NGF), 50 U/ml penicillin and 100 μg/ml streptomycin. NGF was from Harlan Bioproducts. Confluent plates of naïve PC12 cells were split 1:6 immediately prior to NGF stimulation, and confluent plates of naïve TrkA cells were split 1:10 before treatment. After 8 days of incubation, 80% of PC12 cells were differentiated and after 10 days of incubation, all PC12 cells were differentiated. TrkA cells were similarly completely differentiated following 4-6 days of incubation, and because cell division is faster in this cell line, more cells were present in these plates than in PC12 cell cultures. At the end of the incubation cells were pelleted, washed once with phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, pH 7.4), counted, and examined. Because of the differences in kinetics of cell division and differentiation in these two cell lines, we compared equal number of cells, from equivalent populations (ie. naïve PC12 vs. naïve TrkA and differentiated PC12 vs. differentiated TrkA), for these experiments.

For inhibitor studies, desipramine or fumonisin B_1 (FB_1) were diluted in 0.9% NaCl and added to the cultures commencing the first day of treatment. FB_1 and desipramine were purchased from Sigma-Aldrich (St. Louis, MO). Control cells received

0.9% NaCl alone. Specific concentrations used were established from dose-response curves. FB_1 was used at 50 μM and desipramine at 7.5-15μM. Final solvent concentrations did not exceed 0.1% or have detectable effects.

Western Blot Examination of protein expression was conducted essentially as described by Zakeri & Ahuja ('94). Cells were treated and collected at various times after induction of either differentiation or death and lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.15% SDS, 0.1mM sodium vanadate, 1 mM DTT, 20 mM β-glycerophosphate, 2 mM EDTA, 1μg/ml aprotinin, 100 μg/ml phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin). The lysates were cleared by centrifugation and their protein concentrations were determined by assay. Protein assay was from Biorad.

Equal amounts of protein lysates were run on SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. The blots were blocked for 2 hr. in blocker (5% nonfat dry milk in PBST (PBS, 137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4, 0.1% Tween-20, pH 7.4) at RT and then incubated with the primary antibody (p75 1:500; TrkA 1:2000) in blocker overnight at 4°C. The blots were re-washed in PBST, incubated with goat anti-rabbit conjugated to horseradish peroxidase for 1 hr at RT, and again washed in PBST. The immune complexes were detected with an enhanced chemiluminescence (ECF) kit and exposed in a STORM imaging system (STORM). Both nitrocellulose membranes and ECF kit were from Amersham (Chicago, IL). Primary antibodies were gifts of Dr. Raymond Birge (TrkA) and Dr. Moses Chao (p75). Secondary antibodies were from Amersham (Chicago, IL).

Lipid Studies

Ceramide labeling and Quantitation by Diacylglycerol (DG) Kinase Assay

The intracellular level of ceramide was determined by the *E. coli* DG kinase assay using the Bligh-Dyer method essentially as described (Karasavvas et al. '96). Cells were collected by scraping and washed twice with PBS (pH 7.4). Each tube received approximately 3-4 x 10^6 cells, and each point was repeated twice. The cells were lysed in 1 ml chloroform/methanol/1 N HCl (Kill solution; 100/100/1, v/v/v). The organic phase was separated by adding 270 µl of buffered saline solution (BSS, 135 mM NaCl, 4.5 mM KCl, 1.5 mM $CaCl_2$, 0.5 mM $MgCl_2$, 5.6 mM glucose and 10 mM HEPES, pH 7.2) and 30µl of 100 mM EDTA and vortexing. The phases were separated by 5 minutes of centrifuging and then the lower organic phase (total cellular lipids) was removed to a clean tube and dried under a stream of N_2 set at 20-25 psi for 10-15 minutes in a hot water bath.

To eliminate glycerophospholipids, the extracted lipid film was subjected to mild alkaline hydrolysis (0.1 M methanolic potassium hydroxide) for 1 hr at 37°C in a water bath. The organic phase was re-isolated by adding 270µl of BSS (pH 7.2), 30µl of 100 mM EDTA and 500µl $CHCl_3$ followed by vortexing. The phases were again separated by 5 minutes of centrifuging and then the lower organic phase (total cellular lipids) was removed to another clean tube and again dried under a stream of N_2 as above.

Cellular lipids were labeled with reaction mixture (Cardiolipin (10 mg/ml from Avanti Polar Lipids), DETAPAC (1 mM from Sigma-Aldrich, St. Louis, MO), N-Octyl-β-D-glucopyranoside (825 mM from Calbiochem), 2X reaction buffer (NaCl 100 mM,

imidazole 100 mM, EDTA 2 mM, $MgCl_2$ 25 mM; pH 6.5), imidazole/DETAPAC (10mM/1 mM), ATP (100 mM), *Escherichia coli* diacylglycerol (DG) kinase (1 mg/ml from Calbiochem), [γ-^{32}P] dATP (3000 µi/mM from Amersham Corp.) for 30 minutes at room temperature. The reaction was stopped and ceramide-1-phosphate was extracted by adding 1 ml Kill solution and 170 µl BSS, 30 µl 100 mM EDTA to each tube. Tubes were vortexed and spun in a centrifuge for 5 min and the lower organic phase was again transferred to clean tubes and dried under N_2. The lipid film was finally resuspended in 50 µl $CHCl_3$:MeOH (1:1). 30 µl ceramide was loaded into each lane of a TLC plate (6 nm silica gel plates of 0.25 thickness from Whatman) along with known concentrations of ceramide (natural ceramide type III from bovine brain from Sigma-Aldrich, St. Louis, MO) as described (Karasavvas and Zakeri '96). The plates were chromatographed in a tank with $CHCl_3$:MeOH:HAc (65:15:5) for 45-75 minutes until the solvent front was 1-2 cm from the top. The plate was then dried and exposed to X ray film for 24 hours. Finally, the radioactive ceramide bands were excised and counted by scintillation, or scanned with STORM phosphoimager and quantitated using IMAGEQUANT software. Cpm were converted to pmols by reading values off a simultaneously run standard curve consisting of known quantities of ceramide.

The remaining lipid solution was dried under N_2 and used to normalize ceramide levels to cellular phosphate as described (Liu et al.'98). NaH_2PO_4 standards were prepared (0-80 nM) in $CHCl_3$:MeOH (1:1). 600 µl ashing buffer (9:1:40 10N H_2SO_4:70% $HClO_4$:H_2O) is added to each tube and tubes are left at 160°C overnight or 220°C for 2 hours. 900 µl H_2O, 500 µl ammonium molybdate (0.9% w/v) and 200 µl fresh ascorbic acid (9.0% w/v) were added and tubes were vortexed, left at 45°C for 30

minutes and read in a spectrophotometer at 820 mM. All results displayed are the average of at least three independent sets of experiments.

Sphingomyelin Mass Assay

Total cellular sphingomyelin (SM) was determined essentially as described by Lee et al. ('98). SM was labeled with 50μCi [^{3}H] choline chloride (American Radiolabeled Chemicals) for 48 hours before treatment.. After treatment, media was removed and cells (5-6 x 10^5) were collected by scraping in 3 ml MeOH:$CHCl_3$ (2:1).

Lipids were extracted using the Bligh-Dyer method essentially as described above. 800 ml H_2O was added to create a monophase, and tubes were spun for 5 minutes at 2000 g to remove large debris. The supernatant was transferred to a clean tube, $CHCl_3$:H_2O (1:1) added, and tubes vortexed. After spinning for 5 minutes at 2000 g, the upper inorganic phase was partially removed and lower organic phase was transferred to a new tube and dried under N_2 (20-25 psi for 10-20 minutes). The lipid film was subjected to mild alkaline hydrolysis by addition of 250 μl methanolic NaOH (2N) for 2 hours at 37°C in a water bath. The base solution was neutralized by the addition of 250 μl HCl (2N) and the lipids re-extracted in 430μl H_2O, 500μl $CHCl_3$:MeOH (2:1) and 850μl $CHCl_3$ followed by vortexing and centrifuging for 5 min. The lower organic phase was again collected into a fresh tube and dried under N_2 as above. The lipid film was resuspended in 70μl $CHCl_3$, of which 50μl was spotted onto TLC plates.

Plates were developed in a tank with $CHCl_3$:MeOH:HAc:H_2O (50:30:8:5) and run until solvent front was 1-2 cm from top. The plate was then dried and exposed to iodine vapor and the radioactive sphingolipid bands excised and counted by scintillation. Because radioactive sphingomyelin standards are not employed by this assay, results can

not be directly quantified to pmols/cells and are therefore represented as percent of control values. All results are the average of at least three independent sets of experiments and have been further normalized to total cellular phosphate as described above using the lipid solution remaining after the TLC plates were loaded.

C. Results

a. NGF stimulation induces differentiation in PC12 and TrkA cells. When PC12 cells are stimulated with NGF, they stop dividing, flatten slightly and produce neuritic extensions (Figure 7A-E, pp. 126-7). This morphological change is visible by light microscope by day 4 (Figure 7C), and as differentiation continues, the cells flatten and extend long thin neuritic processes. After 5-6 days in culture, PC12 cells develop a dense axonal network, making it impossible to delineate the entire axon plexus of any one cell, or to follow the development of individual cells. By day 8 (Figure 7D), most of the neurites have formed plexae on other cells, and by day 10 (Figure 7E) all of the cells have extended neurites.

TrkA cells also differentiate in response to NGF stimulation, but with significantly different kinetics (Figure 7F-J). Undifferentiated TrkA cells are larger than undifferentiated PC12 cells, and often possess neuritic or pre-neuritic extensions like those seen on day 4 following NGF stimulation of PC12 cells (Figure 7F). When TrkA cells are stimulated with NGF, they differentiate quite rapidly and a neuronal morphology is evident by day 2 (Figure 7G). Furthermore, although they possess a neuronal morphology by day 4 (Figure 7H), TrkA cells do not completely exit the cell cycle and cell division is apparent throughout the entire period of NGF stimulation. We have also

examined PC12 and TrkA cells by Western blot as described in "Materials & Methods" and found that naïve TrkA cells express 10-20x more TrkA receptor, and 5-10x less $p75^{NTR}$ receptors than PC12 cells (Figure 8, pp. 128-9).

b. NGF stimulation induces ceramide elevation in naïve cells.

To determine the involvement of ceramide in differentiation, we measured endogenous levels of ceramide by DAG kinase assay during the differentiation of PC12 cells (Figure 7K, pp. 126-7). It is difficult to get an accurate estimate of cell number for these experiments for two reasons. First, individual PC12 cells are heterogeneous and they exit the cell cycle at their own pace. Thus individual cultures often end up with different numbers of cells after equivalent periods of time. Second, differentiated PC12 cells tend to clump and grow on top of each other as they differentiate. We therefore normalized our raw SL data to the amount of cellular phosphate measured per sample in order to more accurately compare equivalent amounts of membrane from PC12 and TrkA cells.

We found that naïve cells have relatively low levels of ceramide (approx. 20 pmol/μg PO_4). Following NGF stimulation, ceramide began to rise, doubling by two days after initial stimulation and reaching a peak on the fourth day (approx. 400 pmol/μg PO_4) by which time neuronal morphology was evident (Figure 7C). After day 4 ceramide levels declined and leveled off, remaining at 5X the level found in naïve cells (approx. 100 pmol/μg PO_4). These results suggest that ceramide plays an active role in the initiation of differentiation in PC12 cells.

Since increases in ceramide might result from increases in SM hydrolysis, one

might label cellular SM and identify alterations in SM location following NGF stimulation. However location of cellular SM is limited by its role as an important structural component of all neuronal membranes, as demonstrated by Harel and Futerman ('93). We therefore instead measured SM levels by SM mass assay (Figure 7K). SM increases substantially during the first two days of differentiation to reach a peak of nearly 3.5X original content on the second day ($p<<0.05$). SM subsequently returns to baseline, where it remains ($p>0.05$) as ceramide accumulation increases. This inverse correlation suggests that the accumulated ceramide results from SM hydrolysis.

To determine the relationship between the TrkA receptor and ceramide during differentiation, we examined ceramide and SM following NGF stimulation of TrkA cells. Naïve TrkA cells can be described as "primed", that is they already possess a distinct pre-neuronal morphology before NGF stimulation even though their ceramide content (20 pmols/μg PO_4) is not greater than that of naïve, non- neuronal PC12 cells (Figure 7L). Following NGF stimulation, TrkA cells progress in differentiation following a 2 fold increase in ceramide during the first two days of stimulation. This increase in cellular ceramide is similar to that observed during the first two days of NGF stimulation in PC12 cells (compare Figures 7K-L). However in contrast to PC12 cells, ceramide accumulation in TrkA cells does not increase steeply nor does it continue to accumulate after day 2.

Kinetics of SM accumulation also differ in TrkA cells, with SM beginning to increase after day 2 and gradually increasing to nearly 4X original levels by day 8 ($p<<0.05$) without returning to baseline. This lack of correlation between ceramide and SM levels suggests that ceramide is not generated by SM hydrolysis following NGF

stimulation in TrkA cells. The differential accumulation of SM may be causally related to differences in PC12 and TrkA cell responsiveness, so that the initiation of differentiation (as exhibited by PC12 cells) and the progression of differentiation (as exhibited by TrkA cells) may have different quantitative and qualitative ceramide requirements as a result of their expression of NGF receptors.

c. Mechanism of ceramide generation

To determine which ceramide pathway is involved in differentiation, we inhibited known generating pathways and then examined the ability of both PC12 and TrkA cells to respond to NGF signaling. As described above, two distinct pathways are involved in the elevation of cellular ceramide (Spence '93; Michell & Wakelam '94; Riboni '95; Heinrich '99; Tsugane et al.'99). Cytokines, hormones or stress can induce the catabolism of membrane bound sphingomyelin (SM) to ceramide by the activity of neutral or acidic sphingomyelinases present on lysosomal or cellular membranes. Desipramine is a specific inhibitor of acidic sphingomyelinase, and thus prevents the hydrolysis of membrane bound SM to ceramide (Albouz et al. '83; Hurwitz et al. '94). Alternatively, ceramide may be synthesized *de novo* in endomembranes such as the endoplasmic reticulum, or in mitochondrial or nuclear membranes via the condensation of acyl-CoA and sphinganine, which is catalyzed by ceramide synthase (sphinganine N-acyltransferase). Since fumonisin B_1 (FB_1) is a specific inhibitor of dihydroceramide synthase (sphinganine- N-acyl-transferase), it is thus an inhibitor of *de novo* ceramide generation. Percent inhibition of ceramide was verified for our experiments by adding

different concentrations of the two inhibitors to untreated cells and measuring ceramide generation.

When we treated PC12 cells with desipramine at the same time as NGF, both differentiation and cell division were inhibited (Figure 9 A-D, pp.130-1) without affecting viability ($p<0.05$). Furthermore less ceramide accumulates in desipramine treated cells (5 pmols/μg PO_4) compared to controls (Figure 9I). As confirmation that SM hydrolysis is inhibited by desipramine, we measured SM contents in control and treated cells and found it to increase to day 2 levels as in control cells, however they continue at a higher level than in controls up to day 6 when they decrease to the level of control cells (Figure 9J). These results suggest that SM hydrolysis leading to generation of ceramide in PC12 cells may be required for both initiation and progression of differentiation as well as for cell division.

However, when PC12 cells were stimulated with NGF in the presence of FB_1, neither viability, cell division, nor terminal differentiation were altered (Figure 10 A-D, pp.132-3), although ceramide accumulation was slightly less and SM accumulation was much less than that of control untreated cells (Figure 10J). These results therefore support a role for SM hydrolysis in initiation and progression of PC12 cell differentiation and suggest that ceramide synthesis may be involved in the initiation of differentiation.

Differentiation is initiated in TrkA cells before NGF stimulation and when they are stimulated in the presence of desipramine their ceramide content is even lower than in untreated cells (Figure 9K, pp. 130-1) and both progression of differentiation (Figure 9 E-H) and cell division are blocked without affecting viability. SM mass assay confirms that SM hydrolysis is inhibited in these cells, with significantly higher than normal levels of

SM ($p< 0.01$) accumulating in treated cells during the first few days of differentiation (Figure 9L). These results suggest that SM hydrolysis leading to generation of ceramide may be required for both initiation and progression of differentiation as well as for cell division. However these requirements are less in TrkA cells than in PC12 cells.

Furthermore and in contrast to PC12 cells, although cell viability was unaffected by FB_1, the ability of TrkA cells to both complete differentiation as well as to divide was lost following treatment with FB_1 (Figure 10E-H, pp.132-3). Much less ceramide is also accumulated in FB_1 treated TrkA cells compared to control cells (Figure 10K) and less SM accumulates as well (Figure 10L). These results support a role for SM hydrolysis in the progression of differentiation in TrkA cells.

D. Discussion

The $p75^{NTR}$ ceramide-signaling pathway, mediated by N-SMase-induced hydrolysis of SM to ceramide, has been proposed to influence the outgrowth of hippocampal neurons (Brann et al. '99). Other investigators have reported that NGF stimulation of T9 glioma cells activates SM hydrolysis through the $p75^{NTR}$ receptor and that NGF stimulation of TrkA receptors inhibits both $p75^{NTR}$ activity and SM hydrolysis (Bamji et al. '98). Our results reveal that naïve and differentiated PC12 and TrkA cells possess different levels of neurotrophin receptors. Although PC12 cells possess $p140^{trkA}$ receptors, both naïve and differentiated TrkA cells express nearly 20x more $p140^{trkA}$ than they do. Similarly, although differentiated PC12 cells exhibit 10x more $p75^{NTR}$ than naïve cells, both naïve and differentiated TrkA cells possess fewer $p75^{NTR}$ receptors than naïve PC12 cells. In fact, it should be noted that it is quite likely that the characteristics of

the TrkA cell line are due not only to the overexpression of $p140^{trkA}$ but to the underexpression of $p75^{NTR}$ as well.

Our results further demonstrate that undifferentiated PC12 and TrkA cells respond differently to NGF stimulation. We confirm here that NGF stimulation activates SM hydrolysis as well as ceramide neosynthesis in both cell lines. Since we have found that small amounts of ceramide are generated by SM hydrolysis in TrkA cells, our results suggest that either TrkA receptor-mediated inhibition of SM hydrolysis does not occur in this system, or that TrkA-mediated inhibition of SM hydrolysis is leaky. Alternatively, this might suggest that both aSMase and nSMase are active in PC12 cells, while only one pathway is inhibited in TrkA cells that overexpress $p140^{trkA}$ and underexpress $p75^{NTR}$.

We also report that although TrkA cells differentiate in response to NGF, they are unable to generate as much ceramide as PC12 cells. PC12 cells exhibit a 2-fold increase in ceramide prior to initiating differentiation in response to NGF stimulation, and they complete differentiation only after an additional 5-fold increase in cellular ceramide. In contrast, naïve TrkA cells have already initiated differentiation even before administration of NGF. Glassman et al ('97) reported that overexpression of TrkA led to receptor dimerization and potentiation of the NGF signal. We report here that TrkA cells complete differentiation with only a 2-fold increase in cellular ceramide. We conclude that only minimal amounts of ceramide are required for progression of differentiation, while larger amounts are required for the initiation of differentiation.

We also report here that NGF-induced initiation of differentiation in PC12 cells is regulated by SM hydrolysis and not ceramide neosynthesis, since only desipramine blocks both ceramide elevation and differentiation. This is in accord with results recently

reported by Herget et al. ('00) that retinoic acid-induced differentiation is not regulated by ceramide neosynthesis. However we have found that NGF-induced progression of differentiation in TrkA cells is regulated by both SM hydrolysis and ceramide neosynthesis, since both desipramine and FB_1 block both ceramide elevation and differentiation. These results suggest that during differentiation most ceramide is derived from plasma membrane SM, with smaller quantities neosynthesized in cytoplasmic organelles. Furthermore, while both forms of ceramide can mediate progression of differentiation, only plasma membrane-derived ceramide can initiate NGF-induced differentiation.

We considered that a large accumulation of SM might also be required for neurite extension since SM is an important structural molecule in cellular membranes. During the first two days of differentiation in control PC12 cells, SM increases substantially until it is nearly 300-350% of original content. However as ceramide levels increase SM levels drop by a third. SM content in NGF-induced TrkA cells also increases as differentiation progresses, reaching nearly 300-350% of original content by day 4 but there is no comparable drop in SM as ceramide levels increase. The increased accumulation of SM in both PC12 and TrkA cells is most likely fueled by ceramide neosynthesis since the presence of FB_1 in either cell blocks SM accumulation. Nevertheless, FB_1 inhibits progression of differentiation only in TrkA cells. Thus SM accumulation in itself appears unnecessary for progression of differentiation and furthermore the capacity to generate ceramide in the absence of ceramide neosynthesis and thus to complete differentiation, is present only in PC12 cells.

Chapter II

The Relationship between Ceramide and Neurotrophins during Apoptosis

A. Objective

Differentiated PC12 cells are similar to adult sympathetic neurons in that both are addicted to nerve growth factor (NGF) and both die by apoptosis in its absence (Greene & Rein '77a, b; Wang et al. '98). Both high affinity ($p140^{trkA}$) and low affinity ($p75^{NTR}$) receptors are involved in the mediation of such NGF withdrawal-induced apoptosis and thus in the modulation of neuronal function in both embryonic and adult organisms. Furthermore, both receptors mediate SM hydrolysis (Dobrowsky et al. '94; Brann et al. '99; Piccinotti et al. '00) and both SM-derived ceramide and ceramide derived from neosynthesis mediate apoptosis (Schutze et al. '92; Cifone et al. '93; Wiegmann et al. '94; Jarvis et al. '94; Suzuki et al. '97; Garzotto et al. '98). We have also reported in Chapter I that differentiated PC12 cells possess much more ceramide than do differentiated TrkA cells and that much of this ceramide is generated by SM hydrolysis. However, we still do not know whether ceramide is a factor in PC12 cell death and if so, whether one or both ceramide-generating pathways (ie., SM hydrolysis or ceramide neosynthesis) are used. Furthermore, although neuronal PC12 cells are also sensitive to and die via apoptosis following treatment with specific cytokines or toxic agents (Mills et al. '96; Hartfield et al. '97; Park et al. '97; France-Lanord '97; Oberdoerster & Rabin '99) it is unclear whether the same ceramide mediated pathway is used during NGF withdrawal-induced apoptosis as well as in response to other stimuli which induce

apoptosis. Moreover since, in contrast to PC12 cells, TrkA cells are resistant to NGF deprivation (Glassman et al. '97), the role of the TrkA receptor in determining the use of a particular ceramide pathways in the induction of cell death is unclear. To answer these questions we have: a) evaluated the relationship between NGF withdrawal, cellular sphingolipid (SL) content and apoptosis in both PC12 and TrkA cells, b) asked whether the resistance of TrkA cells is a general response to cell death inducers or is specific to NGF withdrawal, and c) determined whether cytotoxic agents like ethanol or camptothecin induce apoptosis via the same ceramide pathways as does NGF withdrawal in either cell line.

B. Materials and Methods

Cell Culture and Treatment

PC12 and TrkA cells were obtained and cultured and examined as described in Chapter I. For NGF withdrawal studies, cells were washed once in PBS (pH 7.4), and replaced in DMEM alone or DMEM containing 2% FBS, 1% horse serum, and antibiotics as above. Cells were incubated in the presence of inducing agents and/or inhibitors for specified times as described below in Results.

N-octanoyl-D-erythro-sphingosine (D-e-C_8-cer; Biomol) was added to the cells as an ethanolic solution. Lyophilized TNF-α (R&D Systems) was reconstituted in 0.1% bovine serum albumin (BSA, 10 ng/μl). Camptothecin (Sigma-Aldrich; St. Louis, MO), dissolved in DMSO and added to the cells in solution. Cycloheximide (Sigma-Aldrich; St. Louis, MO) was added to the cells as an ethanolic solution. For inhibitor studies, desipramine or fumonisin B_1 (FB_1) were diluted in 0.9% NaCl and added to the cultures

simultaneously to other treatments. Specific concentrations used were established from dose-response curves (data not shown). D-e-C_8-cer was used at 10 μM concentration; CAM at 15μM; TNF-α at 10 ng/ml; CHX at 50 μg/ml; ethanol (ETOH) at 220mM; FB_1 at 50 μM and desipramine at 15μM. Control cells received either 0.9% NaCl, DMSO or ethanol alone. Final solvent concentrations did not exceed 0.1% or have detectable effects.

Characterization of Apoptotic Cells

Trypan blue exclusion. To assess the viability of treated cells, we directly counted cells with a hemocytometer after exposure to trypan blue (Mossman '83). The percentage of cells excluding the dye was calculated with respect to the corresponding control and confirmed by 3-4 independent trials.

LIVE/DEAD® Assay. Viability was further confirmed by commercial LIVE/DEAD® kit obtained from Molecular Probes (Eugene, OR). Treated cells were incubated in assay solution consisting of calcein acetoxymethyl ester (AM) and ethidium homodimer as directed by kit. Living cells contain calcein esterase and are able to exclude ethidium homodimer. Calcein esterase hydrolyzes the ester compound and allows the AM group to fluoresce green. Dead cells show no esterase activity and allow entry of the ethidium homodimer indicating lost membrane integrity; as a result live cells fluoresce green and dead cells fluoresce red.

Staining of cells with *bis*-benzimide. To assess the degree of apoptosis in treated cells

we treated them with the DNA fluorochrome *bis*-benzimide (Hoechst 33258; Sigma-Aldrich St. Louis, MO) as described (Karasavvas et al '96). Briefly, cells either in suspension or on coverslips, were washed in PBS and fixed in 3% paraformaldehyde. The cells were re-washed in PBS and stained with *bis*-benzimide (16 μg/ml) for 25 minutes. Following this staining process, cells were washed again in PBS and examined by fluorescence microscopy with a DAPI filter (excitation 375-380nm; emission 450-460 nm). Nuclei with super-condensed chromatin at the nuclear periphery or nuclei fragmented into smaller dense bodies were considered apoptotic. Nuclei with evenly dispersed chromatin were considered not apoptotic.

DNA fragmentation assessed by in situ labeling of DNA nicked ends (TUNEL) *In situ* detection of DNA fragmentation was performed using the ApopTag® kit (Intergen; Purchase, NY) essentially as described (Zakeri & Ahuja '94). Briefly cells were grown on coverslips or slides. Following induction of cell death, slides were pretreated with terminal deoxynucleotidyl-transferase (TdT) buffer for 10 min at room temperature (RT) and then incubated with TdT for 1 hr in a humidified chamber at 37°C. After the slides were washed in PBS, they were incubated with anti-digoxygenin conjugated with peroxidase for 30 min at RT. The slides were washed again in PBS and then incubated with 0.8 mg/ml diaminobenzidine and 0.01% hydrogen peroxide in 0.1 M Tris, pH 7.2. The sections were counterstained with methyl green, dehydrated and coverslipped. Permount mounting media was obtained from Fisher Scientific. For negative controls, slides were incubated without TdT.

Western Blot. Examination of protein expression was conducted essentially as described in chapter I. Primary antibody (PARP) was obtained from Santa Cruz and used at 1:500, secondary antibody was obtained from Amersham.

Lipid Studies

Ceramide labeling and Quantitation by Diacylglycerol (DG) Kinase Assay. The intracellular level of ceramide was determined by the *E. coli* DG kinase assay as described in Chapter I.

Sphingomyelin Mass Assay. Total cellular sphingomyelin (SM) was determined as in Chapter I.

C. Results

a. TrkA Cells are Resistant to NGF Withdrawal. We first identified the dead cells as apoptotic and not necrotic, by depriving differentiated PC12 and TrkA cells of NGF by replacement of media for up to 24 hours. Since PC12 cells develop a dense axonal network after 5-6 days in culture, it is impossible to delineate the entire axon plexus of any one cell, or even to count individual cells *in situ*. For this reason and because the biochemical and morphological aspects of cell death can be variable, we used a combination of methods to identify cell death. We were able to confirm that differentiated PC12 and TrkA cells die by apoptosis following NGF removal (Figure 11A-F). Furthermore, since PARP is cleaved by caspases 3 and 6 during the final stages of apoptosis (Affar et al. 00), we further verified the nature of death by the presence of PARP cleavage fragments on Western Blots (Figure 11G).

We next confirmed previously published work that TrkA cells are resistant to NGF deprivation. Twenty-four hours after PC12 cells are deprived of NGF, nearly 30% of the cells are apoptotic (Figure 11H), by contrast only 8% of TrkA cells are apoptotic at this time ($p < 0.01$). Surviving cells show uniform uncondensed nuclei similar to control untreated cells by Hoechst assay and are poorly stained with ethidium homodimer but well stained with calcein am (LIVE/DEAD® assay) indicating good membrane integrity.

b. NGF Withdrawal Results in increased Endogenous Ceramide. Two pathways, only one of which reduces cellular SM, can generate ceramide. Since one pathway involves the hydrolysis of SM and the other the neosynthesis of ceramide, identifying alterations in total cellular SM and ceramide during apoptosis can give a good indication of which pathway is being used. We therefore investigated this relationship by depriving PC12 and TrkA cells of NGF, and measuring cellular SL content.

We found that apoptotic cell death induced by NGF depletion in PC12 cells correlates with increases in endogenous ceramide (Figure 12A). Although apoptosis is minimal before 12 hours, and has only begun to increase by 18 hours, by 24 hours the number of dead cells has tripled. Similarly, while ceramide levels are unaffected during the first 12 hours, slight increases in ceramide are seen by 18 hours and ceramide accumulation has increased nearly 3 fold by 24 hours. Furthermore despite a lack of correlation between apoptosis and cellular SM accumulation (Figure 12B), between 12 and 24 hours post treatment, SM and ceramide accumulation are inversely related (Figure 12C) and SM which has accumulated during the first 6 hours after NGF removal

gradually disappears as ceramide is generated. These results suggest that the ceramide generated following NGF withdrawal from PC12 cells is derived from SM hydrolysis.

By contrast, there are no changes in viability, cellular ceramide or SM accumulation during the first 24 hours of NGF depletion in TrkA cells (Figure 12D-F), which suggests that TrkA resistance to NGF withdrawal results from a lack of ceramide generation.

c. NGF Withdrawal-Induced Apoptosis is Mediated by Ceramide Neosynthesis. To more specifically resolve whether ceramide was required for apoptosis in PC12 cells and if so which pathway was involved in NGF deprivation-induced apoptosis, we next examined viability and SL content in cells deprived of NGF following inhibition of either SM hydrolysis (with desipramine) or ceramide synthase (with FB_1). When we deprived PC12 cells of NGF for 24 hours in the presence of desipramine, although accumulated ceramide was reduced by half (Figure 13A) while SM accumulation increased 2-fold (Figure 13B), viability was unaltered (Figure 13C). In contrast, when we deprived PC12 cells of NGF for 24 hours in the presence of FB_1, ceramide accumulation was reduced to 10% of controls (Figure 13A), SM accumulation was reduced to 20% of controls (Figure 13B) and apoptosis was effectively blocked (Figure 13C). These results suggested that while NGF withdrawal activates SM hydrolysis, the induction of apoptosis following NGF depletion is due to ceramide neosynthesis alone.

Although TrkA cells depleted of NGF for 24 hours did not exhibit an increase in cellular ceramide, cell depleted of NGF in the presence of the aSMase inhibitor desipramine display a slight increase in ceramide and a nearly 2-fold increase in SM

accumulation (Figure 13A-B). Furthermore, in TrkA cells depleted of NGF for 24 hours in the presence of ceramide synthase inhibitor FB_1, we found a dramatic reduction in both ceramide and SM accumulation (Figure 13 A-B). These results indicate that ceramide of both aSMase and neosynthetic origin is present in TrkA cells following NGF depletion.

d. TrkA Cells are Resistant to Other Cytotoxic Stimuli. Differentiated PC12 cells are often used to model neuronal sensitivity to various cytotoxic agents. To examine if the resistance to cell death was specific to NGF withdrawal, we tested the response of TrkA cells to other cell death inducers. Accordingly, we treated both differentiated PC12 and TrkA cells with either 15 μM camptothecin (CAM), 10 μM ceramide, 10 ng/ml tumor necrosis factor (TNF)-α, 50 μg/ml cycloheximide (CHX), or 220 mM ethanol for 24 hours and quantified the type and rate of cell death by fluorescence and non-fluorescence assays as described above for cells deprived of NGF and in "Materials & Methods." Surviving and untreated cells were poorly stained with ethidium homodimer, bright green with functional calcein esterase activity by LIVE/DEAD® assay, or negative for Hoechst and the apoptotic nature of cell death was again verified by the presence of PARP cleavage fragments on Western Blots.

We found that PC12 cells were sensitive to all the agents that we tested. Approximately 30% of cells treated with CAM, ceramide, TNFα, or CHX and 60% of cells treated with ethanol were apoptotic (Figure 14). Furthermore there was no significant difference between PC12 and TrkA cells in the amount of apoptosis induced by exogenous ceramide, CAM or CHX. However, TrkA cells were resistant to apoptosis induced by either TNFα or ethanol. We found that of TrkA cells, only 13% treated with

TNFα, and 39% treated with ethanol were apoptotic after 24 hours ($p < 0.01$), suggesting that the resistance of TrkA cells to these inducers of apoptosis might be downstream of ceramide generation.

e. Ethanol Stimulation Results in Increased Endogenous Ceramide. To determine whether ceramide accumulation mediates apoptosis induced by ethanol, we exposed PC12 and TrkA cells to ethanol (220 mM) for up to 24 hours, and again measured viability and cellular SL content at specific intervals (Figure 15). We found that 15% of PC12 cells treated with ethanol are apoptotic after just 1 hour, 30% after 6 hours and 55% after 12 hours (Figure 15A). We also found that ceramide levels increased and SM accumulation decreased as viability declined in the first 6 hours of treatment (Figure 15A-B), but that SM began to accumulate again after the first 6 hours while ceramide levels continued to climb, so that both ceramide and SM were tripled by 24 hours (Figure 15C). These results suggest that increased ceramide levels play a role in ethanol-induced apoptosis in PC12 cells, that the increase in ceramide is derived from SM hydrolysis, and that some SM is also synthesized during apoptosis.

We found a similar response to ethanol in TrkA cells. Although TrkA cells generate much less ceramide in total than do PC12 cells, and although there are fewer dead TrkA cells (40%) than dead PC12 cells (60%) 24 hours after treatment, their relative accumulation of SLs following ethanol treatment was almost the same. Like PC12 cells, at least 10% of TrkA cells treated with ethanol are apoptotic after 1 hour, 30% after 6 hours, and nearly 40% after 24 hours (Figure 15D). However, within the first 6 hours of treatment ceramide levels double, nearly all the accumulated SM is lost and the number

of dead cells increases (Figure 15D-E). SM begins to be accumulated again by TrkA cells after the first 6 hours, so that almost half is recovered by 24 hours (Figure 15F). These results suggest that in TrkA cells as well as in PC12 cells, increases in cellular ceramide play a role in ethanol-induced apoptosis, and further that this increase in ceramide may be derived from SM hydrolysis, but that some of the ceramide appears to be synthesized during the course of treatment.

f. Ethanol-Induced Apoptosis is Mediated by SM hydrolysis and Ceramide Neosynthesis in PC12 Cells but only by SM hydrolysis in TrkA cells. To more specifically identify the pathway involved in the induction of apoptosis by ethanol, we treated PC12 cells with 220 mM ethanol for 24 hours in the presence of either desipramine or FB_1 (Figure 16A-C). We found that blocking aSMase with desipramine significantly blocked apoptosis by one third, while reducing by two thirds the amount of ceramide generated and increasing by three fold the accumulation of SM in PC12 cells ($p<<0.05$). However we found that viability was similarly improved in the presence of FB_1 which blocks neosynthesis (Figure 16A), when ceramide was reduced to $1/6^{th}$ original levels (Figure 16B). These results suggest that ethanol-induced apoptosis in PC12 cells is different from that induced by NGF withdrawal in that it is mediated by ceramide that is derived from both neosynthetic and hydrolytic pathways.

When we examined TrkA cells similarly treated with 220 mM ethanol for 24 hours in the presence of either desipramine or FB_1 (Figure 16A-C), we found that desipramine significantly improved viability in TrkA cells following ethanol treatment in that fewer than 20% of treated cells were dead after 24 hours. Furthermore, TrkA cells

treated in the presence of desipramine generated roughly 2/3 the amount of ceramide as cells treated with ethanol alone but possessed nearly 5 times the amount of SM as controls. In contrast, cells treated with ethanol in the presence of ceramide synthase inhibitor FB_1 show no improvement in viability despite almost complete loss of ceramide content and a nearly 3-fold increase in SM accumulation. These results strongly suggest that in contrast to PC12 cells, induction of apoptosis following ethanol-induced cell death is dependent only on SM hydrolysis in TrkA cells.

g. Treatment with CAM Results in Increased Endogenous Ceramide. We next extended our analysis of the involvement of ceramide in apoptosis to camptothecin (CAM), which is often used as a model of direct nuclear fragmentation. We had previously found that both PC12 and TrkA cells are equally sensitive to CAM and that approximately 20% of the cells are apoptotic after 24 hours. To determine whether CAM-induced apoptosis is mediated by cellular ceramide, we treated PC12 and TrkA cells with 15μM CAM for up to 24 hours, and measured viability and cellular SL content as described in Chapter I and in "Materials and Methods." We found that although apoptotic cells are evident within the first 6 hours, only 20% of PC12 cells are apoptotic by 24 hours. However, in contrast to cells either depleted of NGF or treated with ethanol, there is no correlation between ceramide levels and apoptosis in CAM-treated PC12 cells. Although ceramide content of these cells increases by approximately 30% between 6 and 12 hours, it returns to control levels by 24 hours (Figure 17A). Furthermore, SM accumulation does not correlate with apoptosis either, as it is lost almost completely by

the first hour after treatment and is not recovered (Figure 17B). These results suggest that ceramide does not mediate CAM-induced apoptosis.

Like PC12 cells, TrkA cells are not very sensitive to CAM; although apoptotic cells are evident within the first 6 hours, only 10% exhibit apoptotic nuclei by 24 hours and barely 20% at 24 hours (Figure 17D). Here again we find that TrkA cells generate much less ceramide than PC12 cells. In addition, although the ceramide content of CAM treated TrkA cells increases steadily and is nearly quadrupled 24 hours after treatment (Figure 17D), SM is lost early following CAM treatment (Figure 17F). These results confirm that ceramide accompanies CAM-induced apoptosis but does not mediate it and further suggest that the ceramide produced during apoptosis in TrkA cells is of neosynthetic origin, since there is no relationship between ceramide and SM levels.

h. CAM-Induced Apoptosis is not Mediated by Ceramide. To further demonstrate that ceramide does not mediate CAM-induced apoptosis, we treated PC12 or TrkA cells with 15 μM CAM for 24 hours in the presence of either desipramine or FB_1. We found that when PC12 cells were treated in the presence of desipramine, neither viability (Figure 18A) nor ceramide levels (Figure 18B) were affected despite a four fold increase in SM accumulation (Figure 18C). Furthermore, when PC12 cells were treated with CAM in the presence of FB_1 for 24 hours, viability is not improved above that of control cells (Figure 18A) despite a two third reduction in ceramide levels (Figure 18B) and a two-fold increase in SM accumulation (Figure 18C). These results confirm that although CAM-induced apoptosis may result in ceramide neosynthesis it is not mediated by alterations in either ceramide or SM levels. They further indicate that increases in

cellular SM do not always correlate with decreases in cellular ceramide, and that decreases in cellular ceramide do not necessarily correlate with decreased rates of apoptosis.

TrkA cells treated with CAM in the presence of either desipramine or FB_1 yielded similar conclusions; namely that although CAM-induced apoptosis results in ceramide neosynthesis, it is not mediated by it. TrkA cells treated with CAM in the presence of desipramine exhibit no improvement in viability after 24 hours (Figure 18A), despite possessing one third less ceramide (Figure 18B) and twice as much SM (Figure 18C) when compared to control cells treated with CAM alone. Furthermore, if TrkA cells are treated with CAM in the presence of FB_1, there is only a small (10%) reduction in the number of dead cells present (Figure 18A), despite a decline in ceramide levels by half (Figure 18B) and in SM accumulation by three quarters (Figure 18C). These results not only confirm that CAM-induced apoptosis is not mediated by either cellular ceramide or SM, but also indicate that both ceramide and SM neosynthesis as well as SM hydrolysis are activated by this pathway.

D. Discussion:

Differentiated PC12 cells are similar to immature sympathetic neurons in that both are ddicted to NGF and undergo apoptosis after its removal (Mesner et al. '92,'95). However cells overexpressing the TrkA NGF receptor are resistant to such death (Glassman et al. '97). We have reported in Chapter I that NGF-induced differentiation of naïve PC12 and TrkA cells is mediated by ceramide and that PC12 cells are able to

generate more ceramide than similarly treated TrkA cells. We have further demonstrated that this ceramide is of aSMase origin in PC12 cells, but derived from both SM hydrolysis and ceramide neosynthesis in TrkA cells. Similarly, we report here that when NGF is withdrawn from differentiated PC12 cells, the resulting apoptosis is mediated by neosynthetic ceramide, while NGF depletion of TrkA cells also activates both ceramide pathways.

Our initial hypothesis was that the resistance of TrkA cells to NGF withdrawal-induced apoptosis resulted from a defect in ceramide generation. We have found instead that the overexpression of $p140^{trkA}$/underexpression of $p75^{NTR}$ evident in the TrkA cell line seems to affect the capacity of PC12 cells to generate ceramide following comparable stimuli. Nevertheless, although untreated control TrkA cells have roughly 20% as much ceramide as PC12 cells, their resistance to apoptosis apparently is not due to an inability to synthesize ceramide. Although less ceramide is generated than in comparably treated PC12 cells, TrkA cells can generate ceramide and their content doubles after NGF is removed in the presence of desipramine. Furthermore, both ceramide pathways are functional in TrkA cells and both are activated by NGF depletion. Moreover, since increases in cellular ceramide do not necessarily increase the rate of apoptosis, we must conclude that either: a) TrkA cells are less sensitive to ceramide than are PC12 cells, b) it is not the accumulation of ceramide, but the loss of SM that is responsible for apoptosis, c) apoptosis is dependent on the total amount of ceramide generated, d) having large quantities of ceramide is not as important as having enough ceramide at the right time in the right place, or e) that ceramide has nothing to do with apoptosis.

If we take these ideas one at a time, we can dispense with the last issue most directly. When ceramide synthesis is blocked, apoptosis is inhibited in cells depleted of NGF or treated with ethanol, therefore ceramide is either upstream or downstream of the apoptotic pathway in these cells. However, when ceramide synthesis is blocked in CAM treated cells, apoptosis is not inhibited, which indicates that ceramide does not have a role in CAM-mediated apoptosis in PC12 cells.

We further find that TrkA cells are not less sensitive to ceramide than are PC12 cells. In fact, we have determined that both are equally sensitive to exogenous ceramide and die in similar numbers in its presence. However, our results clearly indicate that apoptosis may depend on the total amount of ceramide generated. When similar amounts of endogenous ceramide are generated in PC12 and TrkA cells, they exhibit similar viability; compare for example PC12 cells treated with ethanol in the presence of desipramine and TrkA cells treated with ethanol alone. However, increases in cellular ceramide also accompany CAM-induced apoptosis although we have demonstrated that such apoptosis is not dependent on either ceramide or SM content.

Another possibility that has been recently proposed is that it is the loss of SM *per se* that produces the apoptotic phenotype by profoundly altering the fluidity of the plasma membrane (Tepper '00). Ethanol may indeed cause apoptosis in this manner, and its ability to alter the fluidity of the plasma membrane may be related to such a loss in SM. However, we have not found that apoptosis is caused by loss of SM in either of our cell lines. SM is not lost in great quantity following either NGF depletion or ethanol treatment. We have also found that although SM hydrolysis is activated when PC12 cells are deprived of NGF, only blocking ceramide neosynthesis prevents apoptosis.

Furthermore even when TrkA cells lose nearly all their SM after ethanol treatment, it is recovered within hours. Finally even when loss of SM is inhibited by the presence of desipramine during CAM-induced apoptosis, apoptosis is not prevented.

Perhaps it is the total amount of ceramide generated by a cell that determines whether it will live or die. Ethanol has been demonstrated to alter brain phospholipid levels which correlates to reductions in neuron density (Miller et al. '97). Similarly, we have found that ethanol-induced apoptosis alters SL levels in both PC12 and TrkA cells, and that in PC12 cells both neosynthesis and SM hydrolysis mediate ethanol-induced apoptosis. Furthermore, although both pathways are also activated in TrkA cells, only SM hydrolysis mediates ethanol-induced apoptosis and 24 hours after treatment there are fewer dead TrkA cells (40%) than dead PC12 cells (60%). Thus it is possible that the resistance of TrkA cells to ethanol is the result of an inability to generate sufficient endogenous ceramide in order to trigger downstream targets.

We must also consider that the resistance of TrkA cells to various inducers of apoptosis may well result from a defect in their ability to hydrolyze SM to ceramide. If this were so, we would expect if an inducer could bypass this mechanism and promote apoptosis either through ceramide neosynthesis or through a ceramide-independent pathway, TrkA cells would succumb to apoptosis. In fact, this is not the case, and we find that ethanol induced apoptosis in TrkA cells is prevented only when SM hydrolysis is inhibited.

Finally, we turn our attention to whether having large quantities of ceramide is as important as having enough ceramide at the right time in the right place. Recent studies suggest that the balance between survival and apoptotic signals, which is mediated by

neurotrophins, determines life and death in peripheral neurons. In neonatal sympathetic neurons, TrkA and $p75^{NTR}$ are functionally antagonistic (Bamji et al. '98). TrkA mediates survival signals and suppresses both c-jun and bax activation (Estus et al. '94; Ham et al. '95; Deckworth et al. '96), while $p75^{NTR}$ contains a death domain that stimulates both ceramide generation (Dobrowsky et al. '94; '95; Cassacia-Bonnefil et al. '96) and enhancement of JNK activity (Cassacia-Bonnefil et al. '96; Bamji et al. '98). Since ceramide generated by hydrolysis of SM is in the plasma membrane and that newly synthesized is in the ER, we may speculate that in PC12 cells, ceramide in the membrane signals apoptosis and ceramide in the ER signals differentiation. Indeed, as we have described above ceramide generation via SMase has also been implicated in cell death caused by anti Fas/CD95, TNFα, IL-1, IFN-γ, vitamin D3 ionizing radiation, heat shock and oxidative stress (Schutze et al. '92; Cifone et al. '93; Wiegmann et al. '94; Jarvis et al. '94; Wiegmann et al. '94; Verheij et al. '96). Dawson ('00) has also shown that inhibitors of protein kinase C such as staurosporine induce apoptosis by the formation of ceramide from SM as mediated by nSMase.

We might further speculate that because of the extra TrkA receptors in TrkA cells, they possess a more randomized network of ceramide synthesis, which might result in ceramide in the ER signaling apoptosis and that in the membrane signaling differentiation. Others have also shown that apoptosis may be induced by *de novo* ceramide generation in the ER and a series of observations strongly suggests that ceramide neosynthesis mediates the apoptotic activity of anthracyclines as well as daunorubicin (Bose et al. '95; Suzuki et al. '97; Garzotto et al. '98).

In actuality we have found more gray than black and white; differentiation in PC12 cells is dependent on SM hydrolysis and in TrkA cells it is dependent on both pathways, while apoptosis induced by NGF withdrawal is dependent on ceramide neosynthesis and that induced by ethanol is also dependent on both pathways. We need to look to another aspect of apoptosis to resolve this issue.

So far this discussion has focused on a generalized view of the apoptotic process, while the interactions of signal molecules with caspases actually define two distinct pathways of apoptosis. One pathway involves the activation of death receptors including Fas, TNF and TRAIL receptors. The second pathway is the mitochondrial pathway that induces the release of proteins such as *cytochrome c*, which are normally found in the space between the inner and outer mitochondrial membranes. The two ceramide pathways can be distinguished by these two styles of apoptotic mechanisms. In the first, activation of apoptosis by Fas or TNF receptor mediation results in SM hydrolysis and ceramide generation in the plasma membrane. In the second, ceramide synthase activity occurs in the endoplasmic reticulum and perhaps the mitochondrial or nuclear membrane by the conversion of sphinganine to dihydroceramide (Hirschberg et al. '93; Shimeno et al. '98; Tsugane et al. '99; El Bawab et al. '00). Furthermore, inhibition of the mitochondrial pathway is reported to inhibit ceramide production in cells treated with DNA damaging agents (Tepper '99) and ceramide disrupts mitochondrial respiration (Di Paola et al. '00) even after cytochrome c release (Goldstein et al. '00).

In fact, these two pathways may be connected somewhere further down. Although ceramide induced cell death is independent of the Fas ligand/caspase pathway in specific systems (Bras et al. '00), in others stress induced apoptosis mediated by either

anti-CD95 (anti-Fas), anti CD40 or TNFα is preceded by increased ceramide generation that is not of SMase origin (Segui et al. '00). Recent reports have also indicated that anti-Fas actually causes transient elevation of ceramide followed by downstream sphingosine production, as well as mitochondrial and caspase activation which is then followed by a sustained ceramide accumulation. Furthermore, caspase inhibitors block both early and later ceramide accumulation in these cells (Cuvillier et al. '00) suggesting both that ceramide is not an initiating signal for apoptosis and that ceramide may act as a signal for more than one event during apoptosis. Indeed we believe our evidence confirms that ceramide in and of itself is not an initiating signal for apoptosis, but that a cell must be somehow primed for apoptosis that is triggered by elevations in cellular ceramide, and further that it is not only an issue of how much of a ceramide signal is present but also in which compartment the ceramide signal originated.

CHAPTER III

The Relationship Between Differentiation or Death and Mitochondria

A. Objective

Mitochondrial membrane permeabilization (MMP; a.k.a. mitochondrial permeability transition MPT) and subsequent collapse of mitochondrial potential ($\Delta\Psi_m$) results in the formation of the mitochondrial permeability transition pores (MPTP) and loss of mitochondrial enzyme activity or respiratory function (Susin et al. '98; Skulachev '98; Kroemer et al. '98; Kroemer and Reed '00; Brenner and Kroemer '00). Important consequences of MPT include hyperproduction of superoxide anions, outflow of matrix calcium and glutathione, disruption of mitochondrial biogenesis, and release of soluble intermembrane proteins (Pastorino et al. '96). Swelling of the mitochondria also releases an apoptosis-inducing protein (possibly a protease) that is normally sequestered in the intermembrane space (Susin et al. '97) and *cytochrome c* is also released upon mitochondrial swelling (Yang & Cortopassi '98).

Ceramide and ceramide metabolites are also implicated in contradictory downstream mediation of mitochondrial activation and consequent apoptosis. Chmura et al. ('00) have proposed that mitochondrial function is altered during selection of cells for modification of ceramide production. Exogenous ceramide is reported to cause cytochrome-c release, caspase activation, and apoptosis in melanoma cells and can disrupt mitochondrial respiration even after *cytochrome c* has already been released (Di Paola et al. '00; Goldstein et al. '00). Nevertheless, ceramide appears to have

contradictory effects on different cell types. Although increases in endogenous ceramide are implicated in Fas-induced MMP and apoptosis in melanoma, lymphoid and myeloid tumor cells (De Maria et al. '97; Raisova et al. '00), the inhibition of endogenous ceramide production in Jurkat cells is reported to cause the collapse of $\Delta\Psi_m$ following treatment with Fas, etoposide or γ radiation (Tepper '99).

In Chapters I and II we reported that in PC12 cells, differentiation is mediated by ceramide derived from SM hydrolysis and that, depending on the specific insult, either ceramide pathway can mediate apoptosis. Thus, we found that NGF withdrawal-induced apoptosis was mediated by newly synthesized ceramide but that both pathways mediated ethanol-induced apoptosis. These results suggested that newly synthesized ceramide and ceramide metabolized from SM are not equivalent in the cell and that they may trigger different downstream events. We therefore hypothesized that the origin of ceramide might affect its downstream effects through mitochondrial involvement and activity. To this end we stimulated naïve and neuronal PC12 cells with NGF, which induces differentiation, or inducers of apoptosis such as ethanol, NGF withdrawal and exogenous ceramide, in order to examine the effect on mitochondrial integrity and function.

We also reported in Chapters I and II that overexpression of the TrkA receptor in PC12 cells results in a cell characterized by rapid differentiation (which is mediated by both ceramide pathways) and resistance to apoptosis (mediated by ceramide synthesis). It seemed plausible that if mitochondria were involved in the mediation of either differentiation or death through a specific ceramide pathway, that the TrkA phenotype might result from either an enhancement of normal mitochondrial activity or from protection against loss of mitochondrial integrity or function. To test this idea, we also

examined the mitochondrial activity and integrity of naïve and neuronal TrkA cells following either NGF stimulation or treatment with inducers of apoptosis.

Reports of mitochondrial activation during cell death further suggest the possibility of involvement of a specific mitochondrial component such as ubiquinone (CoQ_{10}) in ceramide mediated-apoptosis. CoQ_{10} has been implicated in both proliferation (Sun et al '92; Crane et al. '94) and apoptosis (Kagan et al.'99; Teranishi et al. '99; Fernandez-Ayala et al. '00; Brancato et al.'00). Although neither its molecular nor cellular mode of action are fully understood, CoQ_{10} has been used clinically for several decades to treat patients suffering from a variety of illnesses (Langsjoen et al. '88; Folkers et al. '88; Topi, et al.'89; Densnuelle et al. '89; Kato et al.'90; Bianchi et al.'99; Park et al.'99; Barbieri et al.'99; Kieburtz '99; Eriksson et al.'99; Khatta et al.'00). In addition, exogenous ceramide induces generation of reactive oxygen species (ROS) at the CoQ_{10} site of the mitochondrial respiratory chain (Quillet-Mary et al. '97), and CoQ_{10} supplementation during serum withdrawal-induced apoptosis of HL-60 cells both inhibits ceramide accumulation and prevents apoptosis (Barroso et al. '97).

These combined observations led us to ask whether CoQ_{10}, either as an antioxidant or through its role in the electron transport chain, could stabilize mitochondria, thus rendering cells more resistant to induction of apoptosis. To this end we stimulated neuronal cells with either NGF or inducers of apoptosis in the presence or absence of CoQ_{10} to determine the effect of CoQ_{10} on mitochondrial integrity and function, ceramide generation and viability in both differentiating and dying cells.

E. Materials And Methods

Cell Culture And Treatment

PC12 and TrkA cells were cultured and maintained as described in Chapter I and cell death was induced as described in Chapter II. Cells were incubated in the absence of NGF or in the presence of inducing agents such as ethanol (220 mM) or exogenous C-8 ceramide (10 μM) with or without coenzyme Q_{10} (CoQ_{10}, Sigma-Aldrich) for times specified below for specific experiments. Commencing the first day of treatment, CoQ_{10} was dissolved in DMSO and added to cell cultures to a final concentration of 100 μM. Control cells received DMSO alone. Final solvent concentrations did not exceed 0.1% or have detectable effects. Specific concentrations used were established from dose-response curves.

Characterization Of Apoptotic Cells

Trypan blue, *bis* benzimide, LIVE/DEAD® and TUNEL® assays were conducted as described in Chapter II.

Lipid Studies

Ceramide was quantitated by diacylglycerol (DG) kinase assay, and we quantitated total cellular sphingomyelin (SM) by SM Mass Assay as described in Chapter I.

Analysis Of Mitochondrial Activity

$DiOC_6(3)$ Assay. Permeabilization of the inner membrane (MMP) can be assessed indirectly by determining loss of $\Delta\Psi_m$ as a function of decreased uptake of the cationic

fluorochrome $DiOC_6(3)$ (3,3'dihexyloxacarbocyanine). $DiOC_6(3)$ is normally driven by $\Delta\Psi_m$ (the difference in potential across the membrane), to accumulate within the mitochondrial matrix inside the inner mitochondrial membrane (Pastorino et al. '98). This technique does not allow us to differentiate between general loss in integrity for each mitochondria, selective loss in some of the cells, or even selective loss in mitochondrial function in individual cells.

Measurement of mitochondrial potential or "energization" was performed essentially as described in Pastorino et al. ('98). At least 5 x 10^5 PC12 and TrkA cells were cultured and allowed to differentiate as described in Chapter I and then treated with inducers of apoptosis as described in Chapter II. During the final 30 minutes of treatment, $DiOC_6(3)$ (3,3'dihexyloxacarbocyanine; Molecular Probes: Eugene, OR) at a final concentration of 100 nM was added to and gently mixed with the culture medium. The plates were wrapped with foil to protect them from light and returned to the incubator at 37°C. After incubation, the cells were scraped into clean tubes with a rubber scraper (Nunc) and washed twice in 1x PBS to remove excess dye. The pelleted cells were then lysed with a Dounce homogenizer in 500 µl ddH_2O. The lysate was spun at room temperature for 1-2 minutes at low speed and the supernatant transferred to glass cuvettes. The concentration of retained dye was read on a fluorescence spectrophotometer at 488 nm excitation and 500 nm emission and compared to control values. Results are the average of at least three independent sets of experiments.

Rhodamine-123 Based Confocal Microscopy. Permeabilization (MMP) or rupture of the outer mitochondrial membranes can also be monitored by scanning confocal

microscopy after immunofluorescence staining with the fluorescent dye rhodamine-123 (Rh-123; Sigma-Aldrich) as described in Lemasters et al. ('98). Rh-123 accumulates electrophoretically in energized mitochondria in response to their negative inside membrane potential relative to the cytoplasm (Emaus et al. '86). Mitochondrial retention of the dye is then used as a measure of mitochondrial polarity (Johnson et al. '81; Lemasters et al. '87). This technique theoretically allows us to differentiate between general loss in integrity for each mitochondria, selective loss in some of the cells, or even selective loss in mitochondrial function in individual cells, however for the experiments described here we have not differentiated between these various possibilities.

Cells, at a density of 2-3 x $10^3/cm^2$ were plated onto non-fluorescent glass cover slips (Fisher Scientific) or cover glass chambers (Nunc) in media and cultured as described in Chapter I. At time of treatment, cells were confluent and Rh-123 at a final concentration of 0.5-1.0μM was added to the culture medium and the culture was left for 30 minutes at 37°C in the dark. The cells were then washed several times in 1x PBS to remove excess dye. Confocal imaging was performed using a Meridian confocal microscope (Lansing, MI) at 20X magnification. An argon-ion laser beam (700 mW) was used for single fluorescein emission after excitation at 450-490 nm with emission at 515-550 nm. At each session, 3-5 similar fields of cells, containing at least 20 cells apiece, were counted, photomicrographs were recorded, and temporal alterations in fluorescence intensity were calculated automatically with a digital imaging processor by a computer assisted image analyzing system (Meridian, Lansing MI). Results are the average of at least three independent sets of experiments.

Inducers of apoptosis were added to the culture medium after the background dye was removed by washing, and mitochondrial potential was measured by fluorescence confocal microscopy. For some experiments we captured electronic images every 10 minutes for several hours, while for others we took images every hour for 24 hours. Rh-123 was localized in control untreated cells for comparative imaging for each time.

MTT Assay. Mitochondrial function can be estimated using the simple and common MTT assay, which measures the dehydrogenase activity normally present in living cells and which was originally designed as a viability assay. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) is a water-soluble salt that is cleaved by active mitochondrial dehydrogenases present in living cells (Mossman '83), resulting in the formation of an insoluble purple precipitate. PC12 and TrkA cells were cultured and differentiated as described in Chapter I and treated with inducers of apoptosis as described in Chapter II. After treatment, MTT (Sigma-Aldrich) was added to the cells at a final concentration of 5 mg/ml and the culture was left at 37°C. After several hours the resulting precipitate was collected by centrifugation, and washed. The dye was extracted from the cells with 0.1N acidic isopropanol (HCl), cleared by centrifugation and the amount of converted tetrazolium was quantified by spectrophotometer (OD_{570}) yielding a measure of absorbency as a function of concentration. Results are the average of at least three independent sets of experiments. This technique also does not allow us to differentiate between general loss in integrity for each mitochondria, selective loss in some of the cells, or even selective loss in mitochondrial function in individual cells.

C. Results

a. Mitochondrial integrity and function during differentiation.

Thus far we have shown that differentiation in PC12 cells is mediated by ceramide derived from SM hydrolysis, while differentiation of TrkA cells is mediated by both SM hydrolysis and ceramide synthesis. To determine whether a downstream mitochondrial pathway operates during differentiation, we stimulated PC12 and TrkA cells with NGF as described in Chapter I, and measured the involvement of mitochondria by two of the three assays described above in "Materials and Methods." We were unable to use confocal microscopy to measure mitochondrial integrity during differentiation, because the experimental protocol requires pre-loading of the cell with Rh-123, followed by confocal imaging at specified intervals during differentiation (which occurs over a period of up to 10-12 days). Such long-term incubation with Rh-123 is toxic to the cells.

In PC12 cells, we found a hyperpolarization of the membrane potential ($\Delta\Psi_m$) as measured by $DiOC_6(3)$ assay, as well as an increase in enzyme activity as measured by MTT assay. Both mitochondrial measurements peaked on the day on which extended neurites become evident (compare Figure 19A, pp. 150-1 to Figure 8 in Chapter I, pp.128-9). By day 10, these values have returned to those seen in naïve, control cells. In contrast, although mitochondrial membrane potential showed a similar pattern in differentiating TrkA cells, with hyperpolarization evident when neurites begin to extend (compare Figure 19B to Figure 8) and a return to baseline control values thereafter, there was no significant increase in mitochondrial enzyme function over this period although saturation levels of the MTT assay had not been reached. Thus differentiation mediated by SM hydrolysis, and accompanying a large increase in intracellular ceramide (as in

PC12 cells) correlated with mitochondrial hyperpolarization and increased enzyme activity but differentiation mediated by both ceramide pathways and accompanied with a relatively smaller total increase in intracellular ceramide (as in TrkA cells) correlated only with mitochondrial hyperpolarization. These results suggest that it is either the ceramide pathway, or the total amount of ceramide generated within a cell that determines the degree of mitochondrial involvement, or perhaps the degree of mitochondrial involvement that determines where and how much ceramide is generated.

b. The effect of components of the electron transport chain such as ubiquinone on differentiation.

Since we found significant variation in mitochondrial activation during NGF-induced differentiation and the mitochondrial respiratory coenzyme and antioxidant CoQ_{10} appears to stimulate cell proliferation *in vitro* (Crane et al. '94; Sun et al. '92), we induced both PC12 and TrkA cells to differentiate in the presence of 100 μM CoQ_{10} and examined: a) the timing and morphology of differentiation, b) the accumulation of cellular ceramide and SM, and c) mitochondrial integrity and function. We determined from a dose-response experiment that CoQ_{10} alone is not toxic to either PC12 or TrkA cells (Figure 20, pp.152-3) and based on this information, we used 100 μM CoQ_{10} for all subsequent experiments.

Addition of CoQ_{10} to the medium did not alter the timing or morphology of differentiation in PC12 cells (Figure 21A-B, pp. 154-5), despite inhibiting both ceramide and SM accumulation (Figure 21C-D). Similarly in TrkA cells, CoQ_{10} did not alter the timing or morphology of differentiation as compared to untreated cells (Figure 22 A-B,

pp.156-7). Although CoQ_{10} nearly completely inhibited SM accumulation and reduced by one third the amount of ceramide generated on day 2 of differentiation, ceramide content on day 4 was the same as in controls. Furthermore, the amount of ceramide in TrkA cells exposed to NGF in the presence of CoQ_{10} was greater that that found in cells in the presence of either FB_1 or desipramine (Compare Figures 9-10 to Figure 22 C-D). These results confirm that neither SM accumulation nor ceramide synthesis is required for the differentiation of PC12 cells, and moreover that the accumulation of SM is not required for the differentiation of TrkA cells. Since ceramide generation is not abolished in the presence of CoQ_{10}, these results do not rule out the possibility suggested in Chapter I that TrkA cells are dependent on ceramide synthesis for the progression of differentiation. Nevertheless, since CoQ_{10} is a mitochondrial component, these results also suggest that ceramide synthesis during differentiation may be mediated by a mitochondrial mechanism. However, since the dose we have chosen is far above physiological levels, we cannot completely rule out secondary, or pharmacological effects.

c. Apoptosis disrupts mitochondrial integrity and function.

Alterations in mitochondrial structure and function are reported to be early events in apoptosis. To determine whether such events play a role in ceramide-mediated apoptosis, we compared, by three distinct assays as described in Materials & Methods, the mitochondrial structure and function of neuronal PC12 and TrkA cells treated for 24 hours with either exogenous C-8 ceramide (10μM), NGF depletion, or ethanol (220 mM).

PC12 cells are relatively insensitive to exogenous ceramide and by 24 hours only 30% of the cells were apoptotic; none of these deaths occur during the first 12 hours post treatment (Figure 23A, pp.158-9). Similarly, in NGF depleted PC12 cells, as we reported in Chapter II, 30% of the cells were apoptotic after 24 hours with almost none of the deaths occurring before 18 hours (Figure 23C). We have determined that such NGF withdrawal-induced death is dependent on synthesis of endogenous ceramide. We also reported in Chapter II that ethanol-induced apoptosis is mediated by endogenous ceramide derived from both ceramide pathways and that such death is rapid; although 80% of ethanol treated PC12 cells were viable within the first hour of treatment, only 50% of them were alive by 6 hours (Figure 23E).

Nevertheless, we found distinct differences in the state of mitochondrial function and integrity depending on the type of insult as well as on the length of exposure. When we assessed MMP by $DiOC_6(3)$ assay after treatment with exogenous ceramide, $\Delta\Psi_m$ was not immediately lost, although there is a 30% decrease in $DiOC_6(3)$ uptake by 24 hours (Figure 23A). At the same time, scanning confocal microscopy of the fluorescent dye Rh-123 indicated that there was an immediate loss of 60% of $\Delta\Psi_m$ within the first hour that was not recovered by 24 hours. This loss of mitochondrial polarity as visualized by Rh-123 microscopy is represented in Figure 24 (A-E, pp.160-1) and we observe both progressive loss of fluorescence and rounding up of cells as apoptosis progresses. Furthermore, when we measured mitochondrial dehydrogenase activity (enzyme function) we found an immediate but transient loss of mitochondrial enzyme activity that was recovered by 3 hours, in spite of the loss of polarity.

Alterations in mitochondrial function and integrity were different following NGF depletion, which is mediated by synthesis of endogenous ceramide. Deprived PC12 cells exhibited rapid MMPT as measured by $DiOC_6(3)$ assay, with a 50% reduction in $\Delta\Psi_m$ within the first hour, of which 50% was temporarily recovered by the third hour, and lost again by the end of the 24 hour treatment period. In contrast, neither polarity, as measured by Rh-123 retention, nor enzyme function were significantly affected by NGF removal (Figure 23C).

In contrast to NGF withdrawal, mitochondrial integrity and function were dramatically reduced following treatment with 200 mM ethanol. $\Delta\Psi_m$ in these cells, as measured by $DiOC_6(3)$, dissipated rapidly and nearly 70% was irretrievably lost after only the first hour of treatment (Figure 23E). Moreover, mitochondrial polarity, as measured by Rh-123 retention, and enzyme activity were also lost during the first three hours of treatment without subsequent recovery.

These results suggest that very early alterations in mitochondrial polarity and/or enzyme function may mediate apoptosis induced by exogenous ceramide, and that MMP is an early event in apoptosis mediated by ceramide synthesis, as seen in NGF withdrawal. These results also implicate rapid destruction of mitochondrial integrity and function mediated by a combination of SM hydrolysis and ceramide synthesis as in ethanol-induced apoptosis.

TrkA cells are less sensitive to exogenous ceramide than are PC12 cells, and by 24 hours they exhibit only a 20% loss in viability (Figure 23B). Similarly, TrkA cells are also resistant to NGF withdrawal-induced apoptosis despite activation of both ceramide pathways by NGF depletion (Figure 23D). In contrast, TrkA cells were somewhat

sensitive to ethanol-induced apoptosis, with nearly 30%-40% dead by 6 hours (Figure 23F).

The degree of response to the specific insults correlated with the amount of observed apoptosis in TrkA cells. As in PC12 cells, the mitochondrial enzyme activity of TrkA cells treated with exogenous ceramide was transiently reduced within the first few hours of treatment. However in contrast to PC12 cells, and beginning at the first hour of treatment, the more resistant TrkA cells exhibit a sharp hyperpolarization as measured by Rh-123 retention and a loss of $\Delta\Psi_m$ as measured by $DiOC_6(3)$ uptake (Figure 23B).

When we measured mitochondrial integrity and function following NGF depletion in TrkA cells, we found a similar but reduced loss of enzyme activity immediately following the insult, with an additional slight loss (<10%) over the remainder of the 24 hour period (Figure 23D). Mitochondrial hyperpolarization was also evident during the first hours of treatment although $\Delta\Psi_m$ as measured by $DiOC_6(3)$ was lost. Furthermore, although both the collapse of $\Delta\Psi_m$ and mitochondrial hyperpolarization were completely ameliorated by the third hour, at this point the mitochondrial depolarization was evident instead.

We found somewhat different kinetics in the integrity and function of mitochondria of TrkA cells treated with ethanol which, as we reported in Chapter II, induces apoptosis mediated by a combination of both ceramide pathways (Figure 23F). In these cells both $\Delta\Psi_m$ and enzyme activity are unchanged until the third hour post treatment, and although $\Delta\Psi_m$ was subsequently reduced by almost 60% at the end of 24 hours, only about 15% of normal enzyme function was lost by this time. Furthermore, despite an early transient increase in mitochondrial polarity, similar to that seen in TrkA

cells depleted of NGF, mitochondrial polarity was reduced to nearly 50% of its original value between the first and third hours and was only somewhat recovered by 24 hours.

These results indicate that mitochondrial enzyme dysfunction was less severe in TrkA cells in correlation with their resistance to apoptosis. Furthermore, MMP was implicated as an early event in NGF withdrawal induced apoptosis in PC12 cells but was a smaller and more transient event in the resistant TrkA cells. TrkA cells also exhibit transient mitochondrial hyperpolarization immediately after induction of apoptosis. These results suggest that resistance to apoptosis induced by exogenous ceramide results from prevention of either early significant losses of enzyme function or loss of mitochondrial polarity. These results further indicate that mitochondrial dysfunction and loss of integrity may play an important early role in the apoptosis of PC12 cells mediated by ceramide synthesis, while the resistance of TrkA cells to such apoptosis might result from protection against such alterations to either mitochondrial structure and function.

d. Exogenous CoQ_{10} can prevent apoptosis.

Since we found both that CoQ_{10} inhibits ceramide synthesis during differentiation and that ceramide synthesis mediates apoptosis induced by specific insults, we tested the ability of CoQ_{10} to render either PC12 or TrkA cells more resistant to apoptosis. We exposed differentiated cells to either NGF depletion, 50 μg/ml CHX, 10 ng/ml TNFα, 10μM ceramide, or 220 mM ethanol in the presence or absence of 100μM CoQ_{10} and used the same assays described in Chapter II to measure the amount and type of cell death. As we reported in Chapter II, PC12 and TrkA cells are differentially sensitive to specific insults. PC12 cells apoptose in response to NGF withdrawal and TNFα, while

TrkA cells do not. Both cell lines are significantly ($p<<0.05$) protected by CoQ_{10} against loss of viability induced by either exogenous ceramide or ethanol (Figure 25 A-B, pp. 162-3). CoQ_{10} also protects PC12 cells against NGF withdrawal or addition of TNFα ($p < 0.05$). Furthermore, although TrkA cells are resistant to apoptosis induced by CHX, PC12 cells are sensitive to CHX, and are not protected by CoQ_{10} (Figure 25A). These results implicate mitochondrial failure in apoptosis and suggest that of all the inducers of apoptosis that we tested, only CHX does not involve a CoQ_{10}/mitochondrial pathway.

e. Alterations in SLs as a result of CoQ_{10}.

As we reported in the previous section, the presence of CoQ_{10} can result in enhanced viability after specific insults. Based on our earlier observations that CoQ_{10} could inhibit ceramide synthesis during differentiation, we hypothesized that the effect of CoQ_{10} on viability results from alterations in cellular SL content. To test this, we examined the ceramide and SM content of neuronal PC12 and TrkA cells that were either depleted of NGF or exposed to 220 mM ethanol in the presence or absence of 100μM CoQ_{10} for 24 hours, as described above in Chapter I and in Materials & Methods. We found that the presence of CoQ_{10} similar to differentiating cells, in neuronal PC12 and TrkA cells exhibit decreased levels of endogenous ceramide (Figure 26 A,C, pp. 164-5) but no change in SM content (Figure 26 B,D). These results suggest that apoptosis induced by either NGF withdrawal or ethanol may be mediated by ceramide via a mitochondrial pathway involving CoQ_{10} or its substrates.

f. Protection by CoQ_{10} correlates with altered mitochondrial function.

To determine how the protection provided by CoQ_{10} against endogenous or exogenous ceramide-mediated apoptosis involves a mitochondrial pathway, we next exposed differentiated PC12 and TrkA cells to either NGF depletion, 220 mM ethanol, or 10 μM exogenous C-8 ceramide for 24 hours in the presence or absence of CoQ_{10}. This time we measured treated cells for all three parameters of mitochondrial function as described above and in Materials & Methods.

We found that in the presence of CoQ_{10} control untreated PC12 cells exhibit an increase in $\Delta\Psi_m$ as measured by Rh-123 and increased enzyme function, but no mitochondrial hyperpolarization as measured by $DiOC_6(3)$ (Figure 27, pp. 166-7). Similarly, cells treated for 24 hours with 10 μM exogenous C-8 ceramide in the presence of CoQ_{10} show no significant mitochondrial hyperpolarization (Figure 27A), but do recover lost $\Delta\Psi_m$ within three hours (compare Figure 24F to 27B) and exhibit improved mitochondrial enzyme activity (Figure 27C). By contrast although we also found that when PC12 cells were depleted of NGF in the presence of CoQ_{10}, they exhibited an early improvement in $\Delta\Psi_m$ (measured by Rh-123), they also revealed an early but transient improvement in enzyme activity, and an improvement in membrane polarity (Figure 27 D-F). In addition, cells treated with ethanol in the presence of CoQ_{10}, display long-term improvements in all three measurements (Figure 27 G-I). These results suggest that CoQ_{10} may have different effects on mitochondrial function and integrity depending on the specific insult and support earlier suggestions that CoQ_{10} acts by either improving or preventing the loss of $\Delta\Psi_m$, and mitochondrial enzyme activity.

It is evident from our experiments that the $DiOC_6(3)$ and Rh-123 assays are measuring slightly different aspects of mitochondrial membrane potential. In fact, in the

presence of CoQ_{10}, control untreated TrkA cells exhibit membrane hyperpolarization as measured by $DiOC_6(3)$ with no corresponding increase in enzyme activity or $\Delta\Psi_m$ as measured by Rh-123 (Figure 28, pp. 168-9). Similarly, when TrkA cells are exposed to either exogenous ceramide (Figure 28A-C), NGF depletion (Figure 28D-F), or ethanol (Figure 28G-I) in the presence of CoQ_{10}, there was no significant alteration either in mitochondrial integrity or enzyme function, despite an early transient hyperpolarization as measured by $DiOC_6(3)$ (compare "Insult alone" to "With CoQ_{10}" in Figure 28 A,D,G).

These results suggest either that the ability of CoQ_{10} to prevent apoptosis induced by exogenous ceramide in TrkA cells was unrelated to mitochondrial function, or that mitochondrial hyperpolarization during the first hour of treatment may be crucial to the induction of apoptosis in this cell line. This suggestion was confirmed with the results from ethanol treated cells which are significantly protected by CoQ_{10} against loss of $\Delta\Psi_m$ during the first hour of treatment. Furthermore, since ceramide synthesis is significantly inhibited by the presence of CoQ_{10}, our results suggest that protection against loss of $\Delta\Psi_m$ results either in or from alterations in ceramide metabolism.

D. DISCUSSION:

Since mitochondria have been implicated as key components of both differentiation and cell death, we investigated alterations in mitochondrial activity following induction of either differentiation or death. We report here that: a) changes in mitochondrial activity are uncoupled and depend on specific stimuli and cell type, b) differentiation and apoptosis are mediated by mitochondria, and c) neurotrophin

expression may prime cells for differentiation or prevent apoptosis by altering mitochondrial activity.

We have found that there are significant increases in both mitochondrial potential and enzyme function during NGF-induced differentiation of PC12 cells, which occurs shortly before neuronal outgrowth is evident and at the same time as cellular ceramide increases. We have also found that the TrkA cells exhibit earlier increases in mitochondrial potential which correlates with both faster neuronal morphogenesis and less ceramide generated. These results suggest that neurotrophin receptors, ceramide and mitochondria interact to prime cells for differentiation. Our investigations led us further to examine the ability of coenzyme Q_{10} (a vital mitochondrial component of the respiratory pathway that has been shown to protect against mitochondrial dysfunction), to affect the differentiation pathway. We found that despite significantly inhibiting the accumulation of cellular ceramide and SM, CoQ_{10} had no effect on the morphology or kinetics of cell differentiation in either cell line. Thus these results confirm that large accumulations of SM or ceramide are not required for differentiation but do not rule out the possibility that small increases in cellular ceramide act as a signal for differentiation.

Mitochondria participate in the control of apoptotic nuclear disintegration (Skulachev '96) and such participation is evident by the reduction of the inner mitochondrial transmembrane potential ($\Delta\Psi_m$) mediated by the opening of mitochondrial permeability transition (MPT) pores prior to apoptosis (Macouillard-Poulletier de Gann et al. '98). Our results suggest that $p140^{trkA}$ overexpression/$p75^{NTR}$ underexpression which we have suggested in Chapter II prevents apoptosis by reducing the amount of ceramide generated in a cell, acts by either delaying or preventing mitochondrial failure. In

general, we report that untreated control TrkA cells exhibit better mitochondrial viability and activity than do PC12 cells in addition to exhibiting protection of mitochondrial viability and activity after exposure to either exogenous ceramide or ethanol. This consequence may explain the resistance of TrkA cells against induction of apoptosis, since apoptosis often starts in the mitochondria, resulting in the release of *cytochrome c* and consequent activation of cysteine-aspartate proteases (caspases) (Zamzani et al. '96). Furthermore, since we have reported in Chapter II that both NGF withdrawal- and ethanol-induced apoptosis are mediated by ceramide, our results suggest that loss of either mitochondrial integrity or function may be part of the apoptotic pathway induced by endogenous ceramide. Thus our results also indicate that the involvement of mitochondria may be common to cell death stimuli that use the ceramide-signaling pathway.

Our results also suggest that neurotrophin receptors may interact with the apoptotic machinery at the level of either ceramide or mitochondria, by inhibiting SM hydrolysis or ceramide synthesis and blocking apoptosis upstream of mitochondrial damage. This effect correlates with the significant depolarization and consequent loss of $\Delta\Psi_m$ exhibited by PC12 cells within the first hour of exposure to either ceramide or ethanol. This loss in membrane potential was not evident in TrkA cells. Furthermore, after 24 hours of NGF withdrawal, PC12 cells also depolarized and exhibited a loss of $\Delta\Psi_m$, which was prevented in TrkA cells deprived of NGF. Thus maintenance of $\Delta\Psi_m$ may be the means by which $p140^{trkA}$ receptor overexpression/$p75^{NTR}$ receptor underexpression elicits its protective effect.

We have previously indicated that PC12 cells which possess both $p140^{trkA}$ and $p75^{NTR}$ receptors are sensitive to inducers of apoptosis, while TrkA cells, which overexpress $p140^{trkA}$ and underexpress $p75^{NTR}$, are insensitive to the same agents. We report here that the mitochondria of PC12 cells depolarize in response to inducers of apoptosis while TrkA cells do not. In fact, the effect of neurotrophin receptors on mitochondrial activation may involve the bcl-2 family of proteins.

Bcl-2 is a 26 kD anti-apoptotic protein which is a product of the *bcl-2* oncogene (de Jong et al. '94; Allsopp et al '93). When bcl-2 is overexpressed it can protect various types of cells from both normal and experimentally induced apoptosis. Bcl-2 has been identified in association with the nuclear envelope, endoplasmic reticulum, and inner mitochondrial membrane, which suggests that it might protect cells by altering mitochondrial function. Although its mode of action is unknown, most researchers believe that it either prevents $\Delta\Psi_m$ depolarization through its interactions with the MPTP, inhibits the release of *cyt c*, or functions as an antioxidant (Marchetti et al. '96; Kluck et al. '97; Armstrong et al. '01). However, Jacobson et al ('93) report that human mutant cell lines that lack mitochondrial DNA (mtDNA), and therefore do not have a functional respiratory chain, or even anucleate cytoplasts ('94) can still be induced to die by apoptosis, and further that they can be protected from apoptosis by the overexpression of bcl-2. This suggests that neither apoptosis nor the protective effect of bcl-2 depends on mitochondrial respiration or the presence of nuclear factors. Nevertheless, ceramide generation is inhibited by the Bcl-2 protein (Yoshimura et al. '98) and the activity of both low and high affinity NGF receptors may be modulated at the level of mitochondria by Bcl-2 (see review by Yuan and Yankner '00). Since Bcl-2 in overexpressing cells is

associated with the nuclear envelope and endoplasmic reticulum, as well as with mitochondria ad ceramide synthase activity is reported in those membranes as well, we therefore suggest that Bc-2 might rather be primarily involved in the inhibition of ceramide neosynthesis and only secondarily involved in a downstream mitochondrial pathway.

We next examined the ability of CoQ_{10} to rescue cells induced to die by various toxicants. Consistent with its ability to reduce the level of ceramide generated by NGF stimulation, simultaneous treatment of cells with CoQ_{10} and toxicants resulted in improved viability in most cases. However CoQ_{10} only protects some cells against some cytotoxic insults. This differential result may imply a selective protective role of CoQ_{10} against specific toxins in specific cell types. Similarly, cell death stimuli do not affect different cell lines equivalently, nor does CoQ_{10} serve as a universal protective agent. PC12 cells are sensitive to most inducers of cell death; they are dependent on the hormone NGF and they die in response to a variety of other cytotoxic stimuli ($p<0.05$). These cells exhibit the most consistent protection afforded by CoQ_{10} after NGF withdrawal, or application of exogenous ceramide, TNFα or ethanol, but are not protected against CHX. Similarly, TrkA cells, which are normally more resistant to a variety of cytotoxic stimuli, are also protected by CoQ_{10} against exogenous ceramide and ethanol. These results suggest that ceramide- mediated apoptosis induced by exogenous ceramide, ethanol, or withdrawal of NGF share a similar pathway that is different from that induced by CHX. Furthermore, CoQ_{10} has a differential ability to suppress apoptosis: cells treated with exogenous ceramide are better protected than are cells treated with ethanol. This may reflect alternative uses for CoQ_{10} within a cell. Although

CoQ_{10} is widely thought of as an antioxidant whose presence is mainly necessary for mitochondrial red-ox activities, it may have additionall functions.

One of those functions may be the mediation of mitochondrial activity. Mitochondrial viability and activity was generally enhanced by CoQ_{10}, and CoQ_{10} protected against loss of mitochondrial viability and activity after exposure to cytotoxins such as ceramide or ethanol. CoQ_{10} could interact with the apoptotic machinery in the mitochondria, preventing the loss of mitochondrial potential by sequestering ROS generated in response to cell death signals and thus blocking cell death. This effect correlates with the significant loss of $\Delta\Psi_m$ exhibited by PC12 cells within the first hour of treatment with either ceramide or ethanol alone. This loss in membrane potential was not evident when cells were treated in the presence of CoQ_{10}. Furthermore, after 24 hours of NGF withdrawal, PC12 cells also exhibited a loss in $\Delta\Psi_m$, which was prevented by CoQ_{10}. Thus protection against loss of $\Delta\Psi_m$ appears to be the means by which CoQ_{10} elicits its protective effect.

Our results also indicate that regardless of significant changes in $\Delta\Psi_m$, CoQ_{10} does not necessarily work at the level of mitochondrial dlehydrogenase activity. Although we report that CoQ_{10} significantly increased $\Delta\Psi_m$ in cells exposed to short term ceramide treatment and long term NGF withdrawal, its presence had no significant effect on mitochondrial dehydrogenase. Thus, mitochondrial dehydrogenases are retained and remain capable of accepting electrons from MTT after depolarization. This implies a substantial limit to the use of MTT as an early assay for cell death. Other investigators have shown that ceramide causes a disruption of the inner mitochondrial transmembrane potential that precedes the nuclear signs of apoptosis in various cell types (Arora et al.

'97; France-Lanord et al. '97). Ceramide may evoke increased ROS generation (Quillet-Mary et al. '97), and oxidative stress may cause $\Delta\Psi_m$ (Satoh et al. '97). Thus, CoQ_{10} may in this instance act as an antioxidant.

However, the protective role of CoQ_{10} in ethanol induced toxicity may also result as an indirect effect and in contrast to its effect on cells depleted of NGF. It is only following ethanol treatment that PC12 cells exhibit rapid and sustained loss of cell viability, $\Delta\Psi_m$ and mitochondrial enzyme activity, which are ameliorated in the presence of CoQ_{10}. Previous investigators have indicated that CoQ_{10} protects against glutamate neurotoxicity *in vivo* (Favit et al. '92) and that N-methyl D-aspartate (NMDA) is a key mediator of glutamate induced cytotoxicity (Balazs et al. '88). Ethanol also inhibits NMDA receptors (Miller '96). Taking all these observations, we may postulate that any benefit against ethanol toxicity gained by treatment with CoQ_{10} may result from protection of mitochondrial enzyme activity.

One of the greatest effects of CoQ_{10} on PC12 cells following NGF withdrawal is an increase in $\Delta\Psi_m$, evident 24 hours after treatment. Differentiating neurons depend on NGF (Batistatou & Greene '91); withdrawal of it activates the c-Jun NH2 terminal kinase (JNK) group of MAP kinases (Xia et al. '96) and the endogenous ceramide signaling pathway (Dobrowsky et al. '94). Since the ceramide signaling pathway is activated 18-24 hours after NGF withdrawal, the late depolarization of the mitochondria may directly result from ceramide generation, as we see after one hour of exogenous ceramide. These results further suggest that the protection afforded by CoQ_{10} against apoptosis induced either by exogenous ceramide or by ceramide synthesis mediated by NGF depletion may reside in its ability to prevent mitochondrial depolarization and loss of enzyme activity

while its ability to protect against SM hydrolysis-mediated apoptosis following ethanol treatment may result from an improvement in $\Delta\Psi_m$. Finally, these results suggest that the prevention of MMP is the most common effect of either TrkA receptor over-expression or CoQ_{10} at the cellular level and thus may be the most important factor in preventing cell death by these inducers.

Finally, yet another effect of CoQ_{10} on both differentiation and survival is an overall reduction in the synthesis of ceramide and accumulation of SM, similar to that seen in TrkA overexpressing cells. As in TrkA cells, this loss does not prevent differentiation, but does correlate with a protection against specific stimuli of apoptosis and with a general enhancement of mitochondrial integrity and viability. Furthermore when TrkA cells are exposed to CoQ_{10}, they exhibit an even greater reduction in their level of ceramide, greater protection against stimuli of apoptosis and better enhancement of mitochondrial integrity and function than in control untreated TrkA cells or PC12 cells. This enhancement suggests the existence of a feedback between the mitochondria and cellular ceramide, whereby TrkA receptors and specific components of the mitochondria might inhibit the synthesis of ceramide and increases in cellular ceramide might result in mitochondrial failure and apoptosis.

CONCLUSION

Apoptosis and differentiation can both be divided into three distinct phases, induction, activation and acquisition of characteristic biochemical and morphological features. Over the last few years, ceramide has emerged as a controversial second messenger that is involved in the induction of diverse signaling leading to differentiation, mitogenesis and apoptosis. Furthermore, since two distinct pathways can generate ceramide (SM hydrolysis or ceramide neosynthesis), its origin is speculated to be vital to its downstream signaling activity. At the same time, the mitochondrion has emerged as an important mediator of the apoptotic signal and has been speculated to be the "central executioner" of apoptosis. In this study we have investigated the modulation of both ceramide and mitochondrial function during the induction of differentiation and cell death in the rat pheochromocytoma (PC12) and TrkA (Trk 6-24 PC12) cell lines, with specific reference to the relationship between neurotrophin receptors and the origin of cellular ceramide. The TrkA cell line was generated from PC12 cells which are a heterogeneous population of adherent cells that can be induced to differentiate to a neuronal phenotype by the addition of NGF. TrkA cells have been stably transfected with a 20 fold overexpression of the $p140^{trkA}$ neurotrophin receptor and they differentiate in response to NGF with both distinct morphology and faster kinetics.

We initiated our studies by asking if and how ceramide is generated in response to NGF stimulation and whether the expression of NGF receptors affected either the cell's response to NGF or its ability to generate ceramide. We showed that the differentiation of PC12 cells in response to NGF activates both ceramide pathways but requires only SM hydrolysis. Over-expression of the high affinity TrkA-NGF receptor resulted in a cell

that differentiated but generated much less ceramide than the parental cells and which moreover required the use of both ceramide pathways to do so. This suggested that the two pathways can substitute for each other and that only minimal amounts of ceramide are required to initiate differentiation. Furthermore, we showed that over-expression of TrkA receptors could alter the relative amount of ceramide produced by either pathway in PC12 cells which correlated with both alterations in cellular morphology and activity.

However, PC12 cells depleted of NGF died by apoptosis mediated by neosynthetic ceramide alone and not SM hydrolysis, which suggests that these two pathways are specific to downstream events in the PC12 cell line. This finding is in general agreement with a reported finding that ceramide generated by distinctly different mechanisms mediates induction of either apoptosis or differentiation by retinoic acid (Herget et al. '00). Nevertheless, we have also found that TrkA cells respond to NGF withdrawal or other cytotoxic stimuli in the same way they respond to NGF stimulation, namely by activation of both ceramide pathways, which again suggested that these two pathways may substitute for one another. Moreover although ceramide neosynthesis is reported to be responsible for apoptosis induced by a variety of cytotoxic agents, we have found its activity to be both cell and inducer specific. Thus a contradiction exists in that when the overall level of cellular ceramide in a cell is low as in TrkA cells, apoptosis appears to be inhibited and the cells are resistant to a variety of insults, which suggests that an increase in intracellular ceramide is required for apoptosis in this system. Despite this, our data clearly confirm that of Karasavvas and Zakeri ('99) which indicate that increases in intracellular ceramide alone are not sufficient to kill cells. We therefore postulate that where ceramide is generated in a cell is as important as the total amount

available, and suggest that downstream targets such as mitochondria may mediate the ceramide signal.

To establish the possible role of mitochondria in ceramide-mediated differentiation and/or apoptosis, we examined the relationship of ceramide-induced and ceramide-mediated apoptosis to mitochondrial integrity and function. Our results suggest that the initiation of differentiation, which we report is accompanied by activation of both pathways, but dependent only on SM hydrolysis in PC12 cells, is also accompanied by increased mitochondrial energization and increased enzyme activity. Furthermore, the progression of differentiation in TrkA cells, which we report is mediated by decreased activation of both ceramide pathways is accompanied only by increased mitochondrial energization and not by increased enzyme activity. Thus, just as TrkA cells are able to complete differentiation in the presence of a very limited amount of ceramide, they are also able to differentiate without increases in mitochondrial enzyme activity. These results suggest that the differences in ceramide metabolism observed in TrkA cells may be reflective of differences in mitochondrial status.

Our results also suggest that the loss of mitochondrial integrity may be part of the apoptotic pathway induced by ceramide. As we demonstrate, apoptosis induced or mediated by ceramide exhibit mitochondrial depolarization within hours of treatment, while TrkA receptors appear to interact with the apoptotic machinery upstream of the mitochondria, blocking apoptosis by preventing mitochondrial depolarization as well as by preventing increases in intracellular ceramide. This finding is intriguing because we report that the antioxidant and mitochondrial coenzyme CoQ_{10} interacts with the apoptotic machinery both by inhibiting neosynthesis of ceramide and by preventing loss

of mitochondrial integrity and function. Our results therefore suggest that at the cellular level the prevention of depolarization is the most common effect of either TrkA receptor ove-expression or CoQ_{10} and is perhaps the more important to preventing apoptosis.

Recent advances in our understanding of apoptosis have fueled debates concerning the relative contributions of caspases, mitochondria, and ceramide. Some scientists conceive of mitochondrial membrane permeability as a decisive step in the apoptotic cascade, while others insist on the primacy of downstream caspases and nucleases or the relative importance of ceramide signaling. For the most part this debate may be futile due to multiple crosstalk and leakiness between these pathways. However, we present here in summary, the following model of neurotrophin-regulated 2nd messenger signaling which is consistent with our data bearing in mind that in our model system PC12 cells possess both $p140^{trkA}$ and $p75^{NTR}$ receptors and respond to NGF with aSMase-mediated differentiation, while TrkA cells possess much more $p140^{trkA}$ and much less $p75^{NTR}$ and respond to NGF with differentiation mediated by both ceramide pathways.

In the model that we can construct based on the research reported here (Figure 29, pp. 170-1), ligation of NGF to Trk receptors would trigger the activation of Ras, and Raf-1, which in turn bind Bcl-2 on the mitochondrial membrane (or maybe even on endoplasmic reticulum or nuclear membrane), both inhibiting the mitochondrial death promoter bax and ceramide synthase (which has been reported in endoplasmic reticulum and nuclear membrane as well). Activated Ras would also recruits PI(3)K, catalyze the formation of PIP2, and regulate the localization and activation of Akt(protein kinase B/PKB) which in turn would support the neuronal survival (Virdee et al. '99) by

sequestering in the cytoplasm a phosphorylated FKHRL1, which would prevent the transcription of the FasL gene, and consequent SM hydrolysis thereby also promoting survival over apoptosis (Brunet et al. '99). At the same time, the MAPK pathway would have been activated by Raf-1 thus resulting in differentiation. In this manner cell survival may be modulated by the expression of neurotrophin receptors on a cell surface through the differential activation of Bcl-2, the Akt/PKB pathway, and ceramide generation.

However, we also report that neuronal PC12 cells are sensitive to NGF depletion or other inducers of apoptosis and respond with *de novo* ceramide synthesis and apoptosis. We may speculate that in the absence of neurotrophin or in the presence of specific inducers, Bcl-2 is not bound and both ceramide neosynthesis and bax are activated, at the same time that FasL is transcribed. In fact as we have noted previously both we and others have observed such transcription in PC12 cells in response to inducers of apoptosis. Since FasL activates SM hydrolysis, ceramide generation increases in this cell by both pathways, and the MPT is induced by increased levels of ceramide resulting in lost mitochondrial potential and enzyme activity, release of cyt c and activation of apoptotic caspases.

The response of TrkA cells to both NGF and inducers of apoptosis fit similarly into this model. Since TrkA cells possess so many more $p140^{trkA}$ than do PC12 cells, homodimer formation and consequent autophosphorylation (Hempstead et al. '92) results in those pathways being selectively activated and significantly greater activation of both Ras, and Raf-1. More Raf-1 would in turn bind more Bcl-2 on the cytosolic membranes, thus effecting a greater inhibition of bax as well as reduced amounts of ceramide

neosynthesis in addition to faster activation of the Raf-1/MAPK pathway with the end results of more rapid differentiation. Furthermore, since bax induces the MPT, $\Delta\Psi_m$ depolarization, and release of *cyt c* would be expected to be inhibited in TrkA cells, and we do indeed find that TrkA cells exhibit better mitochondrial energization than PC12 cells.

Finally, we have also reported that TrkA cells are insensitive to NGF depletion and other agents which induce apoptosis and do not transcribe FasL in response to NGF depletion (unpublished observations) and we suggest that these characteristics as well result from the overexpression of $p140^{trkA}$, since normally activated Ras recruits PI(3)K, catalyzes the formation of PIP2, and regulates the localization and activation of Akt/PKB which in turn sequesters FKHRL1 and prevents the transcription of FasL (Brunet et al. '99). Alternatively or in addition to the effect caused by the overexpression of $p140^{trkA}$, the underexpression of $p75^{NTR}$ by TrkA cells may be responsible for some of the characteristics of the TrkA phenotype. $P75^{NTR}$ expression has been reported to induce the activation of CAPK, NF-κB, JNK, and SMase and thereby generate a constitutive death signal which is active only in the absence of TrkA receptors. Since this neurotrophin receptor is underexpressed in the TrkA cell, its death signal would therefore be absent, less SM would be hydrolyzed and CAPK (kinase suppressor of Ras), NF-κB and JNK activity may not be activated, resulting in a cell which is resistant to a variety of inducers of apoptosis.

From these studies many important questions arise and of them, one of the most important is whether ceramide signaling is upstream or downstream of the mitochondria. Known signal transduction pathways indicate that the mitochondria is well downstream

of both $p75^{NTR}$- and TrkA-mediated ceramide signaling (see Figure 7). In fact, as we demonstrate, mitochondrial and ceramide pathways intersect upstream within hours after induction of apoptosis and continue for at least up to 24 hours afterwards. Furthermore, our studies suggest that the mitochondria is upstream of ceramide synthesis during differentiation as well. This finding is intriguing because ceramide is mostly associated with early signaling events in apoptosis. It is possible that ceramide neosynthesis is downstream of the mitochondria, and associated with the execution phases of differentiation or apoptosis, while SM hydrolysis is upstream of the mitochondria, and associated with the signaling phases of either differentiation or apoptosis. Our studies clearly show the need for a careful analysis to elucidate the relationship between ceramide signaling and mitochondria and thus better understand the role of ceramide in the mediation of differentiation and death.

II. Figures and Figure Legends

Figure 1. The Fas signaling pathway. As discussed in the text, Fas signaling is triggered by receptor trimerization induced upon contact with membrane bound FasL. Subsequent recruitment of FADD and creation of the DISC results in activation of downstream caspases -8 and -3. Furthermore, activated Fas can activate either DAXX or FAN which then, either independently or through NSMase activity, activate DNA destruction through the JNK pathway.

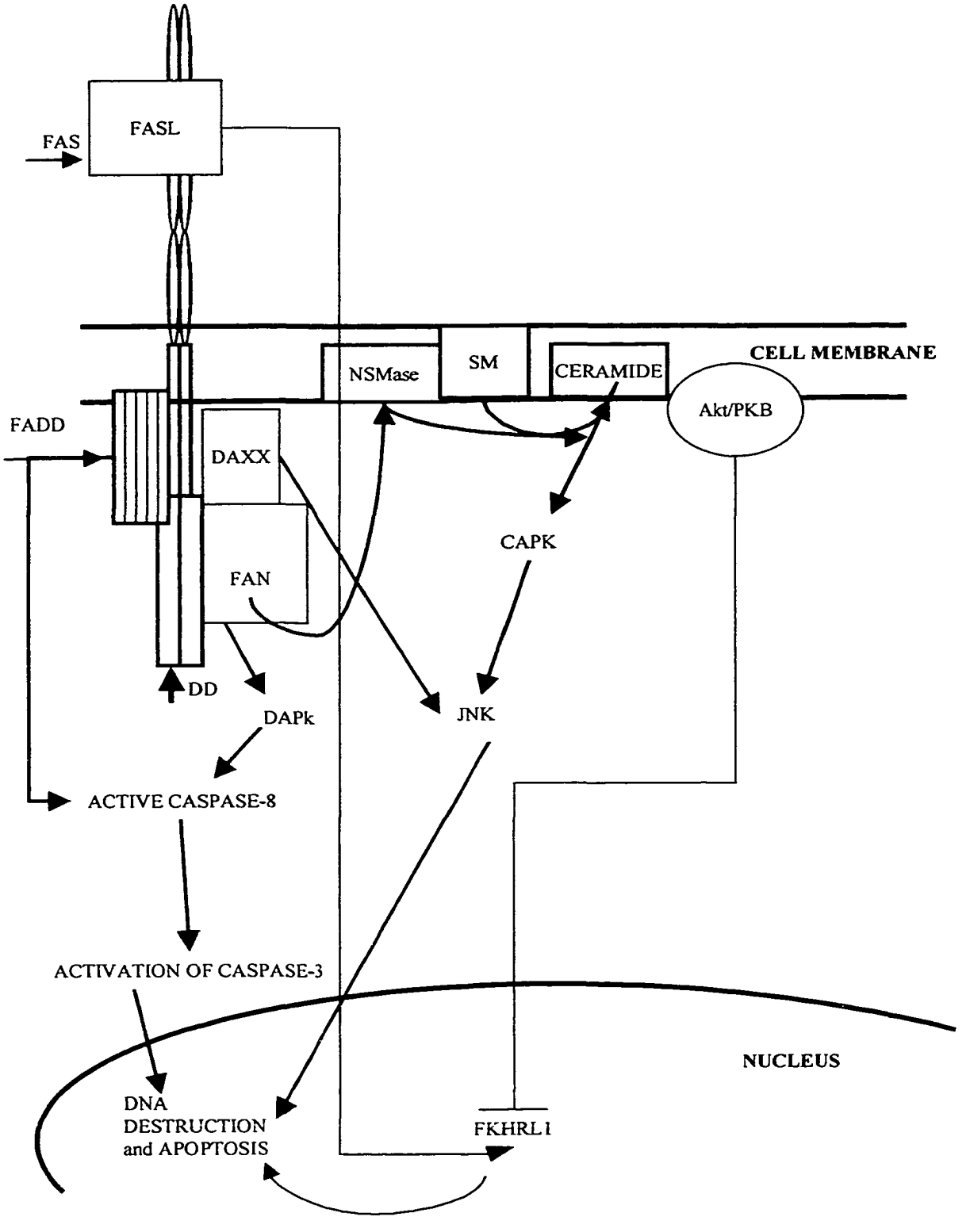


Figure 2. The SAPK1/JNK and SAPK2/p38 signaling pathways. As discussed in the text, the SAPK1/JNK and SAPK2/p38 signaling pathways which induce apoptosis can be activated by (A) ligation of death receptors on the plasma membrane by specific soluble "death" signals; (B) nSMase mediated ceramide generation; or (C) increased cellular reactive oxygen species (ROS) generated by the mitochondria which might be mediated by ceramide neosynthesis.

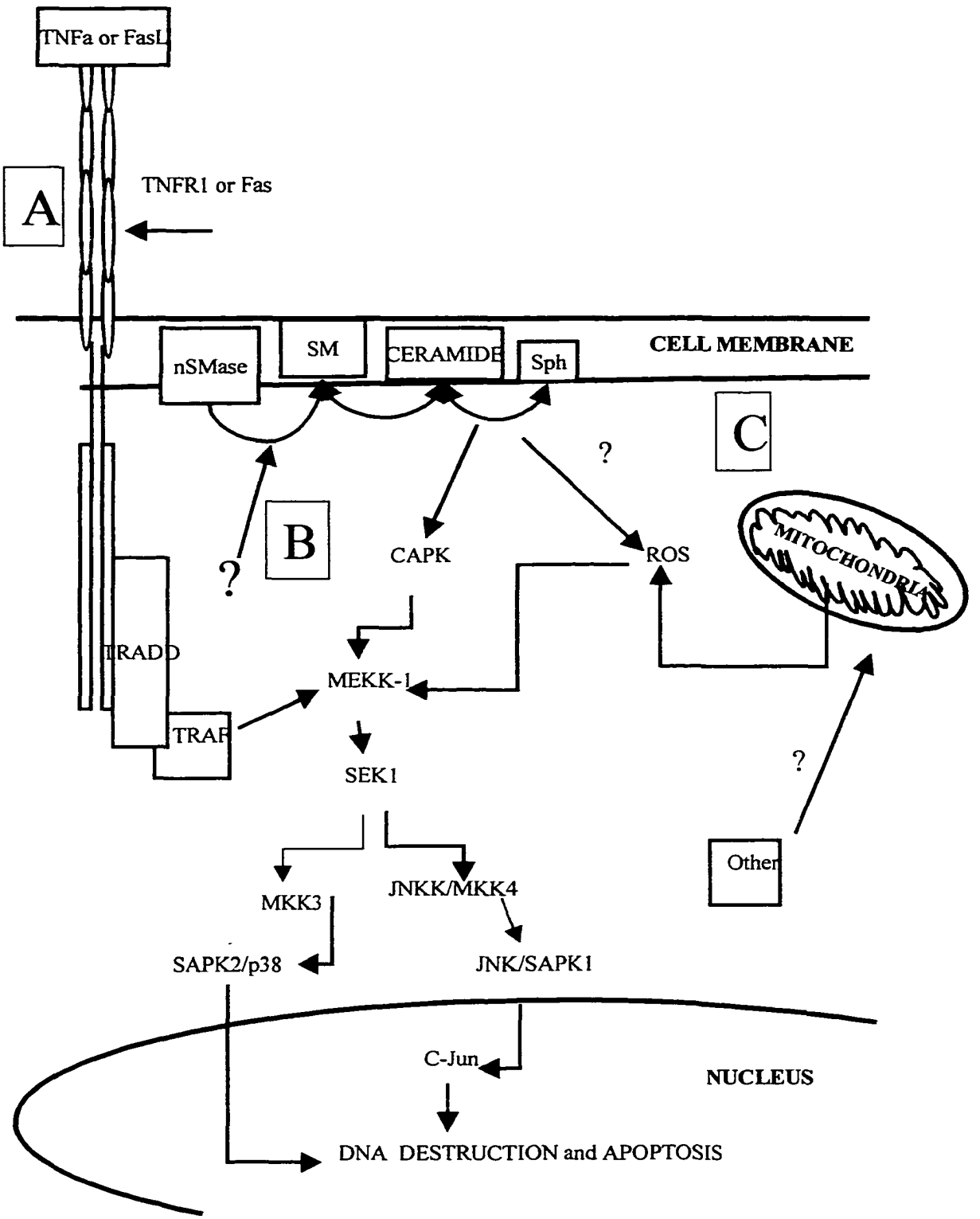


Figure 3. SAPK and MAPK signaling pathways. As discussed in the text, the SAPK pathway mediates apoptosis and is inhibited by the MAPK pathway which mediated differentiation and prevents DNA fragmentation and apoptosis. The MAPK pathway can be initiated by the presence of the ceramide derivative sphingosine (Sph), which can be triggered by the presence of PKC, while the SAPK pathway is activated through nSMase mediated ceramide generation, which is also known to inhibit PKC activation.

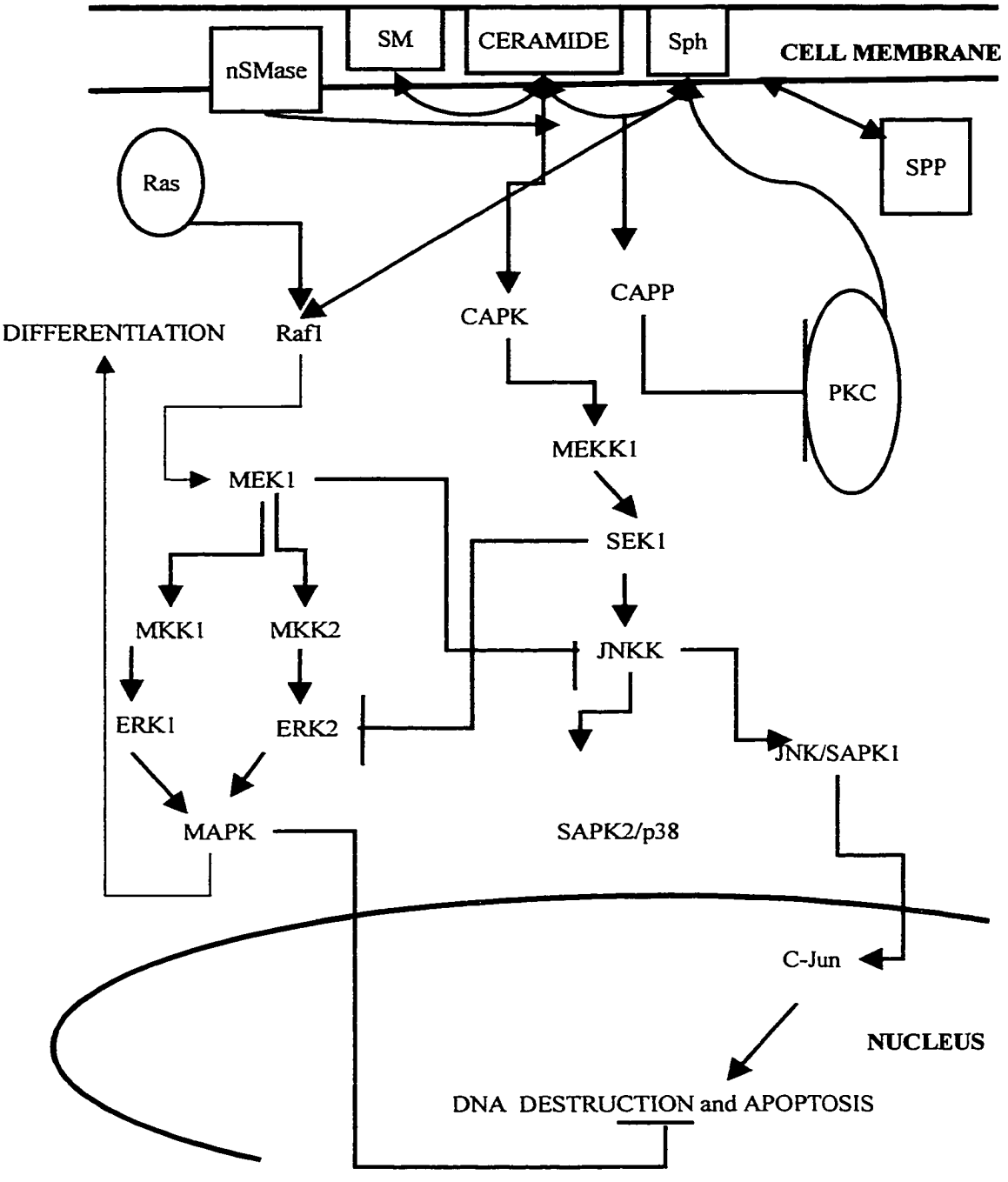


Figure 4. Mitochondrial activation during apoptosis. Insults which can induce apoptosis include a variety of compounds and events as discussed in the text. Many death signals ultimately converge on the mitochondria and are mediated by the Bcl-2 protein and other members of its family, such as bax. Bcl-2 normally inhibits both bax and generation of reactive oxygen species (ROS; peach ovals) by the cell. Activation of bax results in opening of the mitochondria permeability transition (MPT) pores and release of cytochrome c (green spheres) into the cytosol from the inner mitochondrial matrix. Once in the cytosol, cytochrome c binds to Apaf-1 and with caspase-9 forms the apoptosome which activates downstream caspases and DNA fragmentation. Both ROS and apoptosis inducing factor (AIF; pink spheres) are also released from injured mitochondria and they in turn can trigger DNA fragmentation independently of the apoptosome.

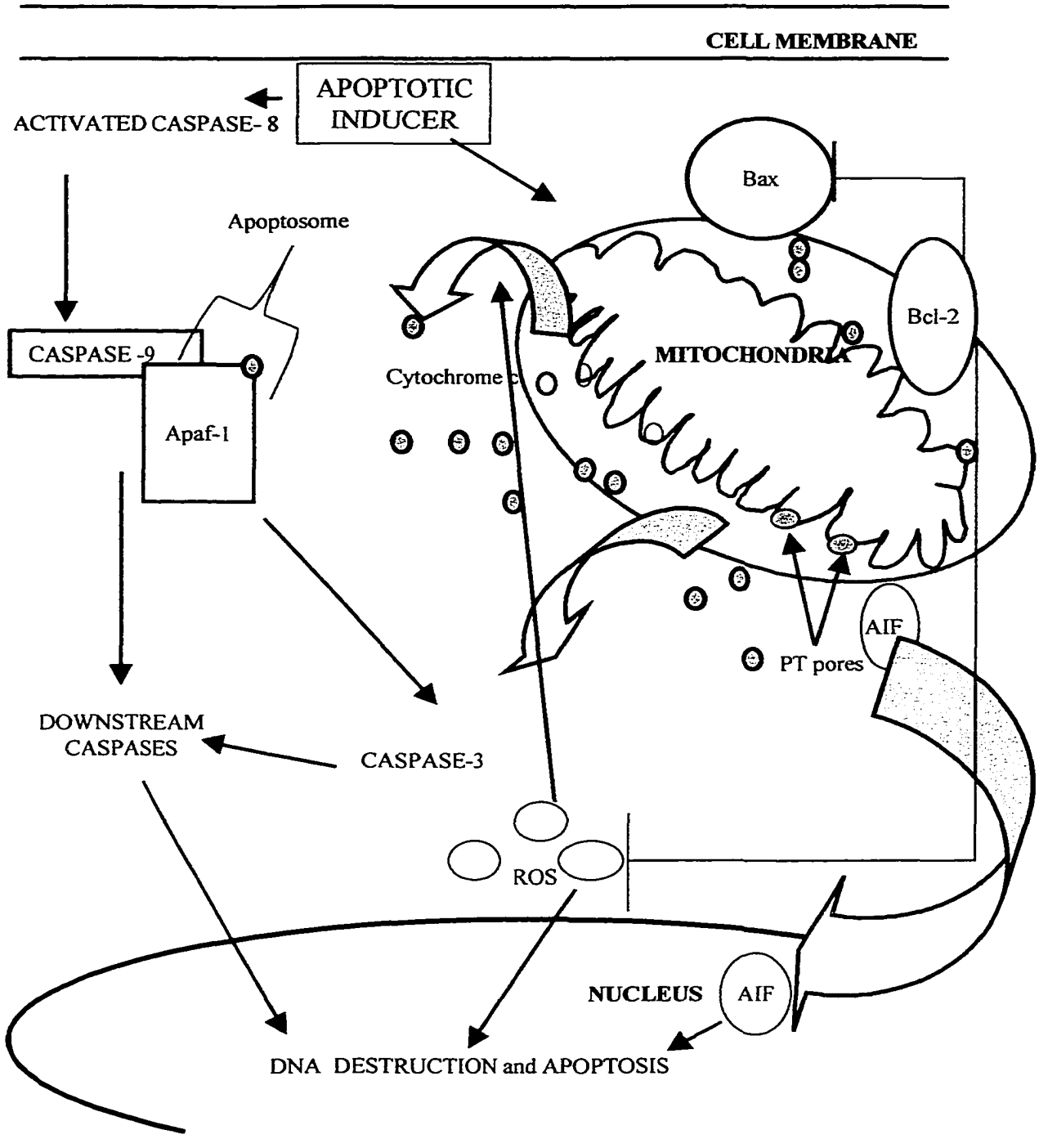


Figure 5. The biosynthesis and metabolism of ceramide. Ceramide is both an end product and penultimate precursor for various sphingolipids, such as sphingomyelin which is synthesized in the plasma membrane and glycosphingolipids. The enzymes involved in ceramide metabolism are shown in italics, compounds found or synthesized in the endoplasmic reticulum are in boldface type those found or synthesized in the Golgi apparatus are in normal type. The inhibitors used in our studies are shown in squares. (Adapted from Schwarz et al 1995).

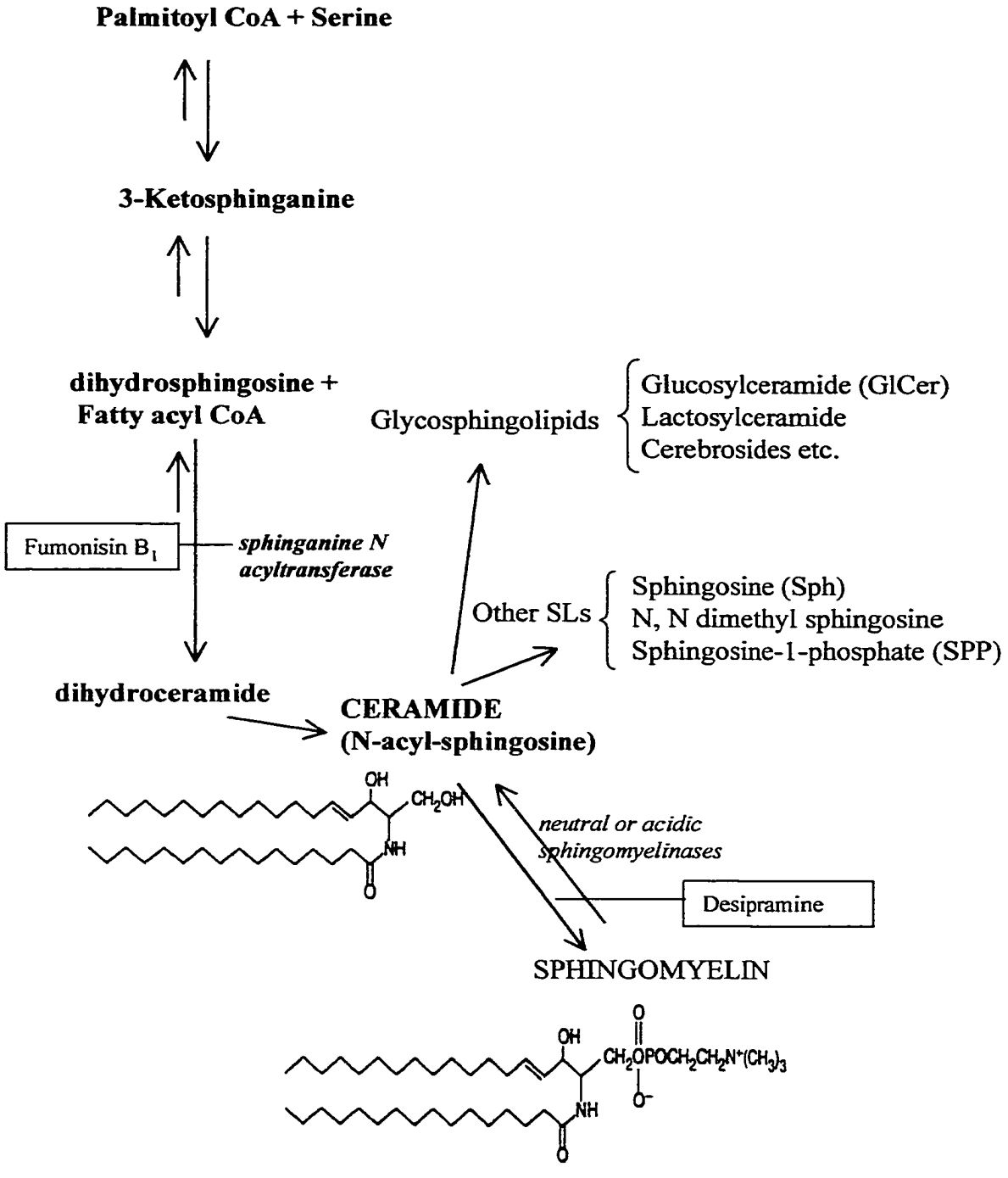


Figure 6. Known schema of the Nerve growth factor (NGF) receptor signaling pathways as described in the text. NGF can be bound by its high affinity ($p140^{NTR}$), or low affinity ($p75^{NTR}$) receptor, which also binds BDNF. NGF binding to the TrkA receptor, initiates receptor auto-phosphorylation, docking of the adaptor proteins Shc and activation of both $p21^{ras}$ (Ras) and PI(3)K, which catalyze the activation of Raf-1, downstream MAP kinase cascade, and suppression of JNK and the formation of PIP2 which regulates Akt (PKB) whose absence results in transcription of FasL Activated Raf-1 also binds Bcl-2 on the mitochondrial membrane, which inhibits pro-apoptotic bax. NGF also induces a 4 fold increase in SM accumulation which may mediated by ceramide synthase (csase) and activates SPP formation. $p75^{NTR}$ induces activation of NF-κB, JNK and the ceramide activated protein kinase (CAPK) which in turn either activate downstream caspases, and DNA fragmentation, or suppress Ras. $p75^{NTR}$ signaling is regulated by the mitochondrial Bcl-2 family and may involve both SM hydrolysis and ceramide synthesis.

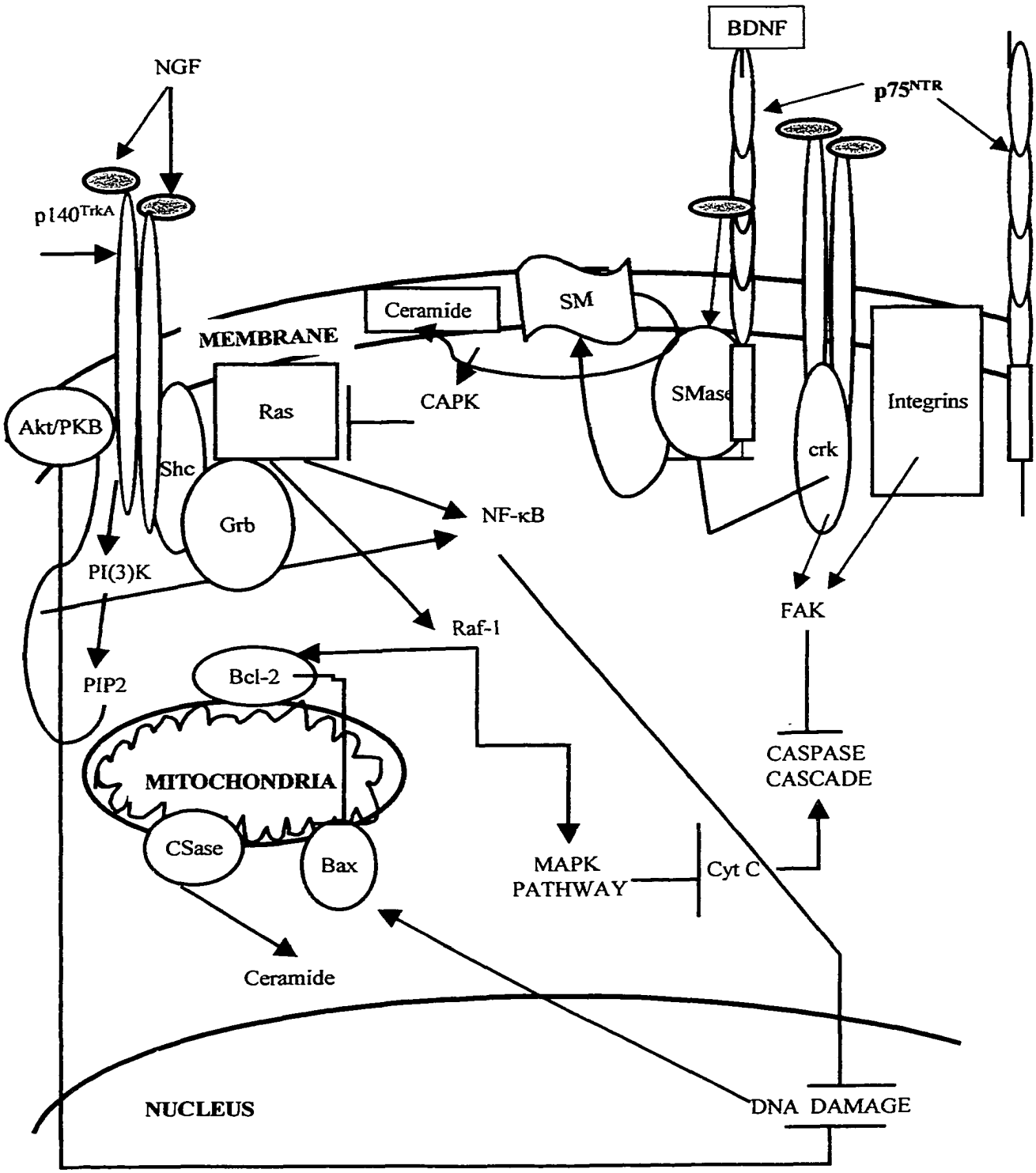
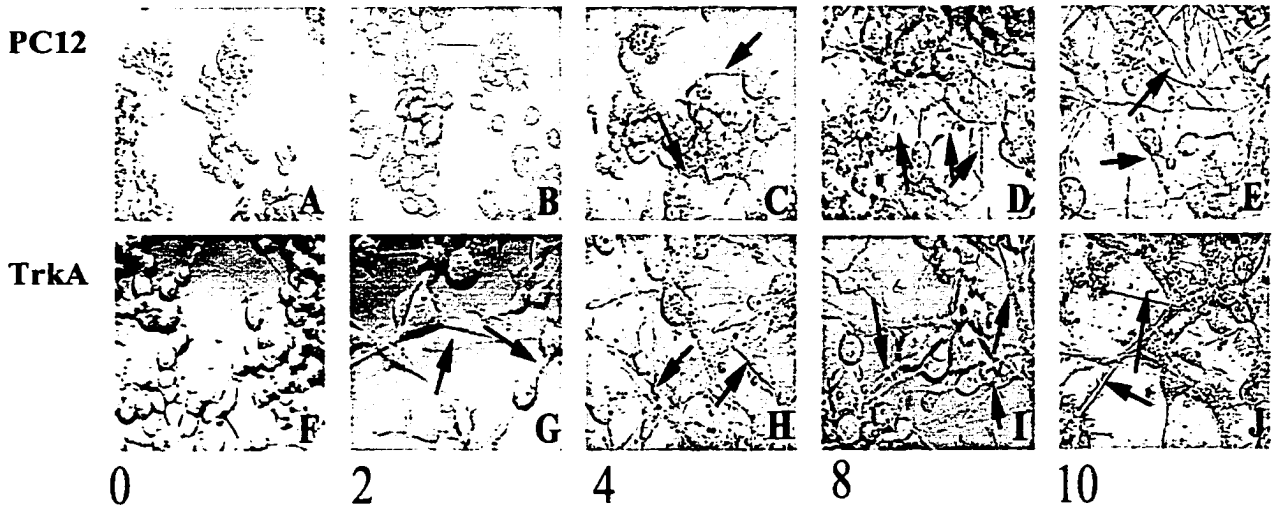
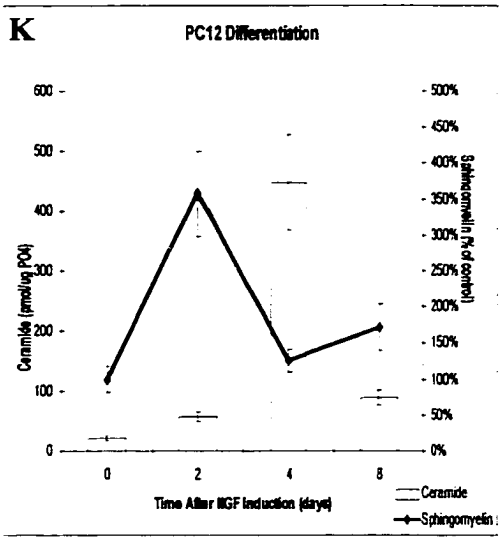


Figure 7. The effect of NGF on differentiation in PC12 and TrkA cells. PC12 (A-E) and TrkA (F-J) cells were induced to differentiate with 0.05ng/ml NGF. Neuronal morphology (shrunken soma and long extended neurites that synapse on other cells) is indicated with arrows (400x magnification). PC12 (K) and TrkA (L) cells were treated as above, lipids were extracted and ceramide and SM levels quantified by the DG kinase assay and SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three experiments; bars, SEM.





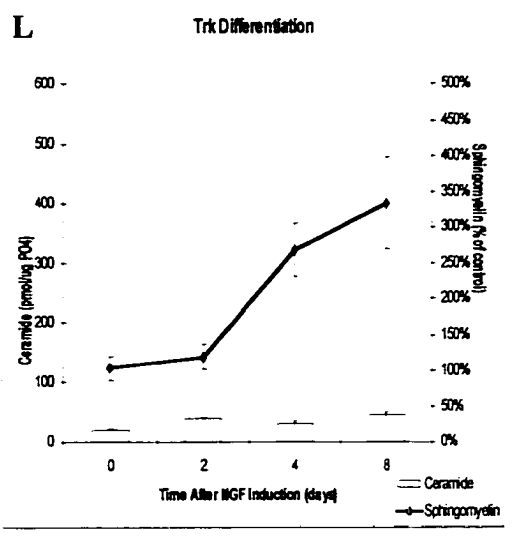


Figure 8. Expression of NGF receptors in naïve and terminally differentiated cells. PC12 (lanes 1-2) and TrkA cells (lanes 3-4) were exposed to 0.05 ng/ml NGF, and cultured until a differentiated phenotype was evident as indicted in text. Whole cell lysates were immunoblotted with antibody recognizing either $p140^{TrkA}$ receptor (A) or $p75^{NTR}$ receptor (B).

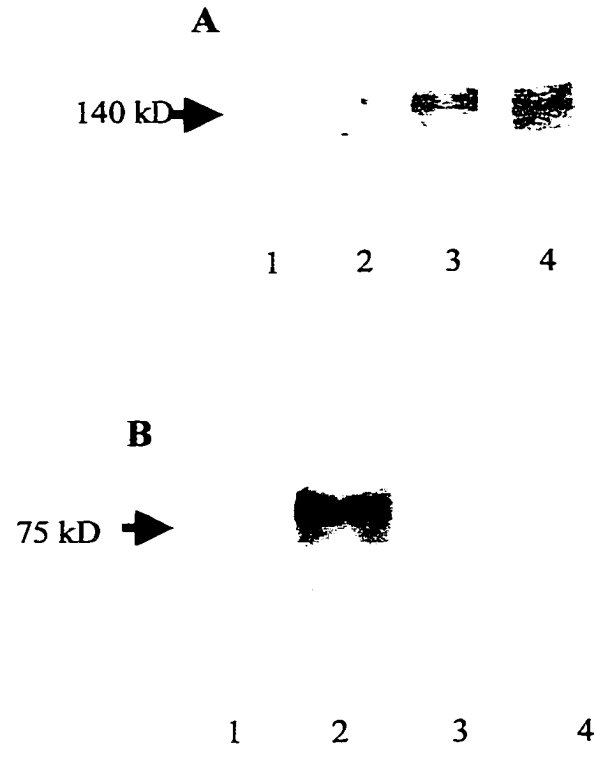
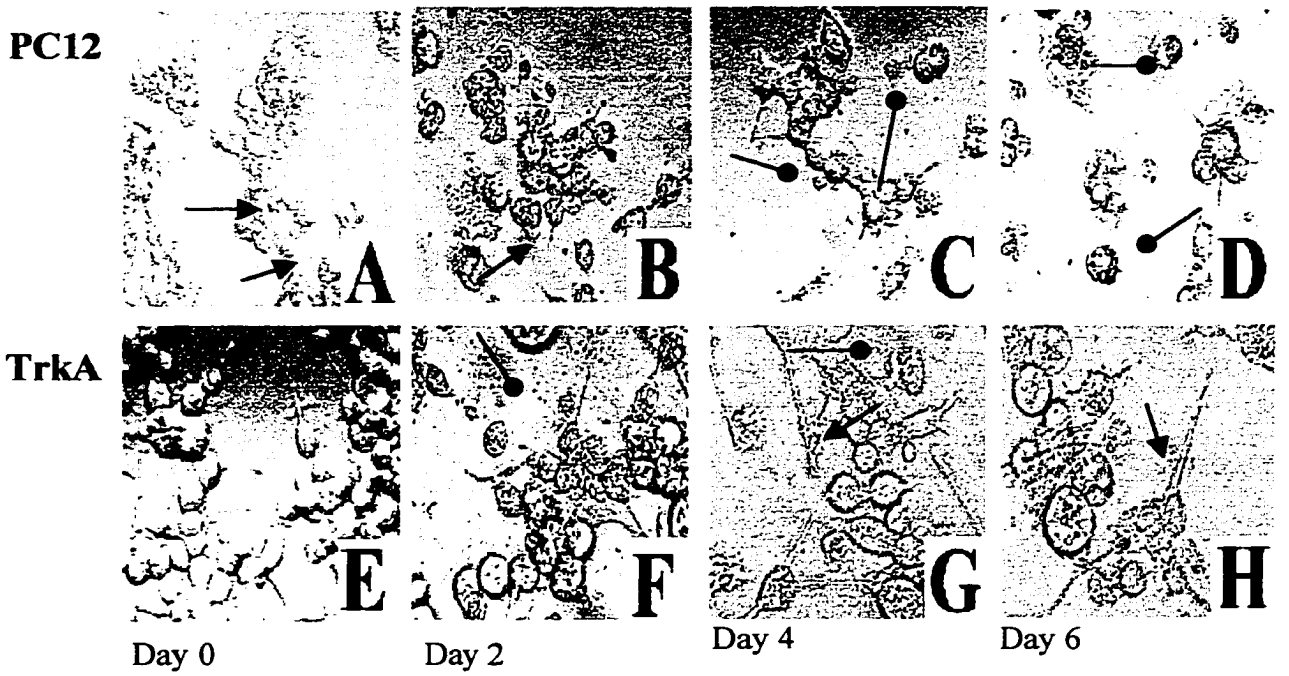
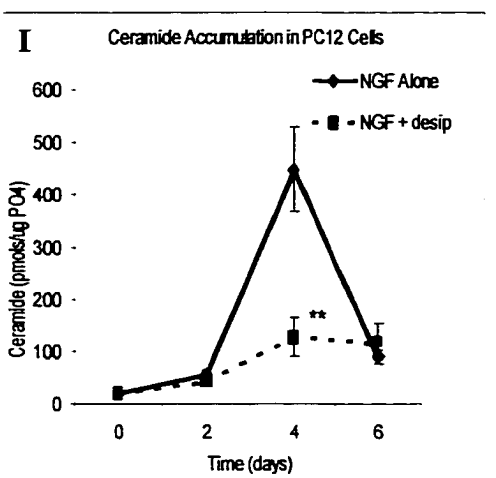
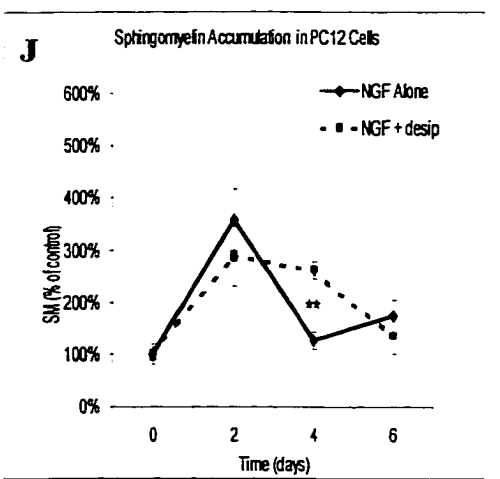


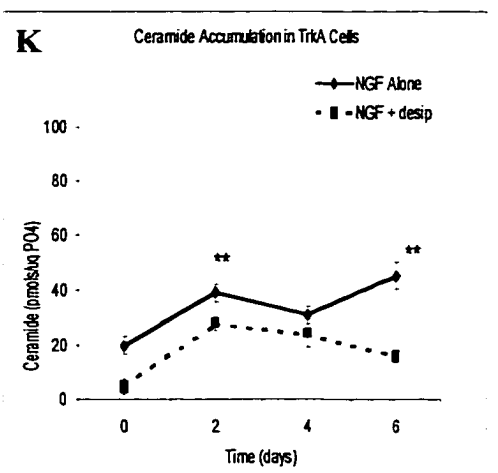
Figure 9. The effect of desipramine on differentiation of PC12 and TrkA cells. A-D, neuronal morphology of PC12 cells stimulated with 0.05 ng/ml NGF in the presence of 15μM desipramine and collected every 2 days for evaluation by light microscopy; arrows, neuritic extensions; clubbed arrows, absent extensions (400X magnification). E-H, neuronal morphology of TrkA cells stimulated with 0.05 ng/ml NGF in the presence of 15μM desipramine and collected every 2 days for evaluation by light microscopy; arrows, neuritic extensions; clubbed arrows, absent extensions (400X magnification).

I, Time course for ceramide generation in PC12 cells treated as above. Lipids were extracted and ceramide levels quantified by the DG kinase assay as described in Materials and Methods. J, Time course for SM generation in PC12 cells treated as above. Lipids were extracted and SM levels quantified by the SM mass assay as described in Materials and Methods. K, Time course for ceramide generation in TrkA cells treated as above. Lipids were extracted and ceramide levels quantified by the DG kinase assay as described in Materials and Methods. L, Time course for SM generation in TrkA cells treated as above. Lipids were extracted and SM levels quantified by the SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three experiments; bars, SEM; ** $p \leq 0.01$.









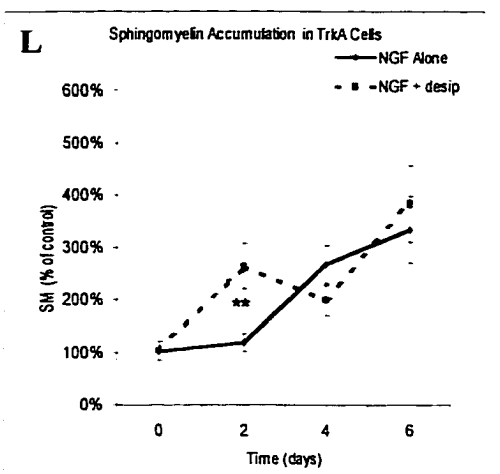
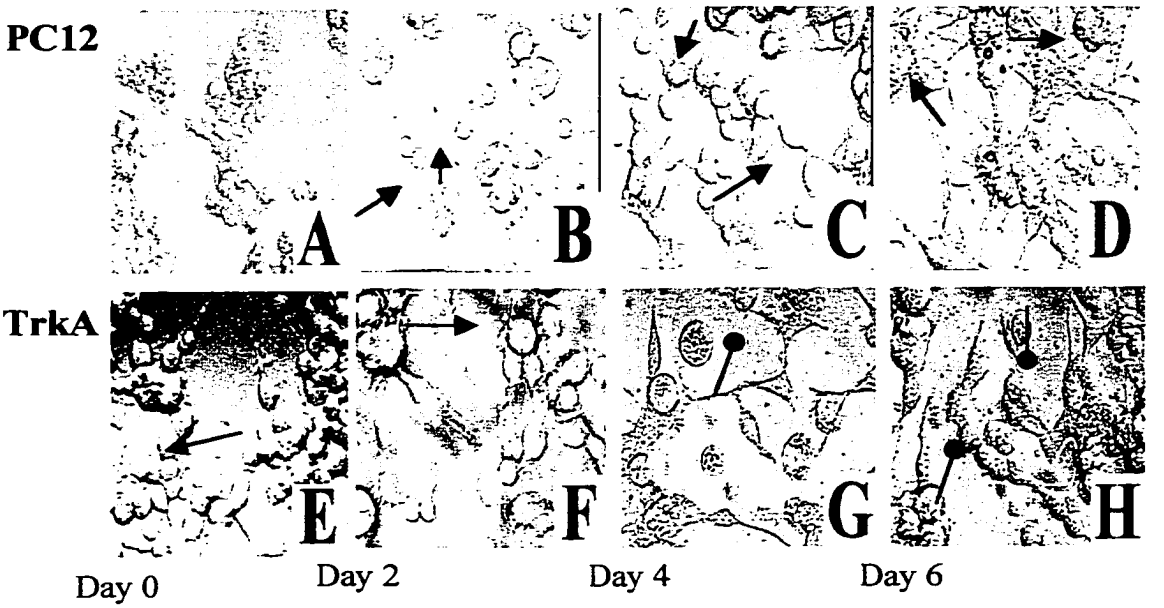
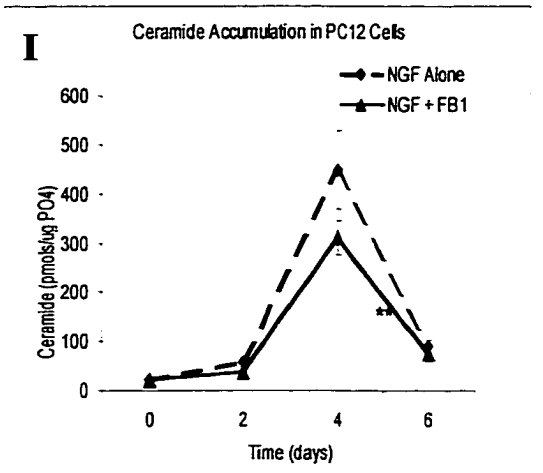
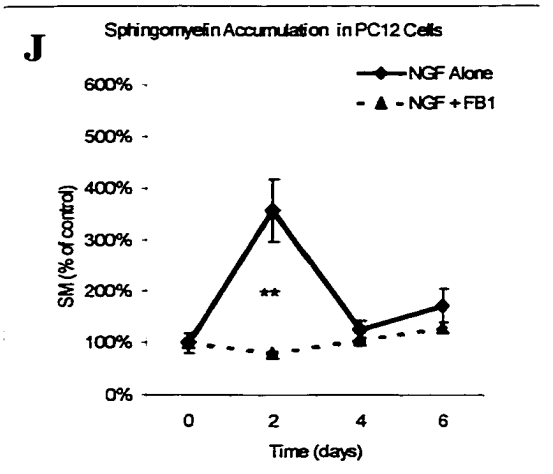
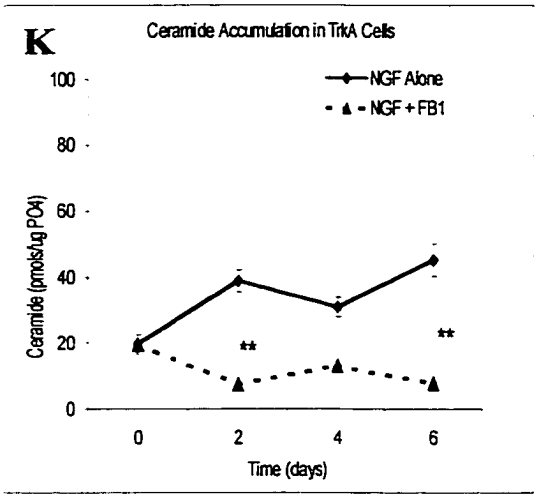


Figure 10. The effect of fumonisin B_1 on differentiation of PC12 and TrkA cells. A-D, neuronal morphology of PC12 cells stimulated with 0.05 ng/ml NGF in the presence of 25μM FB_1 and collected every 2 days for evaluation by light microscopy; arrows, neuritic extensions; clubbed arrows, absent extensions (400X magnification). E-H, neuronal morphology of TrkA cells stimulated with 0.05 ng/ml NGF in the presence of 25μM FB_1 and collected every 2 days for evaluation by light microscopy; arrows, neuritic extensions; clubbed arrows, absent extensions (400X magnification). I, Time course for ceramide generation in PC12 cells treated as above. Lipids were extracted and ceramide levels quantified by the DG kinase assay as described in Materials and Methods. J, Time course for SM generation in PC12 cells treated as above. Lipids were extracted and SM levels quantified by the SM mass assay as described in Materials and Methods. K, Time course for ceramide generation in TrkA cells treated as above. Lipids were extracted and ceramide levels quantified by the DG kinase assay as described in Materials and Methods. L, Time course for SM generation in TrkA cells treated as above. Lipids were extracted and SM levels quantified by the SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three experiments; bars, SEM; ** $p \leq 0.01$; * $p < 0.05$.









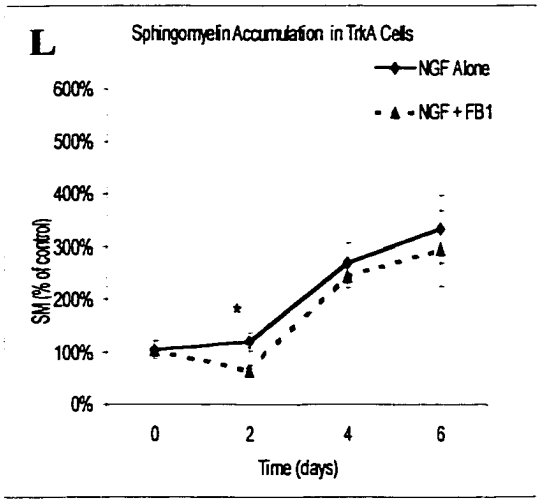
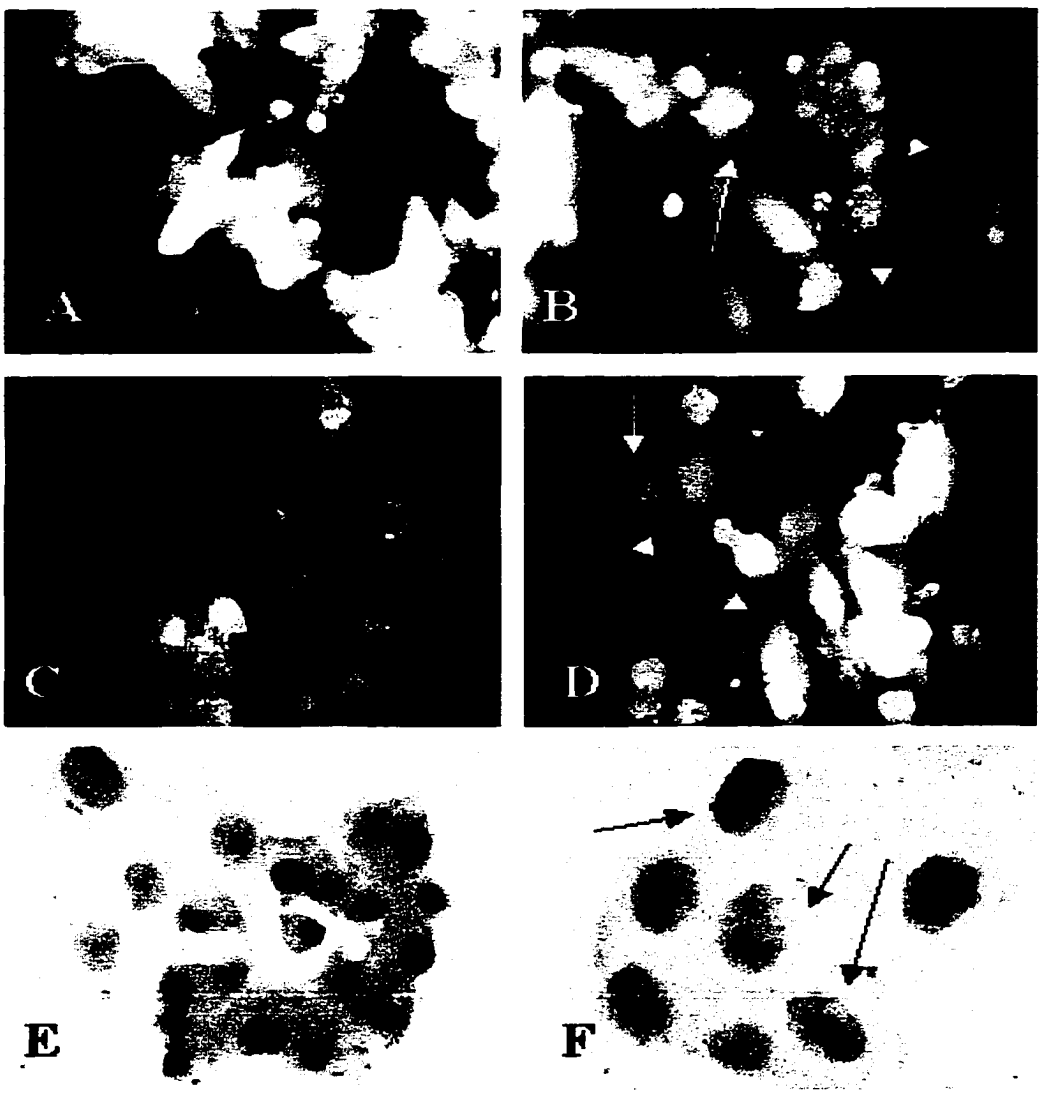
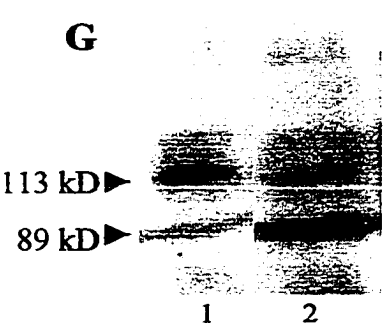


Figure 11. Evaluation of apoptosis in differentiated PC12 and TrkA cells. A, Control untreated cells stained with the DNA specific fluorochrome Hoechst 33258. B, Quantification of morphological changes of nuclear apoptosis was performed by staining cells with the DNA specific fluorochrome Hoechst 33258. Segmentation of the nucleus into three or more chromatin fragments was considered apoptotic as described in Materials and Methods. C, Control untreated cells stained with LIVE/DEAD® assay indicating the presence of calcein esterase activity in viable cells. D, Treated cells stained with LIVE/DEAD® assay indicating loss of functional calcein esterase activity and presence of ethidium homodimer staining of apoptotic nuclei as described in Materials and Methods. E, Control untreated cells stained with ApopTag® TUNEL assay indicating absence of fragmented DNA. F, Treated cells stained with ApopTag® TUNEL assay indicating presence of fragmented DNA by peroxidase activity as described in Materials and Methods. G, Typical western blot illustrating PARP cleavage following induction of apoptosis. Lysates of untreated PC12 cells (lane 1) and cells deprived of NGF for 24 hours (lane 2) were immunoblotted with a serum recognizing PARP. The migration positions of intact PARP (113 kD) and its cleavage fragment (89 kD) are marked. H, number of dead cells by trypan blue assay after 24 hours in cultures of PC12 and TrkA cells following NGF withdrawal as described in Materials and Methods. Each value represents the mean of duplicate determinations from three independent experiments.; bars, SEM. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment.





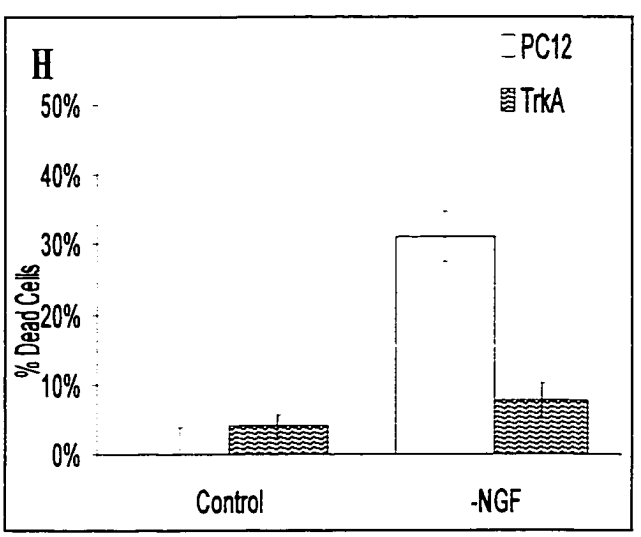


Figure 12. Response to NGF withdrawal in PC12 or TrkA cells. Differentiated PC12 (A-C) and TrkA (D-F)cells were deprived of NGF for up to 48 hours and collected every 3-6 hours for viability assay as well as extraction and quantification of lipids. Quantification of apoptosis (A-B,D-E),was performed by trypan blue staining of dead cells as well as by staining the cells with the DNA specific fluorochrome Hoechst 33258 as described in Materials and Methods. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment. Endogenous ceramide (A,C; D,F) was quantified by DAG kinase assay and sphingomyelin (B-C, E-F) by SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three independent experiments.; bars, SEM.

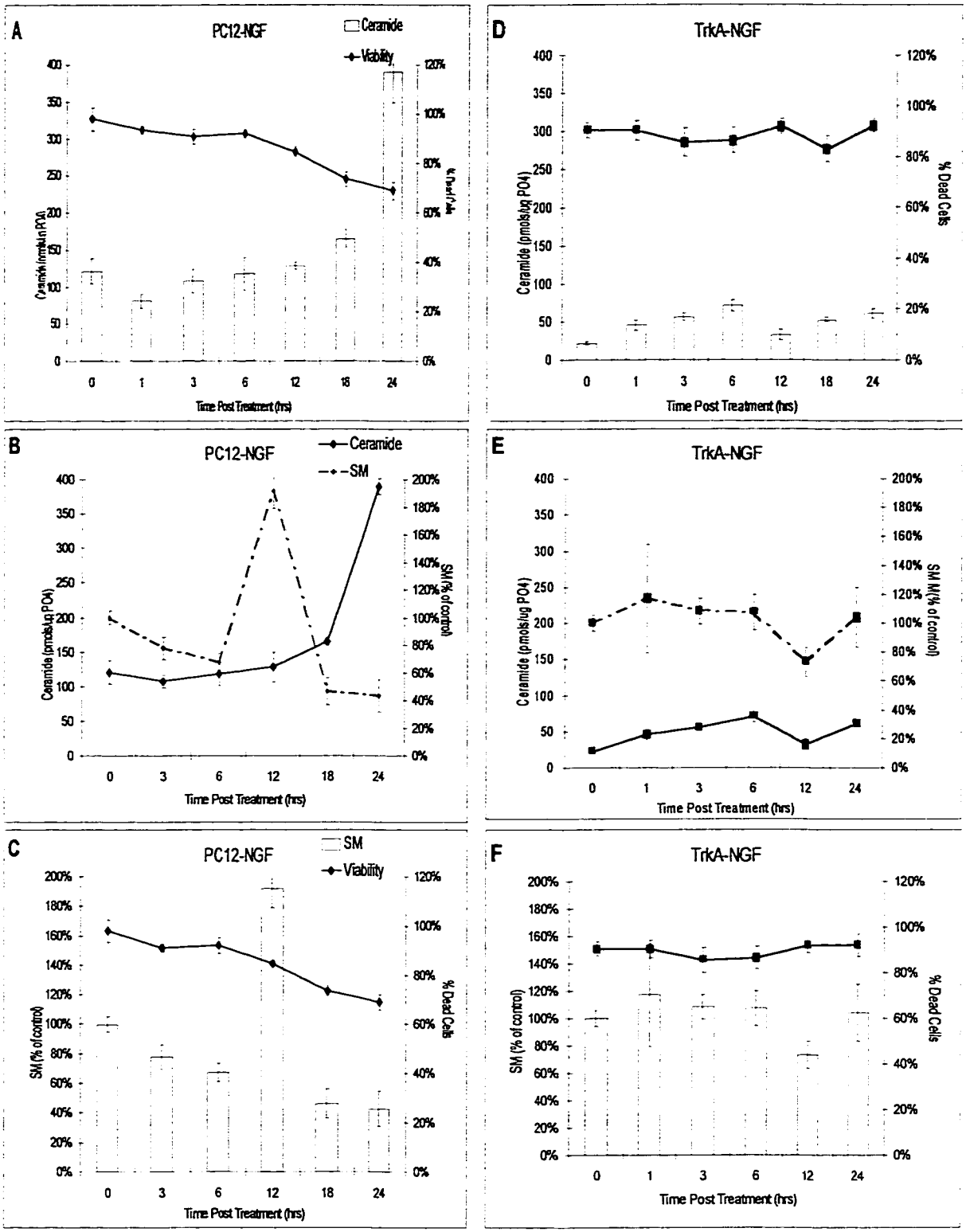


Figure 13. The effect of ceramide inhibitors on PC12 or TrkA cells following NGF removal. Differentiated PC12 and TrkA cells were deprived of NGF for 24 hours in the presence of either 15 μM desipramine or 25 μM FB_1. Control cells treated with vehicle or inhibitors alone are in white, cells deprived of NGF are depicted with wavy lines. Each value represents the mean of duplicate determinations from three independent experiments; bars, SEM; *p< 0.05, **p <<0.05, ***p <<0.01. (A) Measurement of ceramide levels. Lipids were extracted and quantified by DAG kinase assay as described in Materials and Methods. (B) Measurement of SM levels. Lipids were extracted and quantified by SM mass assay as described in Materials and Methods. (C) Measurement of viability. A minimum of 250 cells was scored by trypan blue exclusion for the incidence of apoptosis in each experiment.

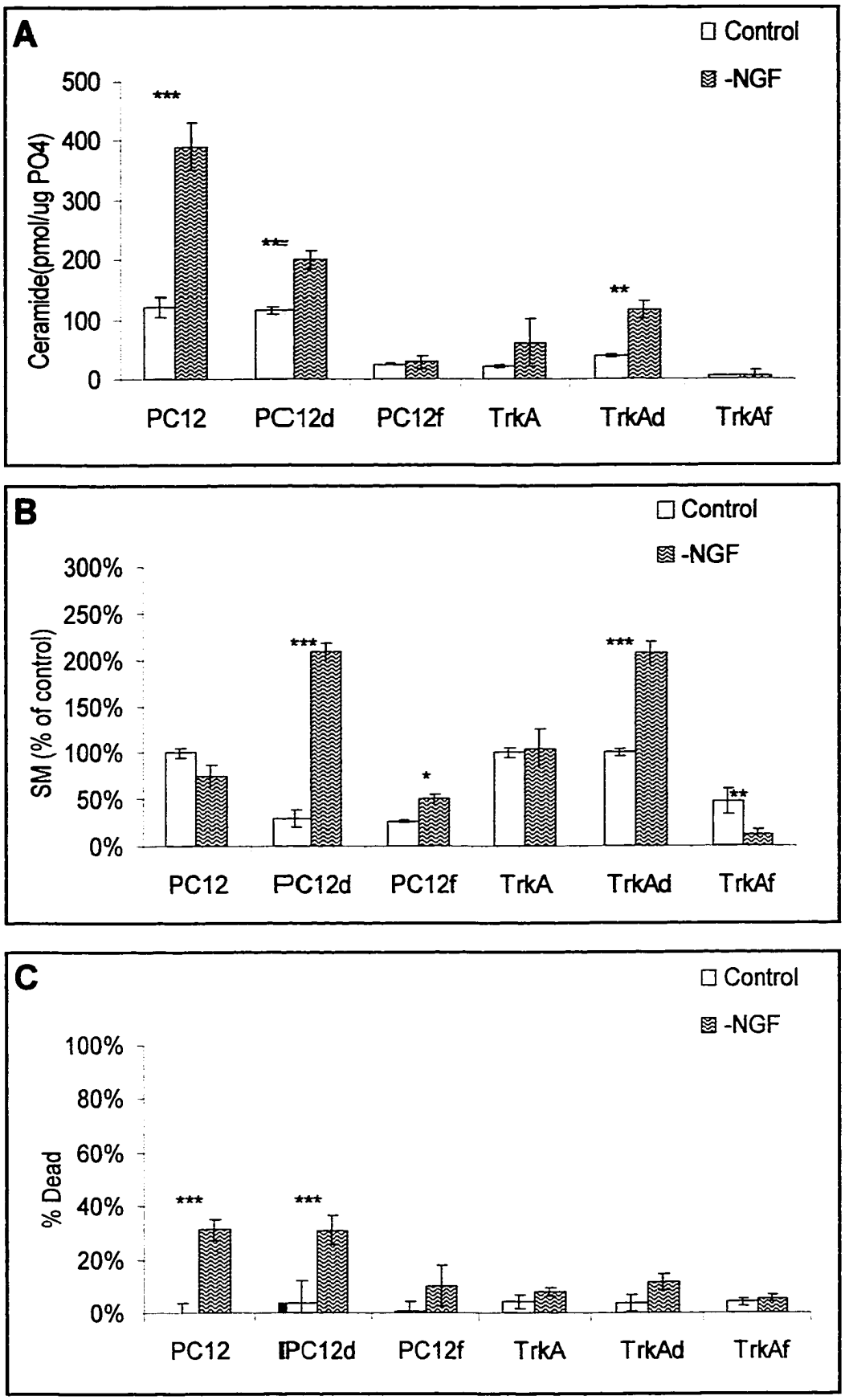


Figure 14. Response of differentiated PC12 and TrkA cells to various cell death inducers. Differentiated cells were either deprived of NGF or treated with 50 μg/ml cycloheximide (CHX), 10 ng/ml tumor necrosis factor (TNF)-α, 10 μM D-e-C_8-ceramide, 15mM camptothecin (CAM) or 220 mM ethanol (ETOH) for 24 hours. Apoptosis was quantified by trypan blue staining of dead cells in addition to the DNA specific fluorochrome Hoechst 33258 as described in Materials and Methods. Each value represents the mean of duplicate determinations from three independent experiments.; bars, SEM; **p <<0.05. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment.

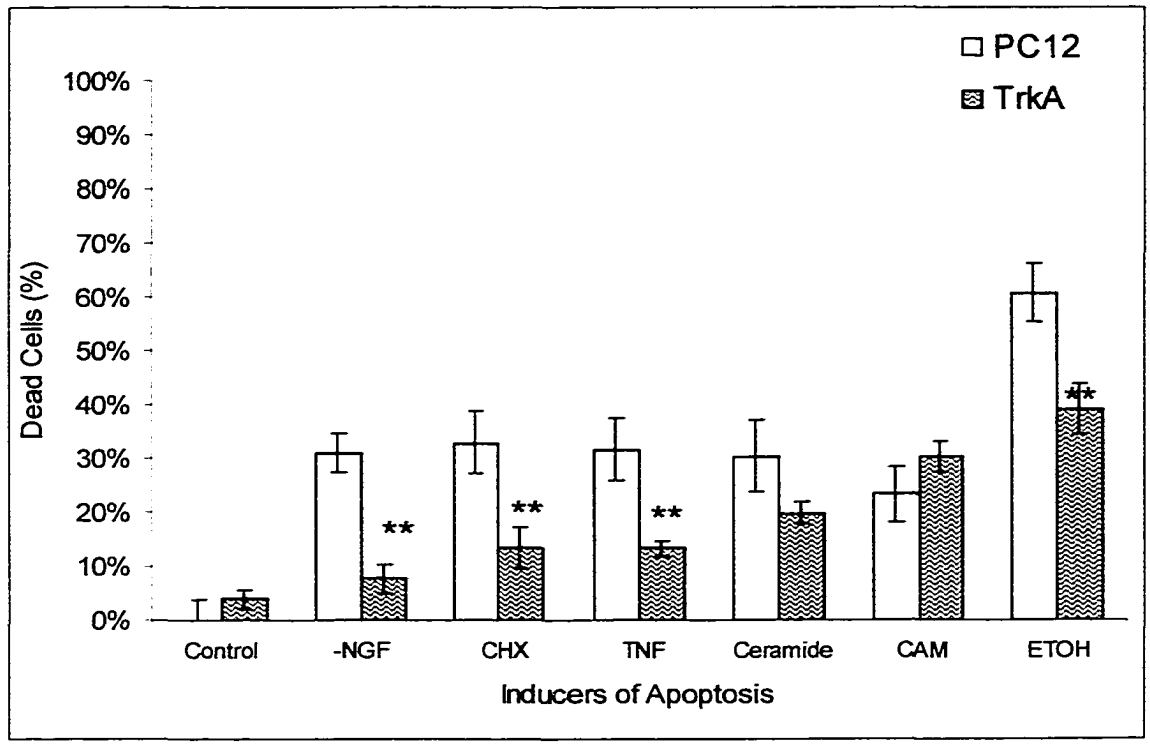


Figure 15. Response to ethanol in PC12 or TrkA cells. Differentiated PC12 (A-C) and TrkA (D-F)cells were treated with 220 mM ethanol for up to 24 hours and collected every 3-6 hours. Quantification of apoptosis (A-B,D-E), was performed by trypan blue staining of dead cells as well as by staining the cells with the DNA specific fluorochrome Hoechst 33258 as described in Materials and Methods. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment. Endogenous ceramide (A,C; D,F) was quantified by DG kinase assay and sphingomyelin (B-C, E-F) by SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three independent experiments.; bars, SEM.

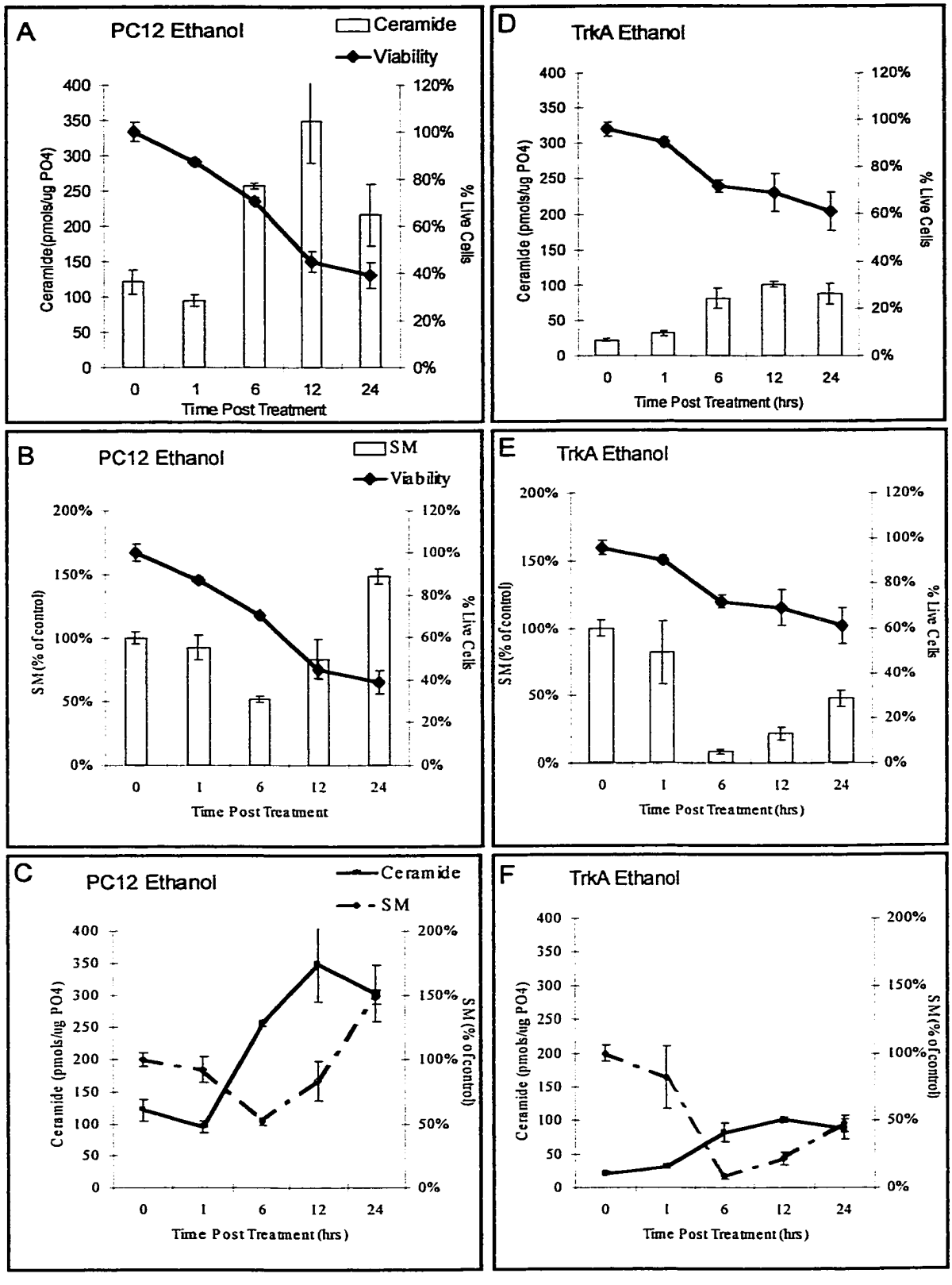


Figure 16. The effect of ceramide inhibitors on ethanol-induced apoptosis. Differentiated PC12 or TrkA cells treated with 220 mM ethanol for 24 hours are compared to cells treated in the presence of either 15 μM desipramine (PC12d, TrkAd) or 25 μM FB_1 (PC12F, TrkAF). Control cells (white) received vehicle or inhibitors alone, experimental cells (wavy lines) received ethanol alone or with inhibitors. Cell viability was determined by trypan blue assay and lipids were extracted and quantified by DG kinase or SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from three independent experiments.; bars, SEM; **p <<0.05, ***p<<0.01.

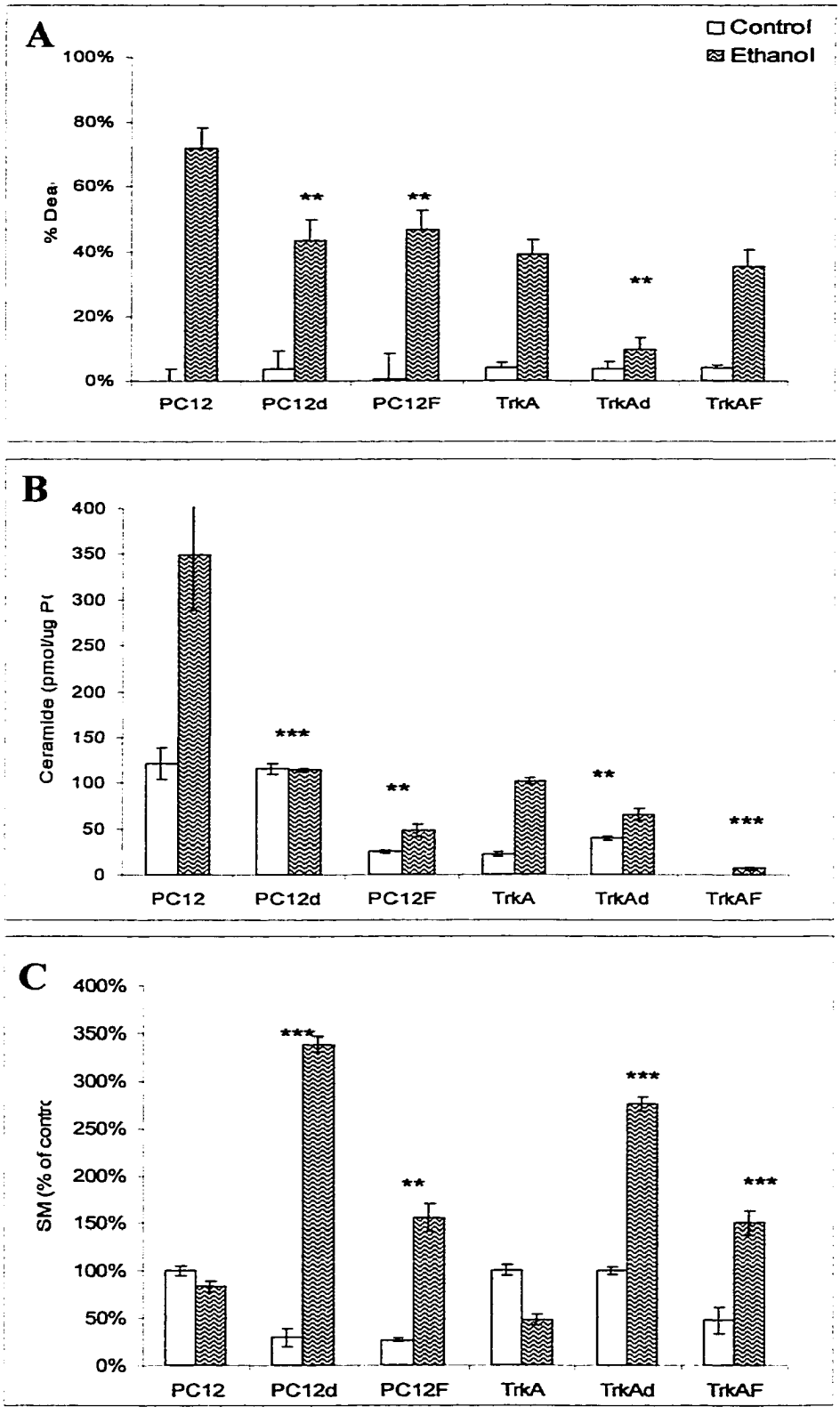


Figure 17. Response to CAM in PC12 or TrkA cells. Differentiated PC12 (A-C) and TrkA (D-F)cells were treated with 15 μM CAM for up to 24 hours and collected every 3-6 hours Quantification of apoptosis (A-B,D-E), was performed by trypan blue staining of dead cells as well as by staining the cells with the DNA specific fluorochrome Hoechst 33258 as described in Materials and Methods. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment. Endogenous ceramide (A,C; D,F) was quantified by DG kinase assay and sphingomyelin (B-C, E-F) by SM mass assay as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three independent experiments.; bars, SEM. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment.

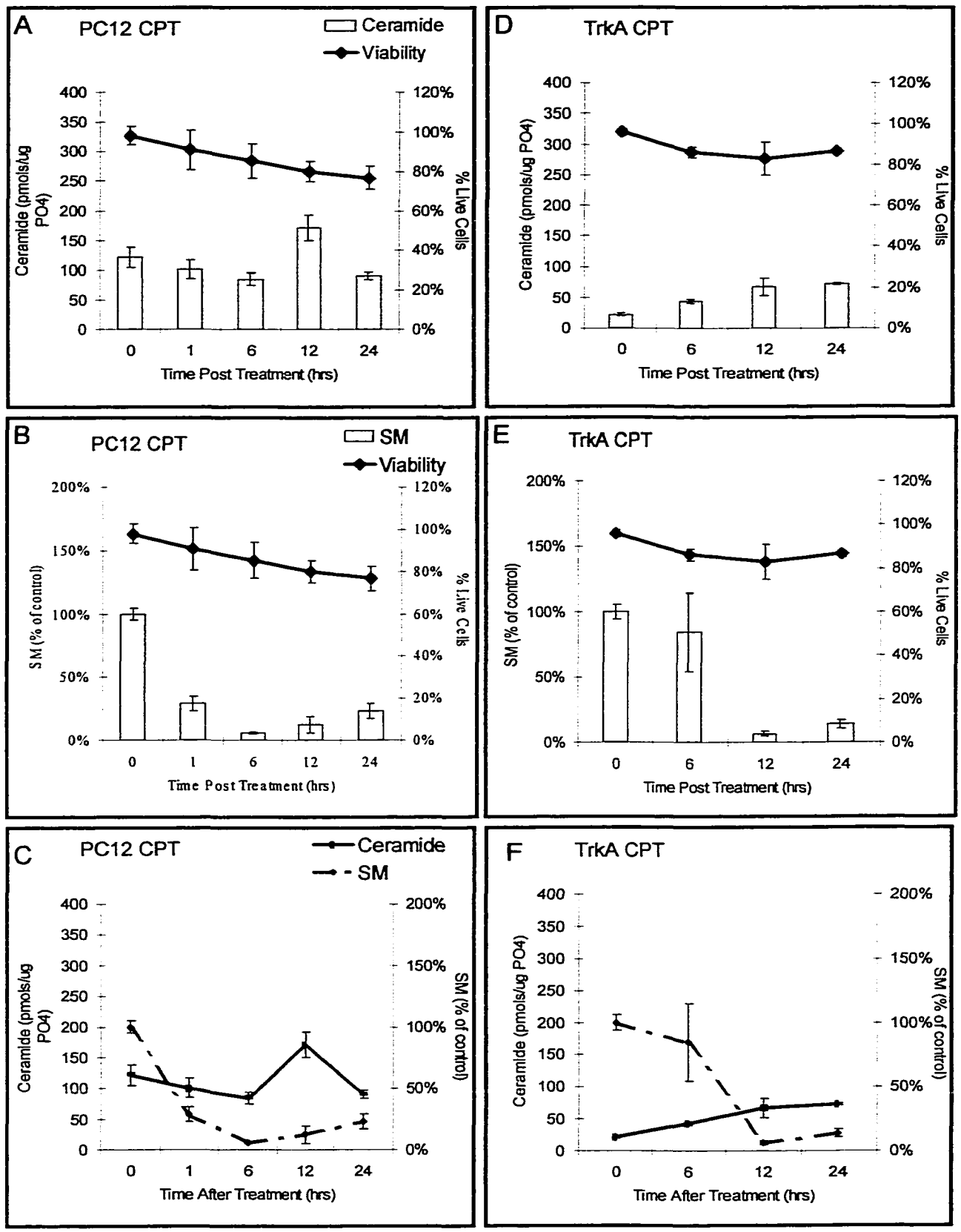


Figure 18. The effect of ceramide inhibitors on CAM-induced apoptosis in PC12 or TrkA cells. Differentiated PC12 and TrkA cells were treated with 15 μM camptothecin (CAM) for 24 hours in the absence (PC12, TrkA) or presence of either 15 μM desipramine (PC12d, TrkAd) or 25 μM FB_1 (PC12F, TrkAF). "Controls" (white) received vehicle or inhibitors alone, "CAMs" (wavy lines) received CAM either with or without inhibitors. Cell viability was determined by trypan blue assay as described in Materials and Methods. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment. Each value represents the mean of duplicate determinations from three independent experiments.; bars, SEM; **p <<0.05.

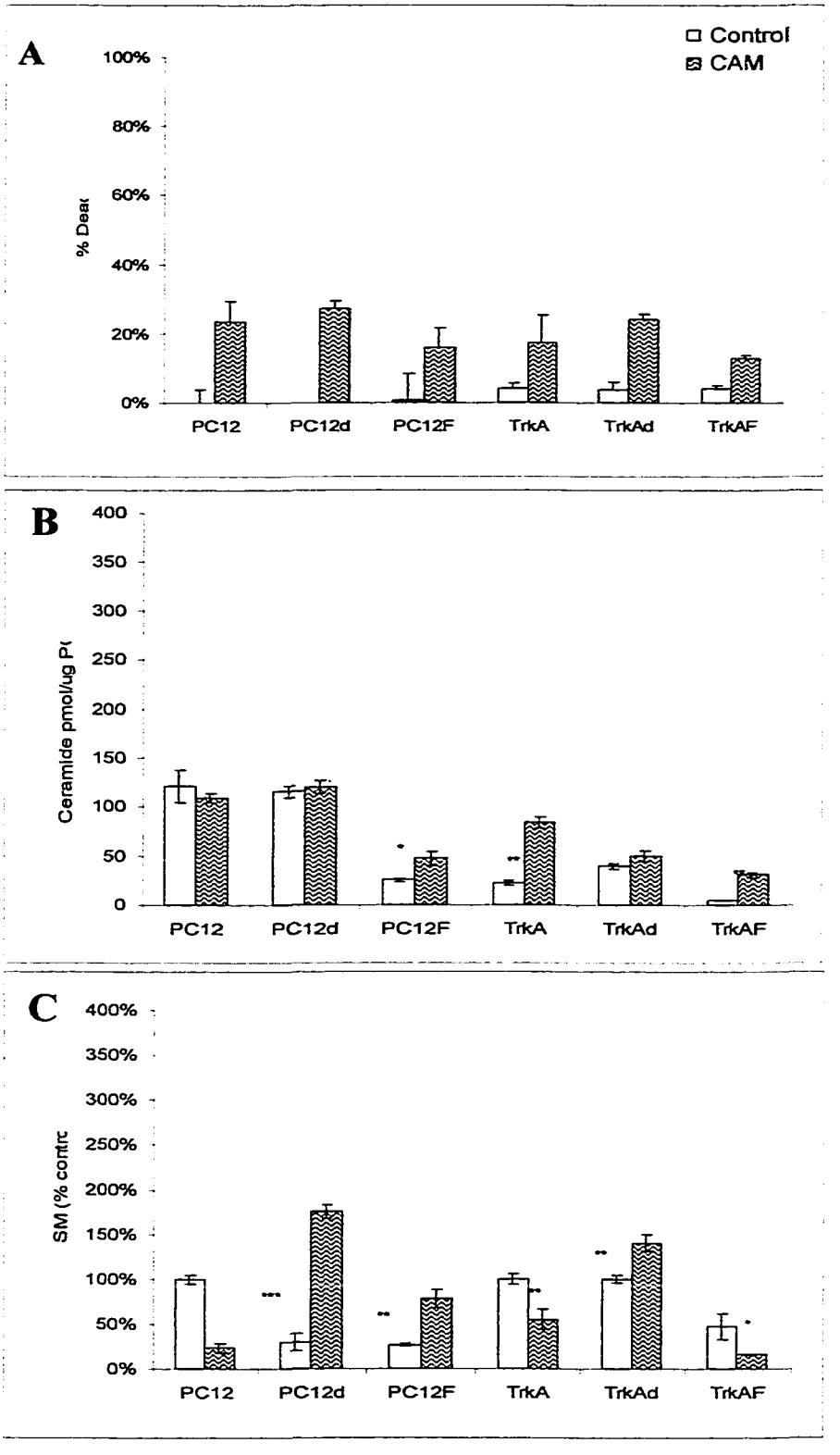


Figure 19. Mitochondrial integrity and function during differentiation. Naïve PC12 (A) and TrkA (B) cells were induced to differentiate by the addition of 0.05 ng/ml NGF. PC12 cells were cultured for a total of 10 days, and TrkA cells for a total of 6 days until differentiation was complete. During this period, cells were collected every 2 days for evaluation of mitochondrial function and integrity as described in Materials and Methods. Mitochondrial membrane permeability (MMP) was measured using the $DiOC_6(3)$ assay and mitochondrial enzyme activity with the MTT assay. Each value represents the mean of duplicate determinations from three independent experiments and is represented as percent of control in order to facilitate comparisons; bars, SEM. See text for explanation.

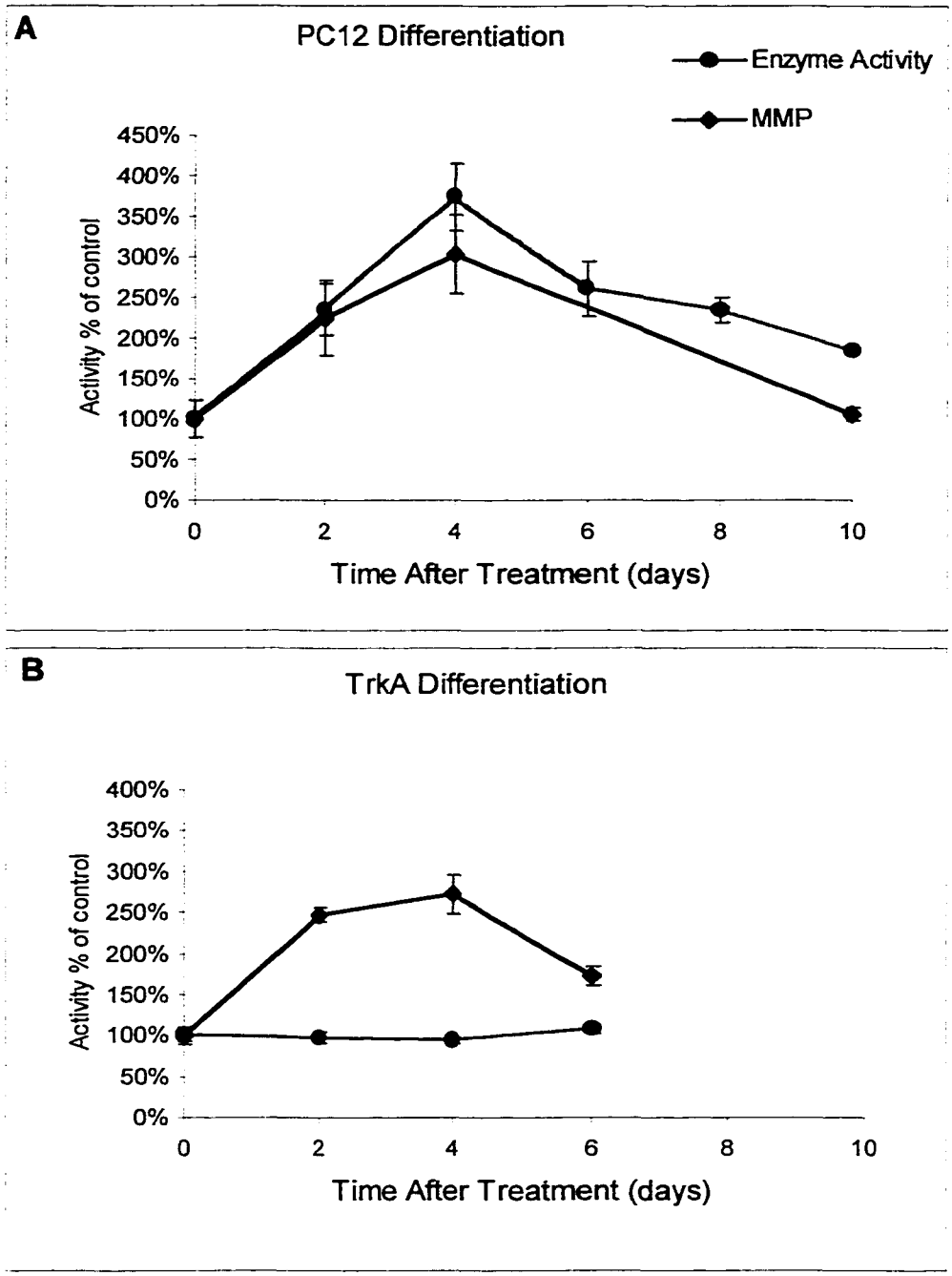


Figure 20. CoQ_{10} is not toxic to either PC12 or TrkA cells.

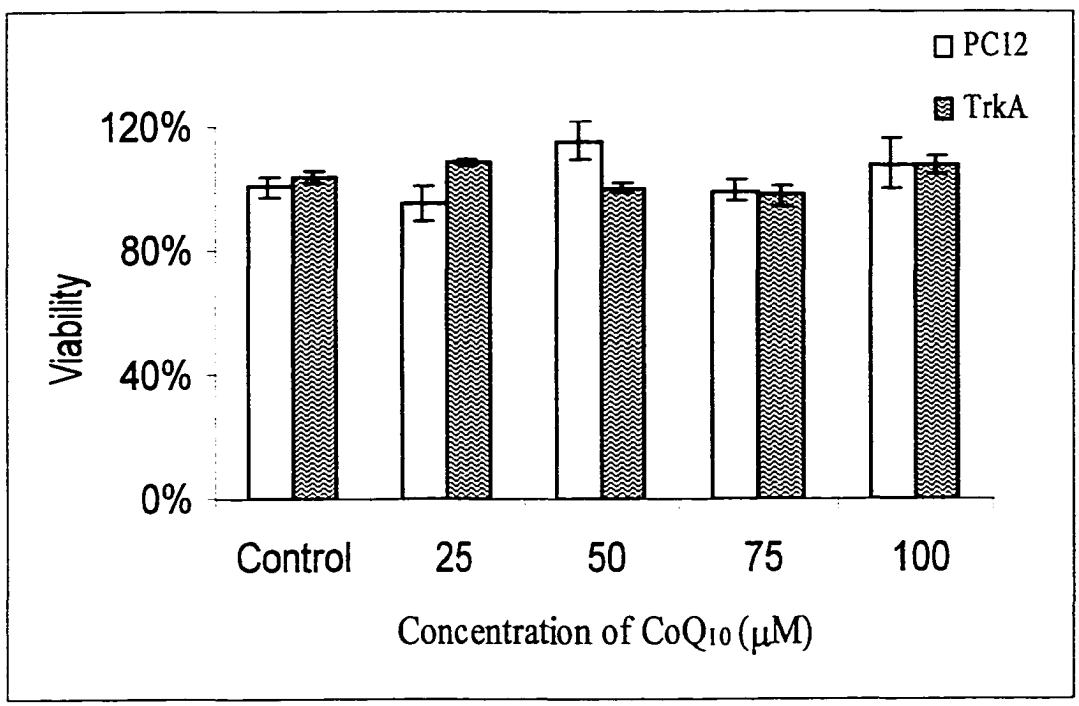
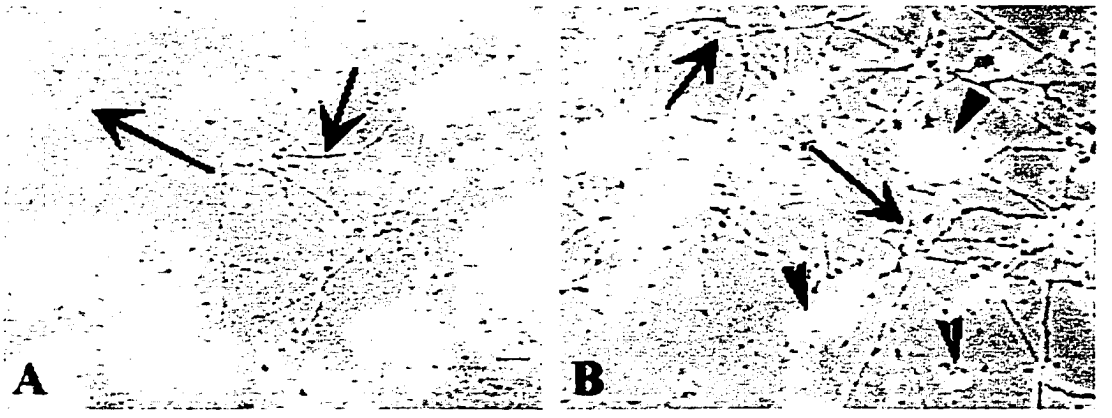
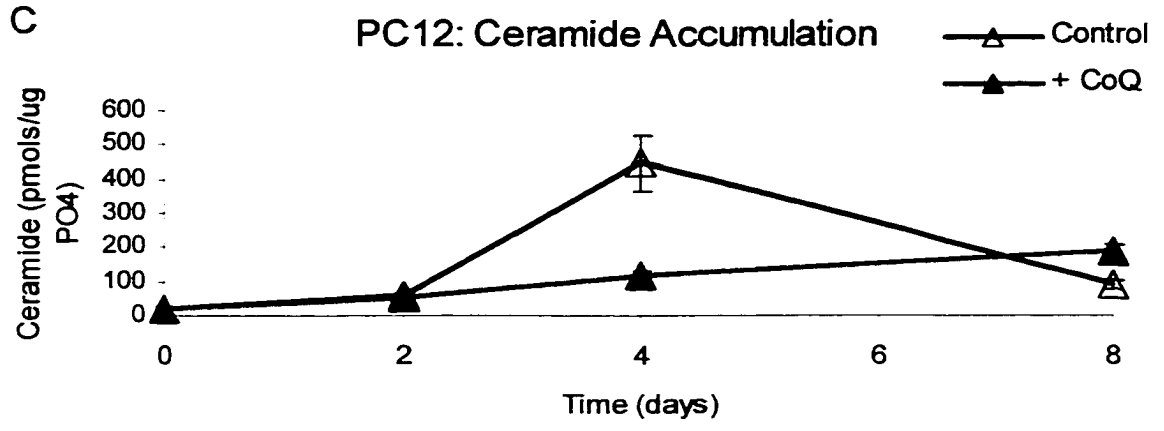


Figure 21. The effect of coenzyme Q_{10} on morphology of PC12 cells. Naïve cells were induced to differentiate for 8 days by the addition of 0.05 ng/ml NGF in the absence (A) or presence (B) of 100 μM CoQ_{10}. (400x). Arrows indicate neurites, arrowheads indicate the presence of crystalline CoQ_{10}. Every two days, lipids were extracted and quantified by DG kinase assay (C) and SM mass assay (D) as described in Materials and Methods. Each value represents the mean of duplicate determinations from three independent experiments; bars, SEM.





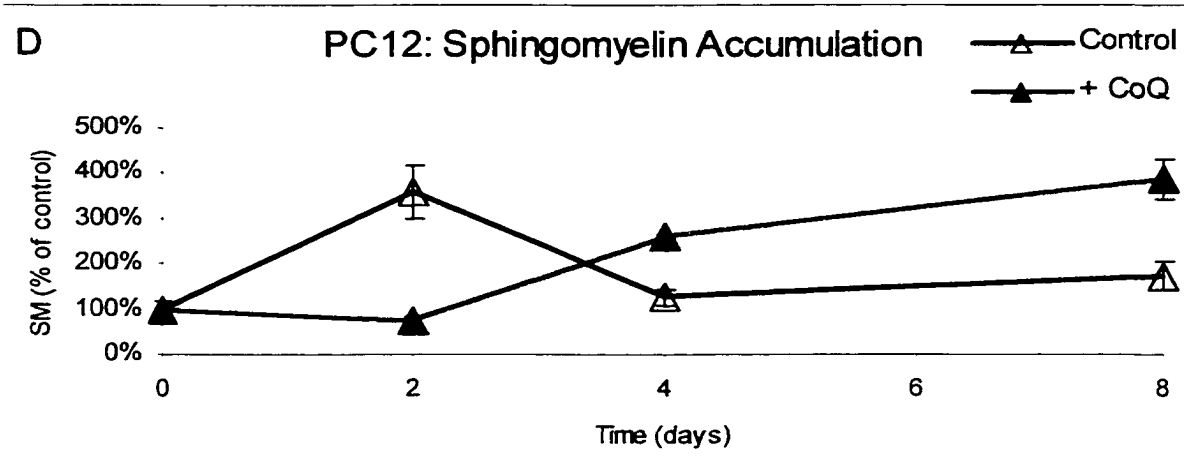
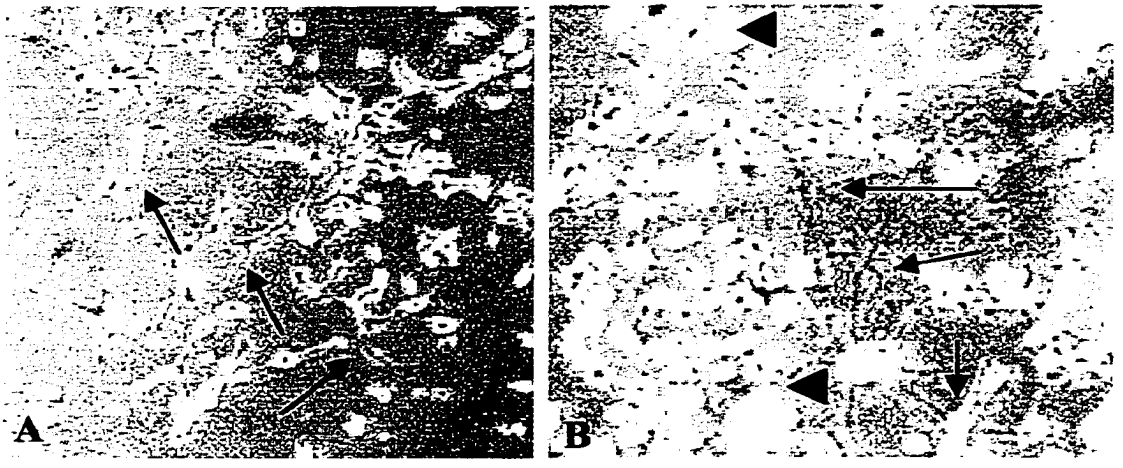
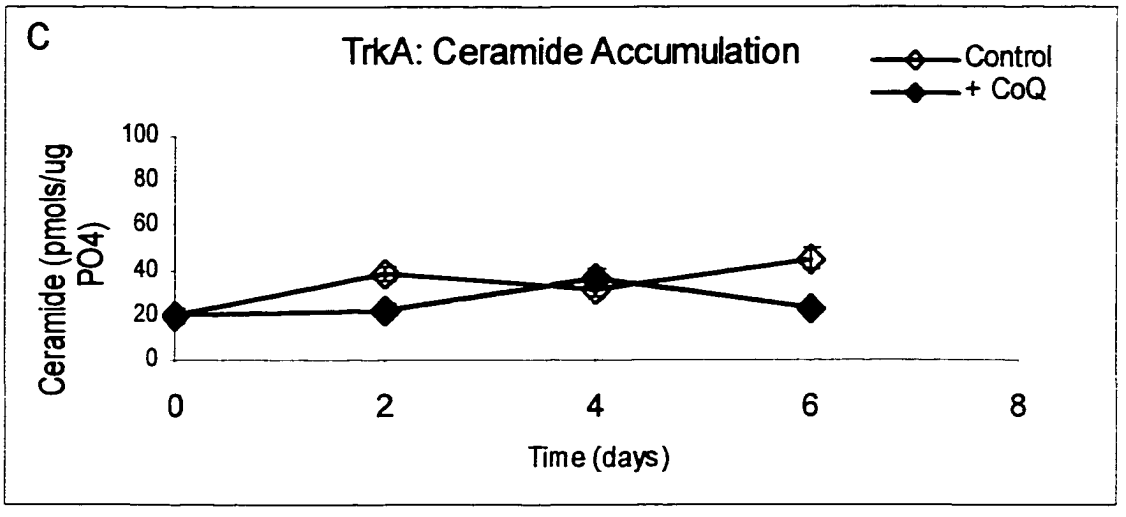


Figure 22. The effect of coenzyme Q_{10} on morphology of TrkA cells. Naïve cells were induced to differentiate for 6 days by the addition of 0.05 ng/ml NGF in the absence (A) or presence (B) of 100 μM CoQ_{10} (400x). Arrows indicate neurites, arrowheads indicate the presence of crystalline CoQ_{10}. Every two days, lipids were extracted and quantified by DG kinase assay (C) and SM mass assay (D) as described in Materials and Methods. Each value represents the mean of duplicate determinations from three independent experiments; bars, SEM.





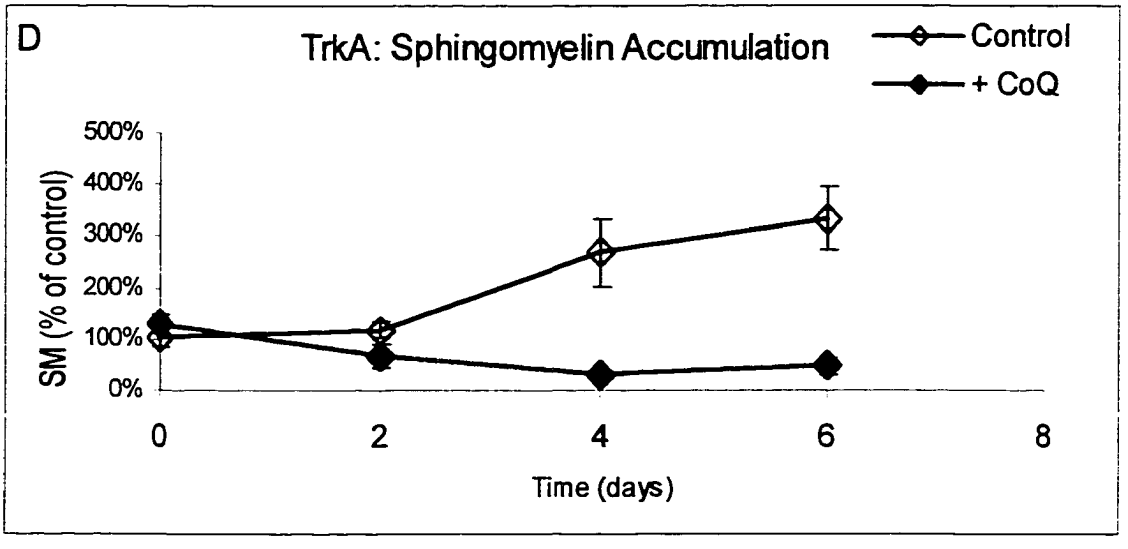


Figure 23. Inducers of apoptosis alter mitochondrial function and integrity in a manner dependent on both the type of insult as well as the length of exposure. Differentiated PC12 (A,C,E) and TrkA (B,D,F) cells were exposed to 10 μM c-8 ceramide (A-B), NGF deprivation (C-D), or 220 mM ethanol (E-F) for up to 24 hours. Viability was determined by trypan blue assay, MMP by $DiOC_6(3)$ assay, loss of mitochondrial membrane potential ($\Delta\Psi_m$) by Rh-123 fluorescence and mitochondrial enzyme activity by MTT assay, as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three independent experiments. bars, SEM; **$p<<0.05$. The mitochondria of PC12 cells generally depolarize immediately following toxic insult, but show a more gradual loss of enzyme activity, while immediately after insult TrkA cells exhibit mitochondrial hyperpolarization and reduced loss of enzyme activity.

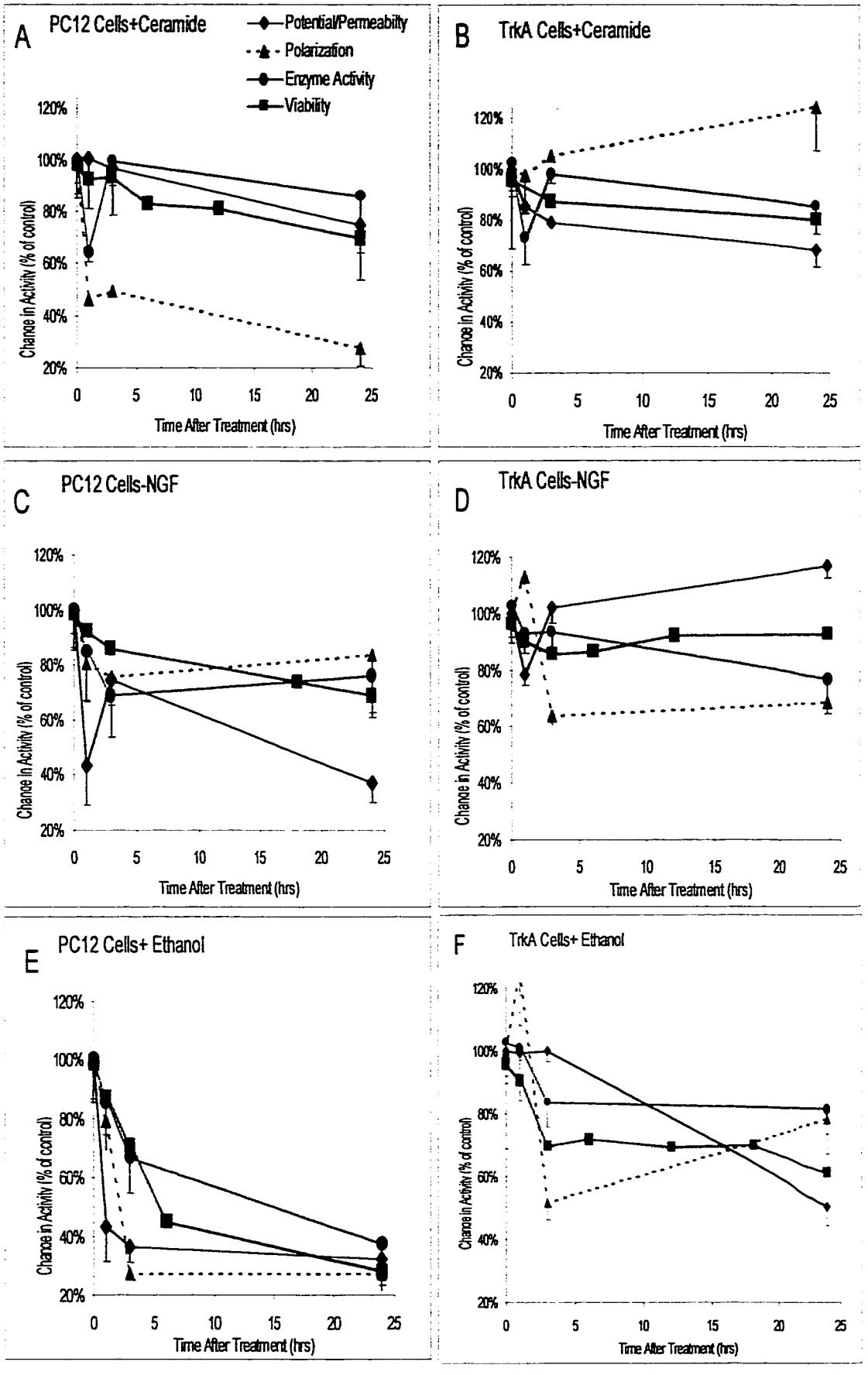


Figure 24. Although there is some variation, we find a general pattern of progressive dissipation of mitochondrial inner matrix polarity as measured by rhodamine-123 (Rh-123) based confocal microscopy. In this example, PC12 cells were grown on slides and differentiated for 10 days, after which they were pre-loaded with the fluorescent dye Rh-123 for 30 minutes, washed and treated with 220 mM ethanol and then examined by confocal microscopy as described in Materials and Methods. Measurements were made of at least three areas per slide and of at least 3 individual slides, and calculated based on total fluorescence/field. Color values range from red/yellow (highest energy value) through violet (lowest measurable energy) and black (no Ψ_m). Loss of fluorescence is not the result of loss of cells from slides. Note that dissipation of polarization is uniform from all mitochondria in all cells, not in individual cells or in individual mitochondria in each cell. All data are presented elsewhere in graph form as % of control values.
(A) untreated cells, (B) after 1 hr, (C) after 3 hours, (D) after 12 hours, (E) after 24 hours, (F) after 3 hours but in the presence of 100 μM CoQ_{10}.

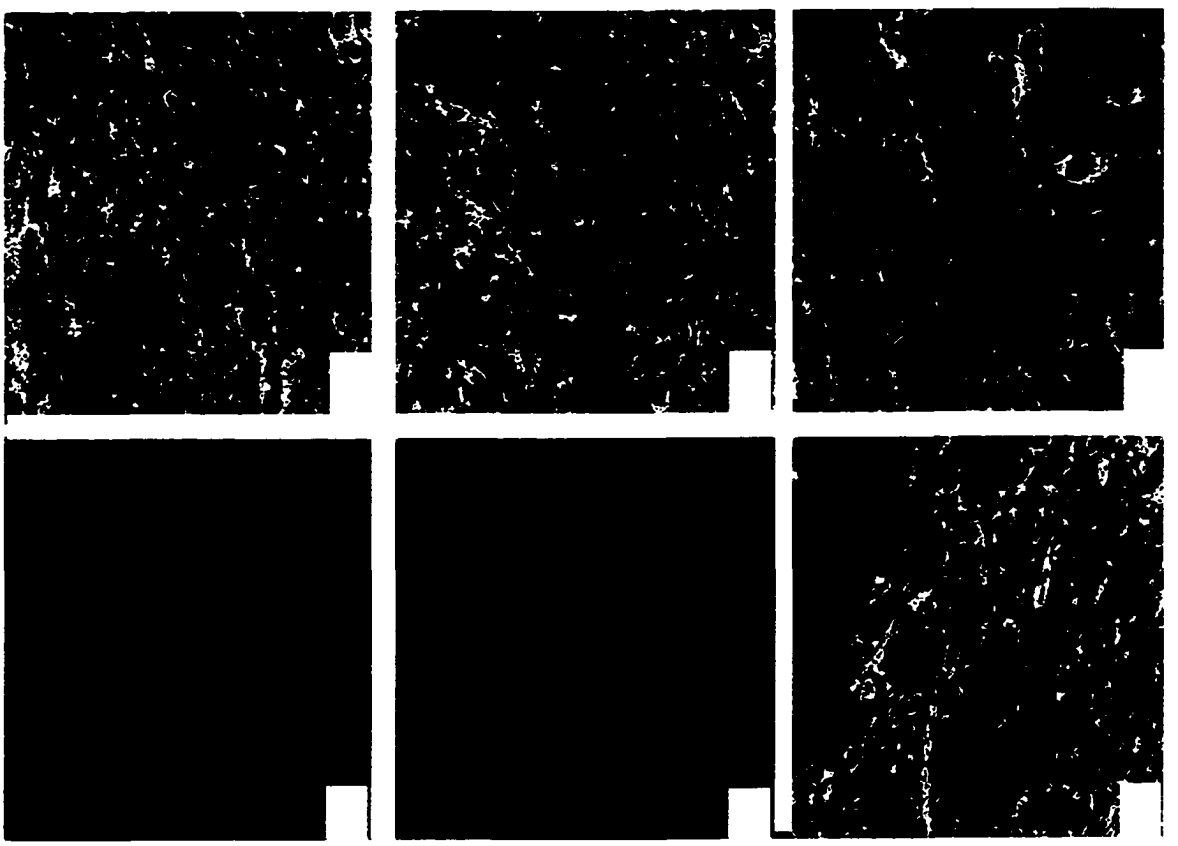
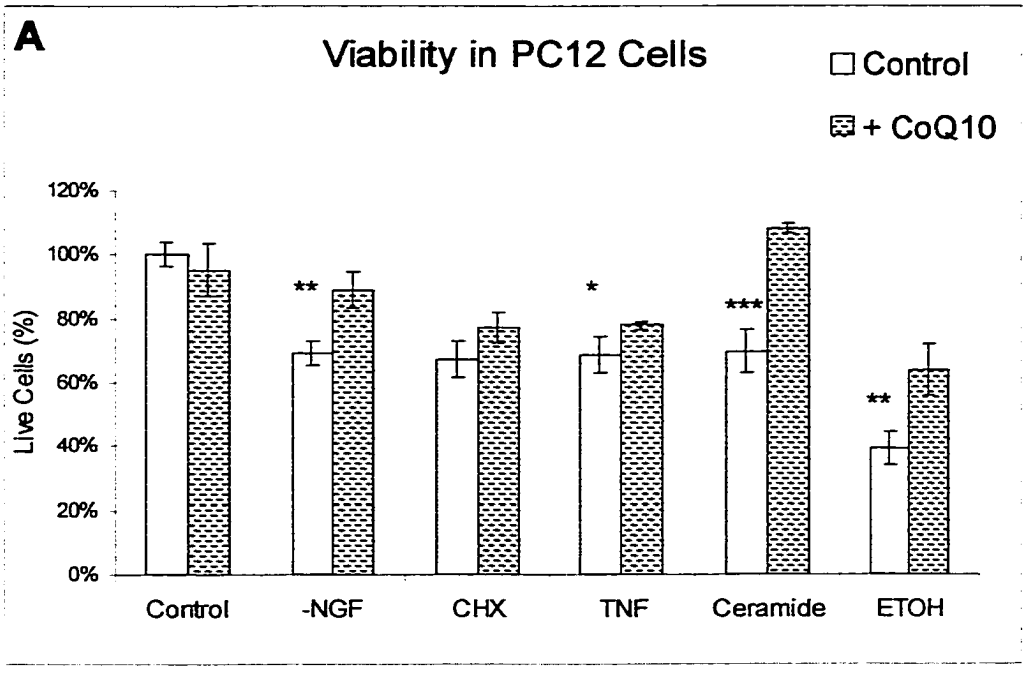


Figure 25. The Effect of CoQ_{10} on Viability. Differentiated PC12 and TrkA cells were subjected to either NGF withdrawal, 25μM CHX, 10ng/ml TNFa, 10mM exogenous C-8 ceramide, or 220 mM ethanol as described in Materials and Methods. for 24 hours in the presence of 100 μM CoQ_{10}, following which the number of dead cells was determined by trypan blue assay. Each value represents the mean of duplicate determinations from three independent experiments; bars, SEM. A minimum of 250 cells was scored for the incidence of apoptosis in each experiment. *$p< 0.05$, **$p << 0.05$, ***$p << 0.01$.



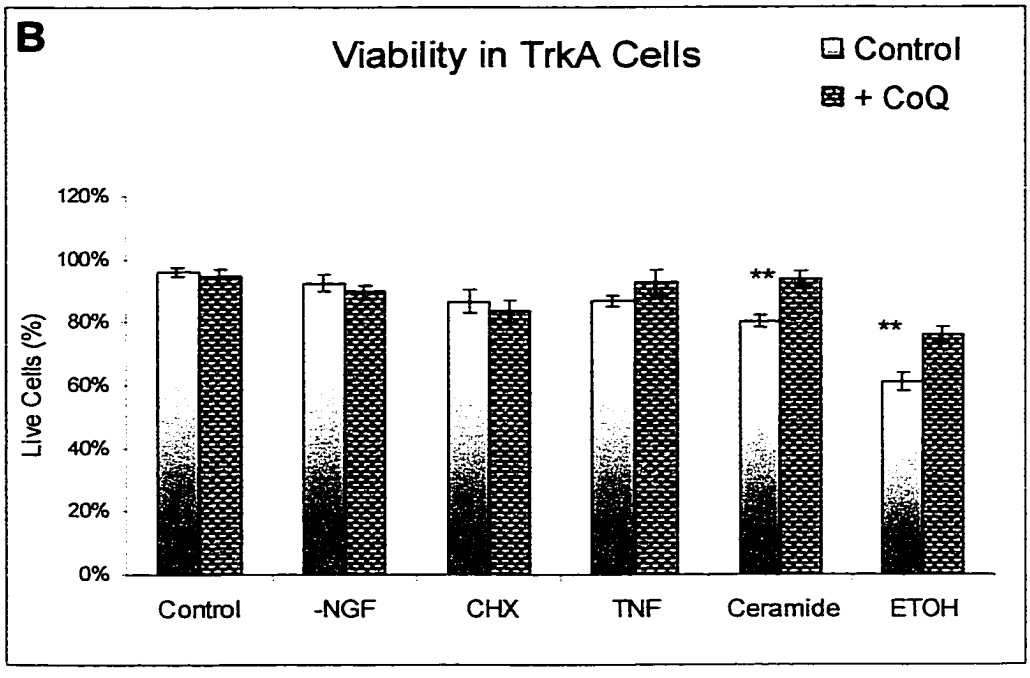


Figure 26. The effect of CoQ_{10} on sphingolipids following toxic insult. Differentiated PC12 (A-B) or TrkA (C-D) cells were either deprived of NGF or exposed to ethanol for 24 hours in the presence of 100 μM CoQ_{10} after which ceramide (C,D) or sphingomyelin (B,D) was extracted and quantified by DAG kinase assay or SM mass assay respectively as described in Materials and Methods. Each value represents the mean of duplicate determinations from at least three independent experiments; bars, SEM; **p <<0.05.

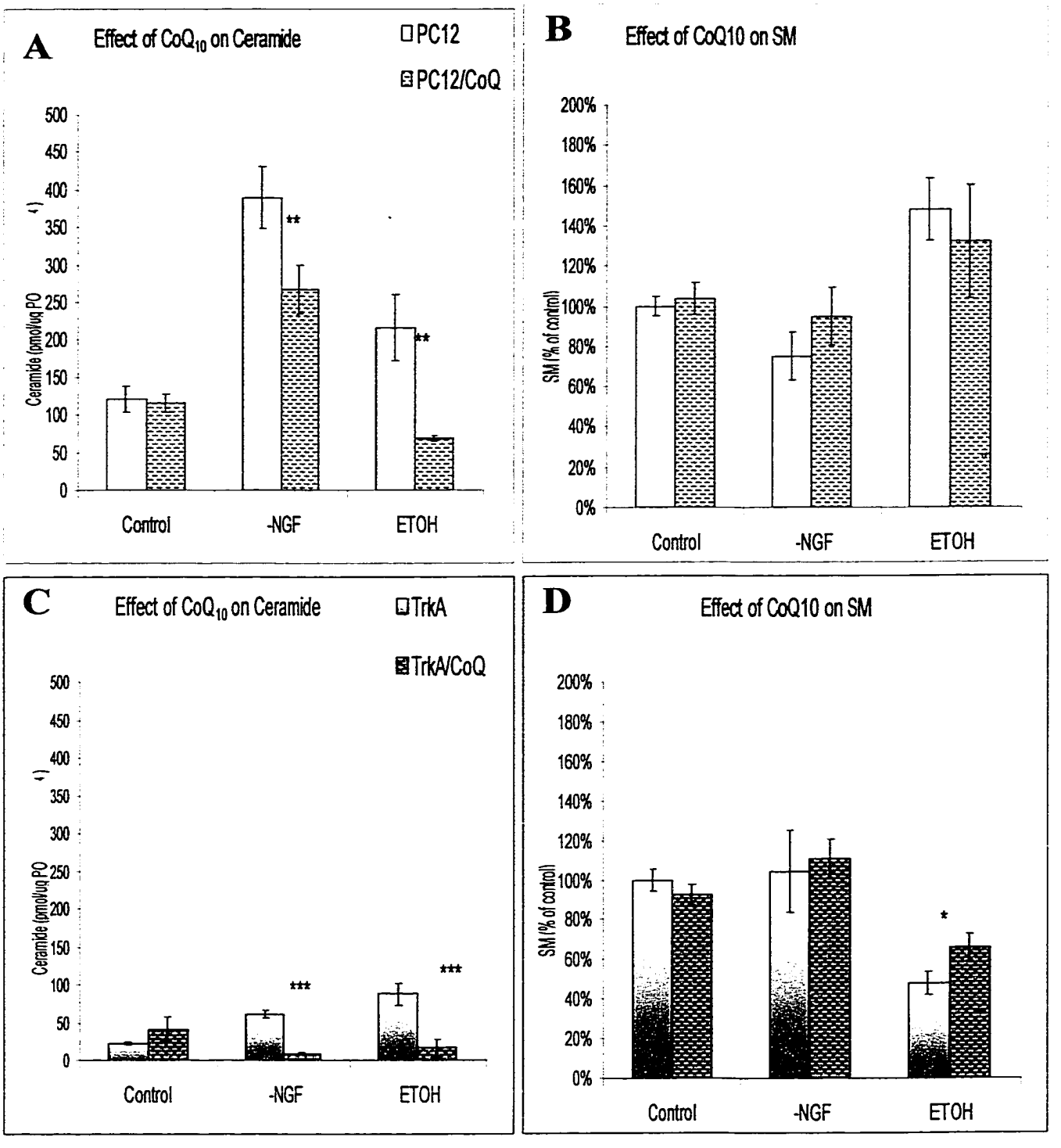


Figure 27. The effect of CoQ_{10} on mitochondrial integrity and dysfunction induced by specific insults. Differentiated PC12 cells were exposed to 10 μM C-8-ceramide (A-C), withdrawal of NGF (D-F), or 220 mM ethanol (G-I) in the presence (red line) or absence (black line) of 100μM CoQ_{10} for up to 24 hours. Control untreated cells were similarly cultured in the presence (dotted blue line) or absence (solid blue line) of CoQ_{10}. Cells were collected at intervals, for measurements of mitochondrial function and integrity as described in Materials and Methods. Mitochondrial membrane permeability (MMP) was measured using the $DiOC_6(3)$ assay (A,D,G), mitochondrial polarization using confocal microscopy of Rh-123 retention (B,E,H) and mitochondrial enzyme activity using the MTT assay (C,F,I). Each value represents the mean of duplicate determinations from three independent experiments and is represented as percent of control in order to facilitate comparisons. bars, SEM.

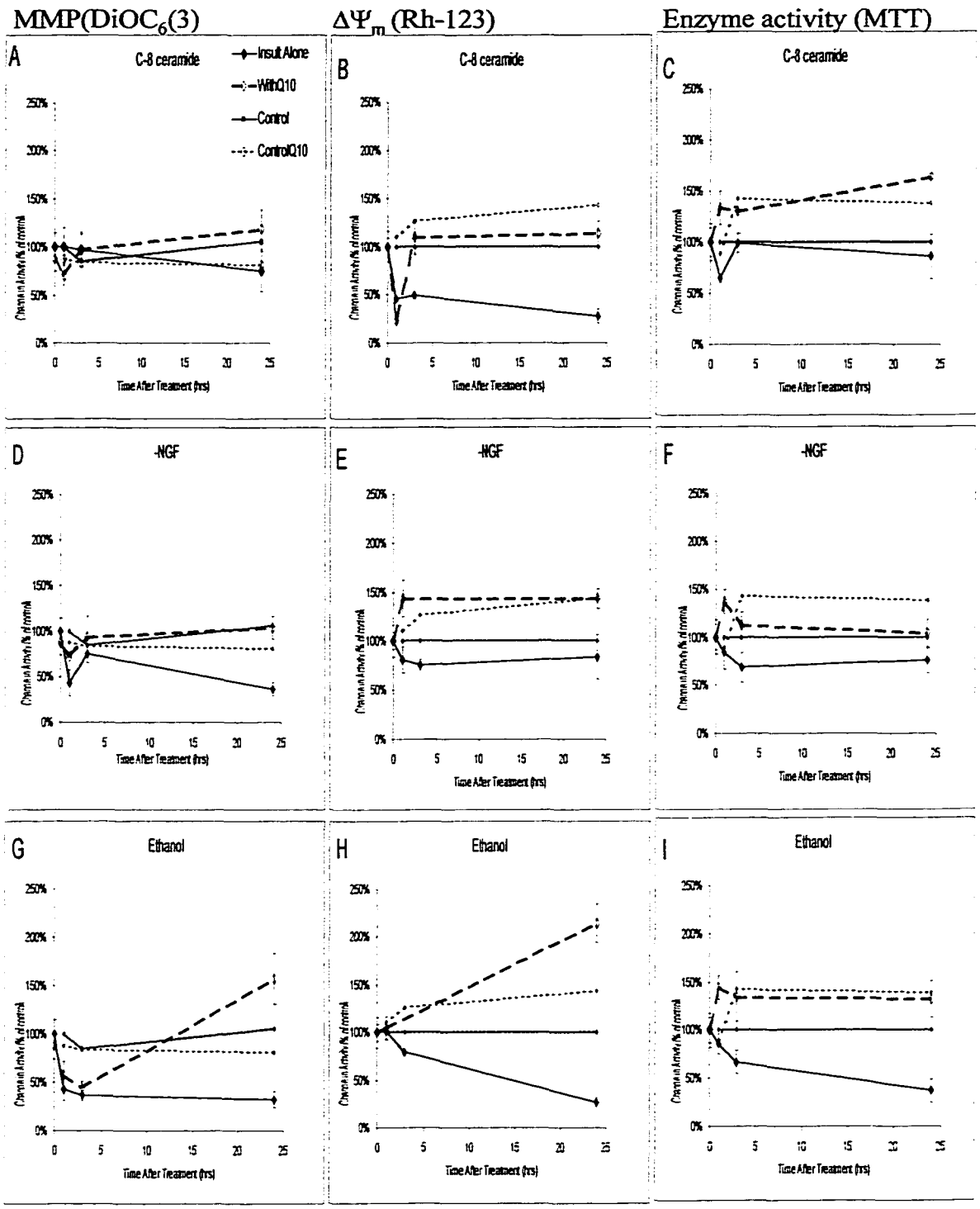


Figure 28. The effect of CoQ_{10} on mitochondrial integrity and dysfunction induced by specific insults. Differentiated TrkA cells were exposed to 10 μM C-8-ceramide (A-C), withdrawal of NGF (D-F), or 220 mM ethanol (G-I) in the presence (red line) or absence (black line) of 100μM CoQ_{10} for up to 24 hours. Control untreated cells were similarly cultured in the presence (dotted blue line) or absence (solid blue line) of CoQ_{10}. Cells were collected at intervals for measurements of mitochondrial function and integrity as described in Materials and Methods. Mitochondrial permeability (MMP) was measured using the $DiOC_6(3)$ assay (A,D,G), mitochondrial polarization using confocal microscopy of Rh-123 retention (B,E,H) and mitochondrial enzyme activity using the MTT assay (C,F,I). Each value represents the mean of duplicate determinations from three independent experiments and is represented as percent of control in order to facilitate comparisons; bars, SEM.

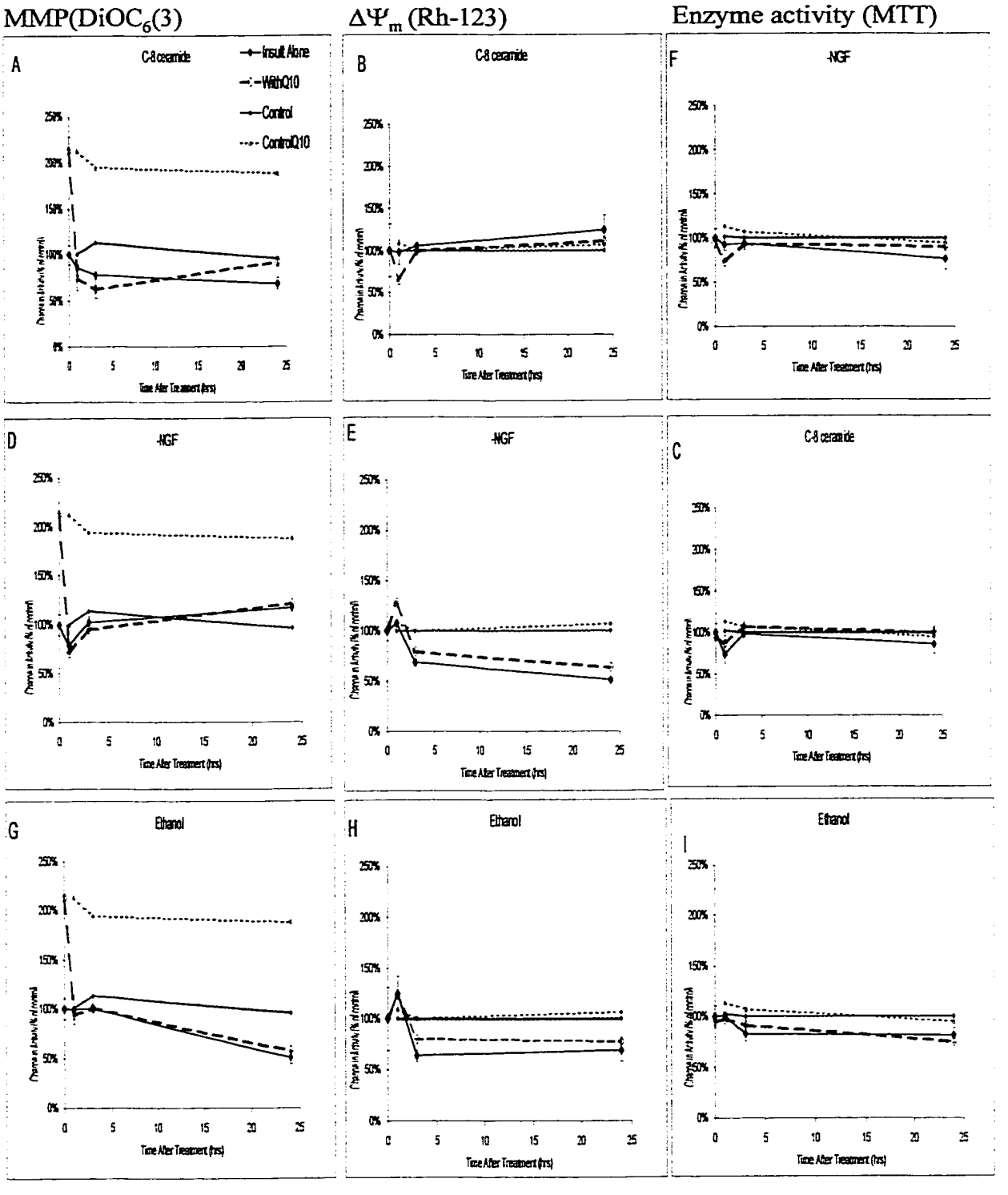
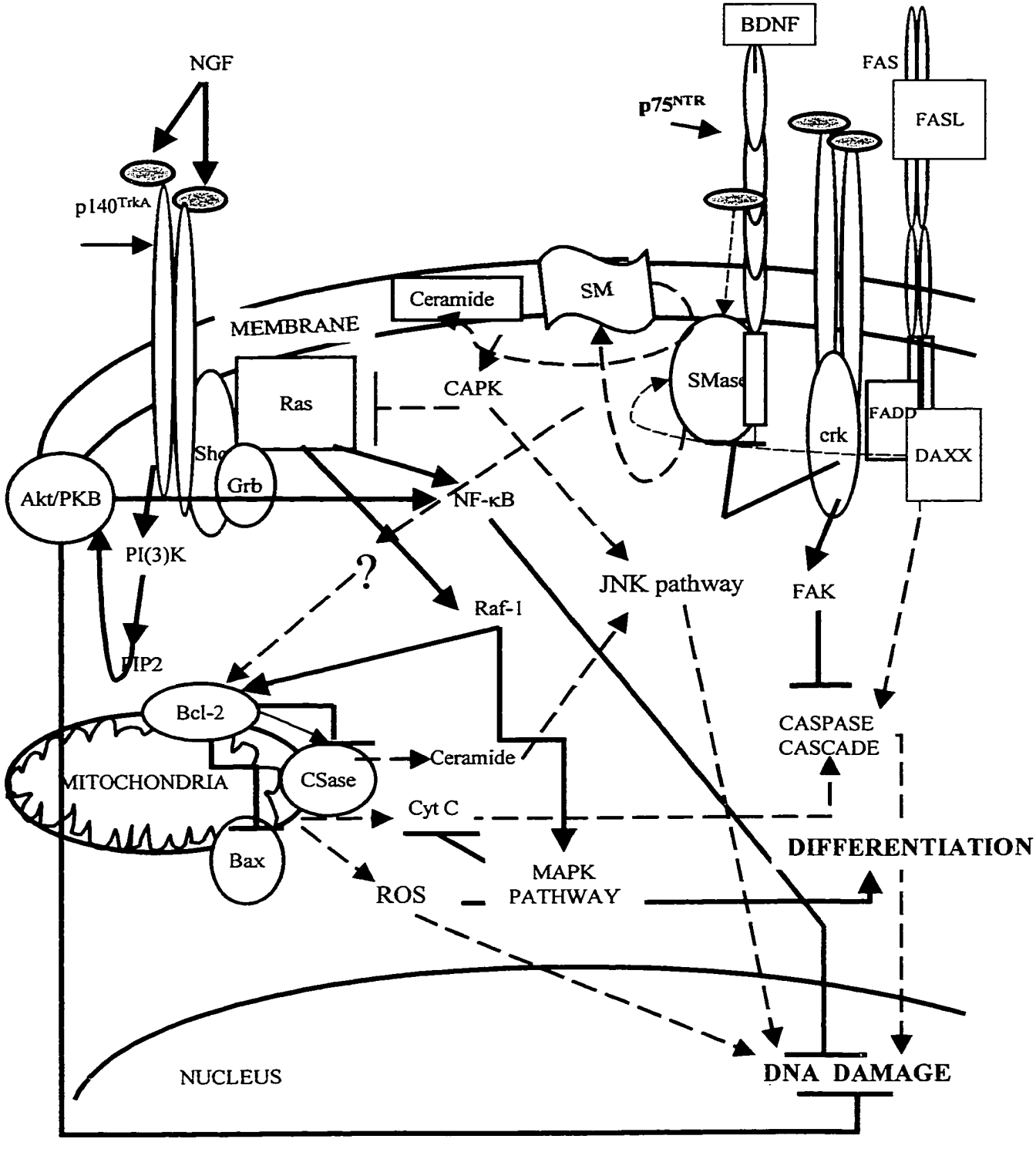


Figure 29. Proposed model of sphingolipid second messengers involvement in neurotrophin receptor signaling pathways. Pathways that result in differentiative are shown in bold, those that result in apoptosis are shown with dashed lines. NGF (grey ovals) can be bound either by the $p140^{trkA}$ receptor, or the $p75^{NTR}$ receptor, which also binds BDNF. See text for the details.



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