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Investigations into the mechanisms of methamphetamine and oxygen-glucose deprivation -induced neurodegeneration: Implications for autophagy and apoptosis

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Investigations into the mechanisms of methamphetamine and oxygen-glucose deprivation -induced neurodegeneration: Implications for autophagy and apoptosis

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biomedical Sciences (Physiology)

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Iowa State University

Ames, Iowa

2010

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LIST OF ABBREVIATIONS

3-MA	3-methyladenine
AIF	Apoptosis inducing factor
ALS	Autophagy lysosomal system
ATG	Autophagy-related genes
AV	Autophagosomal vacuoles
CCCP	Carbonyl cyanide m-chlorophenylhydrazone
CsA	Cyclosporin A
DA	Dopamine
DAG	Diacylglycerol
DAT	Dopamine transporter
dbcAMP	Dibutyral Camp
ER	Endoplasmic reticulum
LAMP-2	Lysosome-associated membrane protein 2
LDH	Lactate dehydrogenase
MA	Methamphetamine
MAP-LC3	Membrane-associated protein- light chain 3
MDC	Monodansylcadaverine
MMP	Mitochondrial membrane potential
MMT	Methylcyclopentadienl manganese tricarbonyl
mTOR	Mammalian target of rapamycin
NOS	Nitric oxide synthase
OGD	Oxygen and glucose deprivation
PCD	Programmed cell death
PD	Parkinson's disease
PI	Propidium iodide
PI3K	Phosphoinositide 3 kinase
PKCδ	Protein kinase C delta
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
SOD	Superoxide dismutase
TEM	Transmission electronic microscopy
TH	Tyrosine hydroxylase
TNF	Tumor necrosis factor
TUNEL	Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling
UPS	Ubiquitin proteasomal system

ABSTRACT

Cellular demise is a well controlled and complex process. The role of apoptosis and their contribution to disease pathogenesis are well known. Yet, emerging evidence indicates that apoptosis interplay with autophagy may be more complex than previously thought. Autophagy may serve a pro-survival or cyto-destructive role depending on the cellular context and type of stressor. In this context the requirement for PKCδ in MA and ischemia-induced apoptotic cell death is briefly discussed.

In the first portion of my thesis I investigated the cell death mechanism(s) underlying methamphetamine induced dopaminergic neurodegeneration. Oxidative stress has been shown to trigger mitochondrial damage and the induction of autophagy, a protein clearance system. We and others have previously demonstrated the induction of autophagy/apoptosis in MA-treated N27 cells; however the precise cellular mechanisms underlying MA-induced stimulation of autophagy/apoptosis remain unclear. The present study explores the following issues: (1) Does mitochondrial impairment precede autophagic induction. If so, does it occur independent of or in association with UPS dysfunction? (2) Does PKCδ, an oxidative stress sensor kinase, play a role in the mechanism of MA-induced cell death? If so, does it activate caspase-3 dependent cell death events? (3) What is the exact contribution of autophagy, is it cytoprotective or prodeath mechanism? Taken together, our results suggest that MA-induced dopaminergic neurotoxicity is likely caused, at least in part, by pronounced proteolytic cleavage of PKCδ and associated oxidative cell signaling events. Additionally, MA, through the induction of mitochondrial stress, activates autophagy as a cytoprotective mechanism to minimize the magnitude of neuronal cell death.

In the second portion of my thesis I evaluated the role of PKCδ dependent cell death signaling events in striatal neuronal degeneration. Striatal ischemia is also often associated with oxidative stress associated cell death following reperfusion post cerebral ischemia. Using an OGD model of ischemia we demonstrated the occurrence of apoptotic cell death in mouse primary striatal neurons. As a key detector of oxidative stress, depolarization of mitochondria membrane potential and cytochrome c release into the cytoplasm are signs of mitochondrial impairment. Additionally, downstream events involve caspase-3 cleavage and PKCδ activation. Caspase-3 mediated proteolytic cleavage of PKCδ in the striatum as a result of ischemic insult is a novel finding which suggests PKCδ plays a regulatory role in striatal neuronal cell death. This finding is further supported from our data showing that cell death is attenuated in PKCδ knockout mice. Previous studies have shown neuron subtype specific susceptibility to ischemic insult in the striatum. Our investigation reveals that GABAergic neurons are more vulnerable to cell death during striatal ischemia, confirming different neuronal subtypes exhibit varying degrees of vulnerability to ischemic damage.

In conclusion, despite the use of two different types of cellular injury models, namely MA and OGD-induced neurotoxicity, our findings underscore the importance of caspase-3 mediated PKCδ dependent cell death events in apoptotic cell death. This study demonstrates that PKCδ might represent a common cell death mechanism, facilitating neuronal demise following exposure to diverse cell stressors that are capable of causing apoptotic cell death.

Chapter I: Background and Literature Review

THESIS ORGANIZATION

The present thesis contains two separate manuscripts, both prepared for submission to Journal of Neurochemistry. Titles include "PKCδ is a key mediator of MA-induced cell death in N27 dopaminergic neuronal cells: Role for autophagy and UPS," and "Proteolytic activation of PKCδ is involved in neuronal death after OGD in mouse primary striatal cultures." Each paper has been incorporated into chapters II and III respectively. Below is a literature review for each manuscript.

PKCδ IS A KEY MEDIATOR OF MA-INDUCED CELL DEATH IN N27 DOPAMINERGIC NEURONAL CELLS: ROLE FOR AUTOPHAGY AND UPS

Methamphetamine-induced Cytotoxicity in Neuronal Cells

Methamphetamine (MA) can cause neurotoxic damage to the monoaminergic system in the rodent and primates. Several dopamine (DA) markers are affected by this drug in the rodent brain, including decrease in tyrosine hydroxylase activities, depletion of striatal dopamine levels and loss of dopamine transporters (Seilden et al., 1987; Cadet et al., 1998; Krasnova et al., 2005). These observations taken together indicate that Methamphetamine causes degeneration of the striatal projections of dopamine neuron terminals.

Both reactive oxygen species (ROS) and reactive nitrogen species (RNS) have been known to be involved in several neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease and amyotrophic lateral sclerosis (Imam et al., 2001; Cadet et al., 1998). Many earlier studies have shown that the role of oxygen-based free radicals may be a critical event in MA-induced neurotoxicity (Yamamoto and Zhu et al., 1998; Imam et al., 1999). Administration of antioxidants, such as ascorbic acid or vitamin E, attenuates MA-induced

neuronal toxicity (Wagner et al., 1985); conversely inhibition of superoxide dismutase (SOD) increases its neurotoxicity (Wagner et al., 1989). MA-induced neurotoxicity is also associated with the production of hydroxyl and superoxide radicals (Krosnova et al., 2001), which are thought to participate in the toxic effects of MA via free radical-mediated protein oxidation and DNA damage. The increase in superoxide radicals is probably due to the release of DA in the brain after MA administration (Baldwin et al., 1993), a secondary effect of the drug–induced redistribution of DA from synaptic vesicles to the cytosol followed by its release into the extracellular space by DA transporters (DAT) resulting in increased DA levels in the synaptic cleft (Sulzer et al., 1995).

The monoamine drug, Methamphetamine, is often used as an experimental model for Parkinson's disease (PD) due the close resemblance to disease pathophysiology. Recently, Fornai et al., showed that MA-induced multilamellar whorls are α-synuclein and ubiquitin positive intracellular whorls, which suggest dysfunction of the ubiquitin-proteasome system (UPS), hence, sharing similarities with the Lewy bodies present in PD (Fornai et al., 2004b; Lazeri et al., 2007). Consistent with previous reports (Chu et al., 2006; Larsen et al., 2002), autophagosomal vacuoles (AV) induced by MA administration in midbrain neurons, appear to play a role in the clearance of these protein aggregates (aggresomes). The AVs in several studies showed engulfment of a variety of cytosolic organelles and contain partially digested materials that are positive for ubiquitin.

Autophagy and Programmed Cell Death (PCD)

Autophagy is characterized by the process of having cytoplasmic organelles or long-lived proteins delivered to lysosomes for enzymatic degradation. In recent years, growing

number of studies have highlighted the relationship of autophagy-induced cytoprotective response with protein aggregation, and its potential in the clearance of damaged proteins. Autophagy has been termed as Type II programmed cell death (PCD) and is characterized by the accumulation of autophagic vacuoles. Increased accumulation of autophagic vacuoles have been described in Parkinson's disease, Alzheimer disease, Huntington disease, prion disease, amyotrophic lateral sclerosis and many other neuronal diseases. Lewy bodies is a major protein in PD, α-synuclein induces autophagic vesicles along with impairment of UPS (Shintani et al., 2004).

Autophagy involves the formation of the double membrane structures called autophagosomes which expands from a phagophore while surrounding a portion of the cytoplasm and sequestering organelles. Subsequently, they fuse with the lysosome to form autolysosomes (Fig. 1). Contents within the autophagosomes are then degraded by the lysosomal enzymes, including acid hydrolase and permease (Klionsky 2007; Chu et al., 2006). Membranes from engulfed organelles become compacted and can form multilamellar whorls, particularly at the autolysosomal stage (Iacovelli et al., 2006). This forms the basis for accumulation of an autofluorescent chemical monodansylcadaverine (MDC), which has an affinity for acidic membrane-rich compartments. Though MDC is often used as an indicator for acidic vacuoles and labeling autophagic vacuole (AV) maturation, it cannot be used as a specific AV marker (Chu et al., 2006). Alternatively, the membrane associated protein light chain 3 (MAP-LC3) is a highly useful AV marker (Mizushima et al., 2008), which is a mammalian analog of Atg 8 (autophagy-related genes).

A significant conundrum that has emerged recently since 1970s when Beaulaton and Lockshin first suggested a role for autophagy in cell death, suggesting that there might be

coordination between apoptosis and type II cell death. Many subsequent studies have shown that although in most situations, autophagy may initially function as a cytoprotective mechanism, but as the damage becomes too extensive, cells may undergo apoptosis or excessive autophagy and may lead to cell death ultimately (Klionsky 2007). Understanding the dual role of autophagy will improve our knowledge on the signal transduction pathway that regulates these processes

Fig. 1

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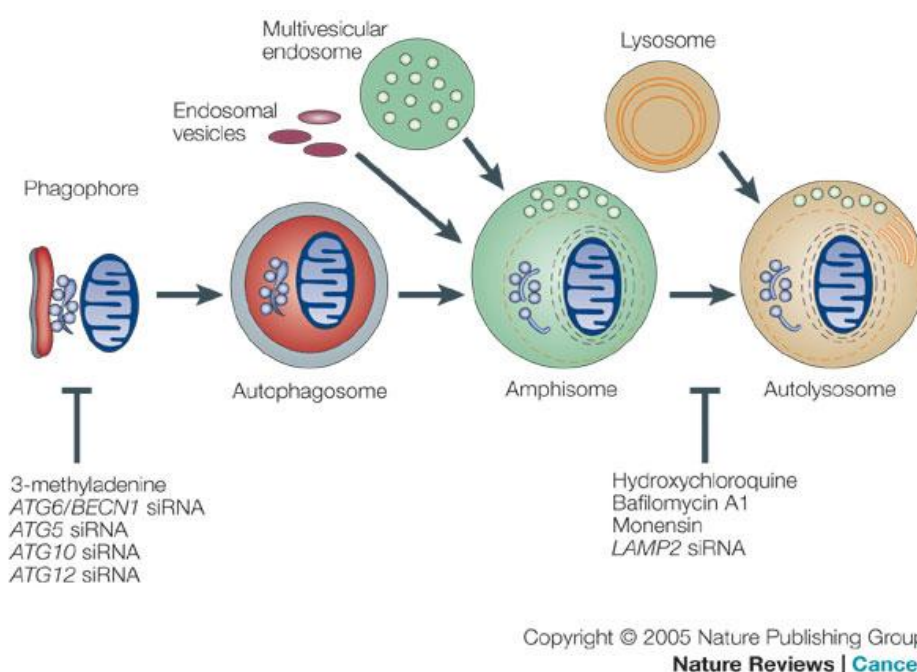
Figure 1. The formation of autolysosomes and inhibitors effective at difference stages in the formation process. (Source: Nature Reviews, 2005)

Genes Involved in Autophagy

Autophagosome formation involves a series of autophagy related genes (ATG genes), which were first discovered in yeast. There are more than 20 genes in yeast that encode proteins essential for the autophagy process (Levine et al., 2006). Many of them participate

in an ubiquitin-like conjugation, and have been categorized into two conjugation systems: the Atg12-Atg5 conjugation system and Atg8 (LC3)-PE conjugation system (Ferraro et al., 2007). Atg12 is an ubiquitin-like protein that is firstly activated by Atg7, which works as an ubiquitin-activating enzyme E1, and covalently links to Atg5 to form conjugates. Atg10 is involved in this process, functioning as an E2-like enzyme for Atg12-Atg5 conjugation (Yang et al., 2005). Multiple Atg12-Atg5 conjugates are then cross-linked with Atg16 to form Atg12-Atg5-Aatg16 complexes for pre-autophagosomal membrane elongation (McCray et al., 2008; Yang et al., 2005).

The other ubiquitin-like protein essential for autophagy is Atg8, the mammalian ortholog of which is MAPL1LC3 (microtubule-associated protein 1 light chain 3). LC3 is proteolytically cleaved by Atg4 before activation by E1-like enzyme Agt7. LC3 is then transferred to E2-like protein Atg3 that catalyzes LC3 to an active form of LC3-II (membrane bound) and conjugates to the lipid phosphatidyl-ethanolamine (PE). This lipidation of LC3 leads to a conformational change essential for its binding to the membranes of pre-autophagosomes and autophagosomes. LC3-PE conjugations are located on the outer surface of autophagosomes and are removed prior to fusion with lysosomes. To date in mammalian cells, LC3 is the most reliable marker for autophagosome formation, and the relative amount of LC3-II reflects the abundance of autophagosomes. Consequently, LC3 staining is used widely as a histological marker for autophagosomes, and accumulation of LC3-II is used for the detection of autophagic activity.

Other genes related to autophagy include Atg6, which its mammalian ortholog is Beclin 1. Atg6 belongs to a class III phosphoinositide 3-kinase (PI3K) complex, participates in early autophagosome formation that might take place before Atg5-Agt12 and LC3-PE in the

autophagosome formation process (Baehrecke, 2005). Atg1 complex and Atg9 complex also plays a role in autophagosome formation mechanism. Phosphorylation of some of the Atg proteins is one of the mechanisms through which autophagy is regulated. On the other hand, a better studied central signaling pathway is through mammalian target of rapamycin (mTOR), a serine/threonine kinase. Rapamycin inhibits mTOR and induces autophagy while activation of mTOR inhibits the process (Chu, 2006). The biochemical mechanism by which mTOR kinase inhibits autophagy is not fully understood, but it involves mTOR inhibiting autophagy by controlling both transcription and translation and by modifying Atg proteins (kinase Atg1 in yeast) and therefore interfering with the formation of autophagosomes (Baehrecke, 2005; Kamada et al., 2000; Klionsky, 2005). Activation of class I phosphatidylinositol 3-kinase (PI3K) leads to the phosphorylation of plasma membrane lipids that recruit and activate Akt/protein kinase B, an upstream regulator of autophagy. Activation of Akt/PKB represses autophagy, tuberous sclerosis complex and small G protein Rheb (Baehrecke, 2005) link tyrosine receptor kinases to mTOR activation and thereby repress autophagy in response to insulin-like and other growth factor signals. The relationship of these genes in autophagy formation process is illustrated in Figure 2.

Fig. 2
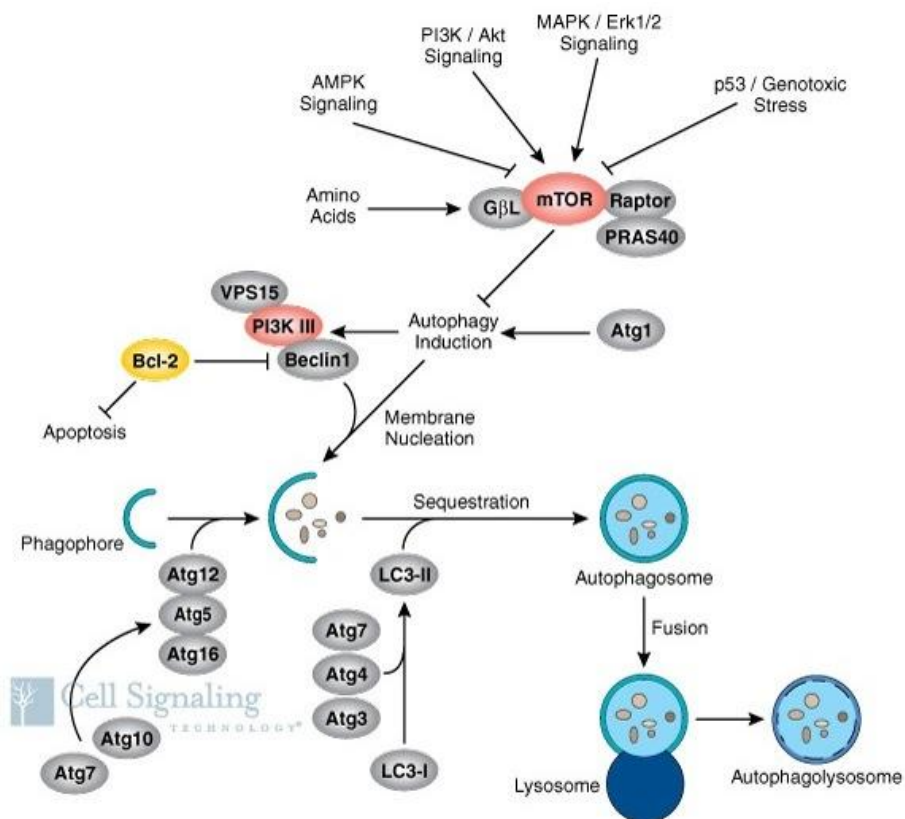
Figure 2. Genes involved in autophagy formation process. (Source: Cell Signaling)

Oxidative Stress and its Role in Autophagy Induction

The role of reactive oxidative species (ROS) has been extensively investigated in many neurodegenerative diseases, including Parkinson's disease, Alzheimer's disease and Huntington disease, as well as in aging and drug abuse in the CNS. In order to prevent damage caused by free radicals generated under these conditions, cells activate different mechanisms in response to oxidative stress (Kaushik et al., 2006). Various defense mechanisms have been discovered to protect cells during oxidative stress, including up-regulation of antioxidants, removal of specific damaged proteins by the ubiquitin-proteasome system and removal of damaged proteins and organelles by the autophagy-lysosomal system

(Scherz-Shouval et al., 2007a). Autophagy occurs at a low basal level in all cells and it contributes to homeostatic functions such as protein and organelle turnover. However, it is rapidly upregulated in stressful situations such as nutrition deprivation, growth factor depletion and hypoxia. Autophagy is also upregulated in cells that are exposed to toxins and stressors and are in need to rid themselves of damaging cytoplasmic components, including oxidative stress, accumulation of aggregate prone proteins (Levine et al., 2006). A study recently provides evidence that generation of hydrogen peroxide during starvation serves as a signaling molecule that initiates autophagosome formation (Scherz-Shouval et al., 2007b). Since defective mitochondria and peroxisomes produce more hydrogen peroxide than normal ones, it is of great importance for cells, especially in long-lived postmitotic cells, to selectively degrade dysfunctional organelles (Liu et al., 2004).

Methamphetamine-related quinone formation is thought to be associated with the generation of superoxide radicals and hydrogen peroxide during quinone redox cycling. Dopamine release caused by MA is accompanied by redox cycling of dopaquinone and consequent formation of oxygen-based radicals such as superoxide radicals. Additionally, MA can induce changes in the levels of glutathione and of antioxidant enzymes, increase lipid peroxidation and induce formation of protein carbonyls, which provide further amplification of oxygen-based radicals induced neurotoxicity in drug treated cells (Cadet et al., 1998).

Mitochondrial ROS and Autophagy

Mitochondria plays a central role in cellular metabolism and apoptosis. During apoptosis, a variety of cellular signals activated from stimuli can be sensed by the

mitochondria and reacts by releasing proapoptotic proteins into the cytosol such as cytochrome c, an upstream regulator of apoptosis (Ko et al., 2005). Upon release, it can then activate downstream effector caspases, leading to apoptosome complex (Apaf-1 and caspase-3, capase-9) formation (Katoh et al., 2004). An indicator of the cell viability is the maintenance of mitochondrial inner membrane transmembrane potential (Iijima, 2006). Electron transport along the respiratory chain generates mitochondrial membrane potential, this electrochemical gradient is essential for ATP synthase activity in the phosphorylative pathway. A frequent occurrence in the mitochondrial electron transport chain is the escape of electrons, results in the production of oxygen radicals, which are often converted into hydrogen peroxide or other reactive oxygen species (ROS). Mitochondria are a major source of intracellular ROS production, and ROS has been implicated as second messengers and are known to participate in physiological processes, such as apoptosis and proliferation (Ko et al., 2005).

Mitochondrial ROS is normally detoxified by superoxide dismutase (SOD), glutathione peroxidase, heme peroxidase and catalase (Bandyopadhyay et al., 1999). SOD lowers the steady state levels for superoxide (O_2^-), while catalase and peroxidases lower the steady state levels for hydrogen peroxide (H_2O_2). In addition to the primary defense against ROS by antioxidant enzymes, secondary defense are free-radical scavengers, such as ascorbate and reduced glutathione (Bandyopadhyay et al., 1999). However, when elimination rates of ROS is overwhelmed by ROS generation, accumulation of ROS results in the mitochondrial damage. It is known that TOR inhibits autophagy, which also regulates mitochondrial biogenesis and activity. By inhibiting autophagy, TOR allows defective mitochondria to accumulate (Blagosklonny, 2007).

## Ubiquitin-Proteasome System (UPS) and Autophagy

Pathways involved in protein degradation are classified into two major pathways, namely, the lysosomal pathway and nonlysosomal pathway. The lysosomal pathway is responsible for degradation of extracellular proteins by endocytosis and for bulk degradation of long-lived proteins and organelles by macroautophagy. The nonlysosomal pathway is characterized by the highly selective degradation of short-lived proteins under basal metabolic conditions. This process involves tagging susceptible proteins with ubiquitin followed by proteasome hydrolysis (Larsen et al., 2002). This pathway is called ubiquitin–proteasome system (UPS), a primary mechanism for degrading and elimination of misfolded intracellular proteins. The presence of ubiquitinated proteins is an important hallmark of neurodegeneration (Iacovelli et al., 2006). The UPS becomes abnormally activated during oxidative stress, inappropriate gene splicing and abnormal proteolytic cleavage. Under these conditions, the UPS may not be able to function normally and degrade the damaged proteins, resulting in protein accumulation in the cells leading to ultimate cell dysfunction and cell death (Chung et al., 2001).

UPS dysfunction has been shown to be prevalent in Parkinson's disease (PD), where pathological changes include loss of dopaminergic neurons in the substantia nigra pars compacta and its characteristic accumulation of intracytoplasmic inclusion bodies. Although the molecular mechanism of the loss of dopaminergic neurons is not fully understood, UPS dysfunction has been proposed as one of the factors in PD pathogenesis (Zafar et al., 2007). We have previously shown that dieldrin, a potential PD producing pesticide, induces neurodegeneration via UPS dysfunction (Sun et al., 2005). Furthermore,

it has also been demonstrated by our research group that proteasome inhibitor MG132 leads to apoptotic cell death in N27 immortalized dopaminergic neuronal cells and the loss of tyrosine hydroxylase-positive neurons in mouse substantia nigra when administered *in vivo* (Sun et al., 2006).

Impairment of the UPS occurs in several neurodegenerative diseases as well as cellular toxicity induced by amphetamine derivatives, such as methamphetamine (MA). Studies have shown that MA induces ubiquitin-positive intracellular bodies within dopaminergic neurons in the substantia nigri (Fornai et al., 2004a). A recent study in chronic MA abusers demonstrates the occurrence of ubiquitin-positive inclusions within nigral dopaminergic cells (Quan et al., 2005). Another study showed that MA-induced ubiquitin inclusions only occur in dopaminergic neurons, and not formed in non-dopaminergic striatal neurons (Fornai et al., 2007). Increased extracellular DA levels induced by MA is coincident with increased levels of ROS (Lazzeri et al., 2007), leading to early stage aggregates often known as autophagic granules seen after MA administration (Larsen at el., 2002). These neuronal inclusions consist of proteins belonging to the multi-enzymatic complex, the UPS (Iacovelli et al., 2006). Recent studies have shown that there is a compensatory relationship between the two protein degradation pathways, UPS and autophagy-lysosomal pathway (Fig. 3).

Fig. 3
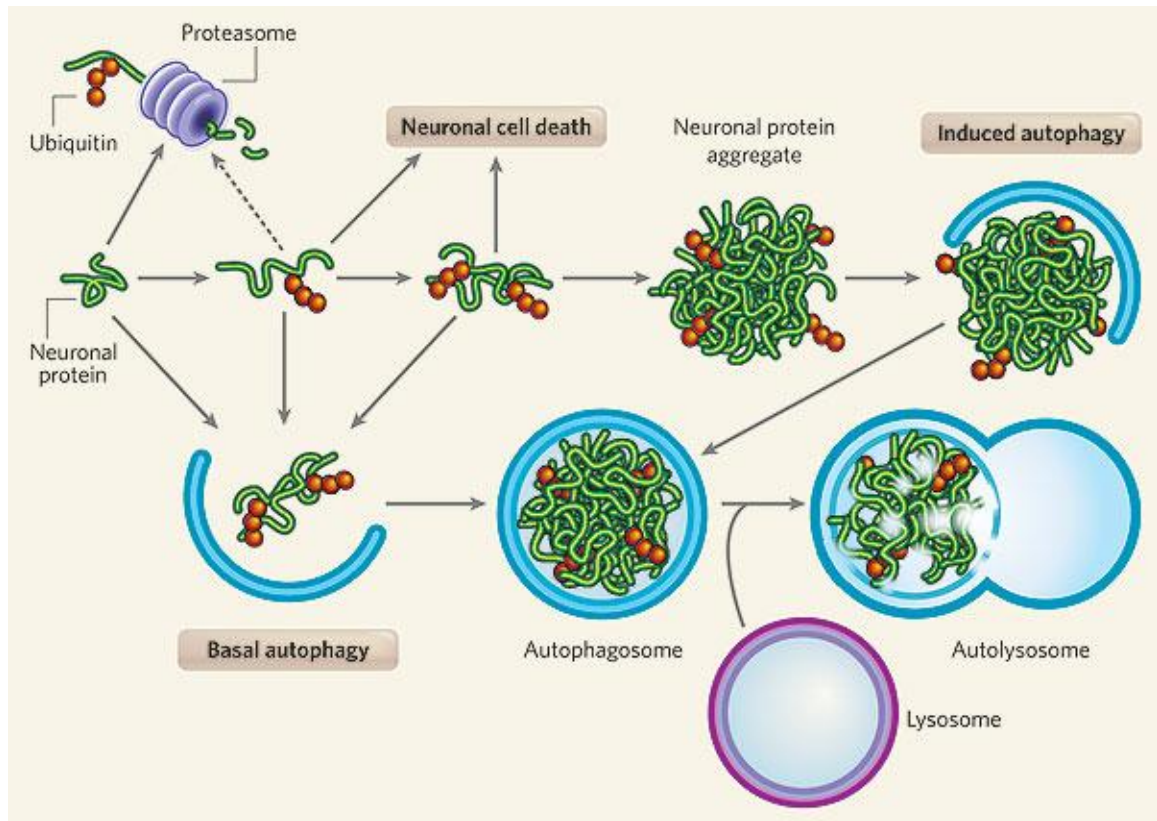
Figure 3. Association between UPS and autophagy suggests UPS as a possible trigger for autophagy. (Bate et al., 2009)

Protein Kinase C Delta and Apoptosis

Apoptosis is a highly regulated cellular suicidal program. It is controlled by a complex cellular signaling network, several members of the protein kinase C (PKC) family have been identified as a substrate for caspases (Griner et al., 2007). PKC belongs to a family of phospholipid-dependent serine/threonine members, which plays a central role in growth factor signaling pathway and regulatory cellular functions, including cell proliferation, cell

differentiation, and cell death (Busa, 2003). PKC family is composed of many isozymes that can be categorized into three groups including the classical PKCs (cPKCs): α, βI, βII and γ; novel PKCs (nPKCs): δ, ε, η and θ; atypical PKCs (aPKCs): ζ, λ and ι (Maher, 2001; Zhang et al., 2007). While cPKCs require Ca^{2+} and diacylglycerol (DAG)/phorbol esters for their activities, nPKCs do not require Ca^{2+} for activation but are still activated by DAG/phorbol esters. aPKCs are independent of both Ca^{2+} and diacylglycerol (DAG)/phorbol esters (Griner et al., 2007). PKCδ, which is a member of nPKC, is primarily activated through DAG in the C1 domain at the plasma membrane. Another activation mechanism involves response to phorbol esters, PKCδ can be activated by proteolysis phorphosphorylation and organellar translocation during apoptosis (Emoto et al., 1995; Griner et al., 2007).

The intracellular distribution of PKCδ is altered upon activation (Kraft et al., 1983). It is demonstrated that PKCδ can translocate from the cytosol to the mitochondria (Li et al., 1999). In that study, PKCδ activation by TPA resulted in cell death in keratinocyte cells, suggesting that PKCδ translocation to the mitochondria serves as a precursor to cell death. Additionally, PKCδ translocation to the nucleus from cytoplasm in response to various apoptotic agents including gamma-irritation and Fas-ligand has been demonstrated (Blass et al., 2002; DeVries et al., 2002). In fact, nuclear localization signal on the COOH-terminal in PKCδ has been shown to be required for both nuclear localization and apoptosis (DeVries et al., 2002). Furthermore, PKCδ is also a caspase-3 substrate, expression of the catalytic fragment cleavage product is a potent inducer of apoptosis in several cell types (Denning et al. 2002; Sitailo et al., 2004; D'Costae et al., 2005). Previously, we have demonstrated that proteolytic activation of PKCδ plays a critical role in oxidative stress-induced dopaminergic cell death (Anantharam et al., 2002; Kaul et al., 2003; Yang et al., 2004). Proteolytic

cleavage of PKCδ (74kDa) by caspase-3 results in a 41kDa catalytic subunit and a 38 kDa regulatory subunit, leading to persistent activation of the kinase (Kaul et al., 2003; Yang et al., 2004). Blockage of proteolytic activation of PKCδ by overexpression of the kinase-dominant-negative PKCδ mutant, cleavage-resistant PKCδ mutant or siRNA directed against PKCδ almost completely prevented the dopaminergic cell death (Kaul et al., 2003; Kitazawa et al., 2003; Yang et al., 2004).

Programmed cell death is mediated, in part, by caspases. Caspases, a family of cysteine proteases that cleave proteins after aspartate residues, are essential for the execution of apoptosis (Cohen, 1997). Caspase-8, -9, -10 participate in the initiation of phase of the apoptosis whereas caspase-3, -6 and -7 are involved in the execution phase of apoptosis, caspase-2 can function as both initiator and executor caspase (Duan, 1997). Activation of the effector caspase leads to cleavage of many cellular proteins that trigger nuclear breakdown, loss of mitochondrial membrane potential and cell death. Caspase-3 is an effector caspase that can be activated by both caspase-8 and caspase-9. Despite PKCδ being a target of caspase-3 and is activated relatively late in the cell death signaling pathway, inhibition of PKCδ can reduce apoptotic activity during oxidative stress and other chemical (drugs) or physical (UV radiation) damage in the cells (Sitailo at al., 2004). In addition, organochlorine pesticide such as dieldrin has been shown to activate caspase-3 that proteolytically triggers PKCδ and induce apoptotic cell death in dopaminergic cells (Kitazawa et al, 2003). These reports suggest a positive feed back amplification loop set by PKCδ activation that amplifies the caspase cascade and helps the cells commit to cell death.

Two major pathways of cell death have been identified: the receptor-mediated or extrinsic pathway and the mitochondrial or intrinsic pathway (Busa, 2003). The extrinsic

pathway is greatly studied in the tumor necrosis factor-alpha (TNF-α) superfamily, associated with the binding of ligand to receptors, which activate caspase-8, an initiator caspase (Ashkenazi and Dixit, 1998). In the intrinsic cell death pathway, characterized by the involvement of the mitochondria, the release of cytochrome c and other proapoptotic events which has been described in the previous sections. In recent years, protein kinase C (PKC) has been implicated to involve in several ischemic and reperfusion insults in multiple organs (Downey and Cohen, 1997; Padanilam, 2001). However, most of this work has been focused on the role of PKC in the cardiac system. Ischemic insult can result in activation of several PKC isozymes, which may mediate different and sometimes opposing functions (Bright et al., 2005).

Growing body of literature support the involvement of PKCδ proteolytic activation in the apoptosis pathway, including caspase activation and regulation of mitochondrial function (Brodie and Blumberg, 2003). This intrinsic pathway has also been shown previously by our group (Anantharam et al., 2002). Treatment of dopaminergic cells with methylcyclopentadienyl manganese tricarbonyl (MMT), an additive to the gasoline in the 1960s, induces caspase-3 mediated proteolytically cleaved PKCδ- dependent apoptosis. The pathway involved cytochrome c release from the mitochondria via caspase-3 dependent proteolytic cleavage of PKCδ. Thus far, several studies from our group (Kanthasamy et al., 2003) have confirmed that PKCδ activation by proteolytic cleavage plays a key role in promoting cell death, especially in neuronal cells.

PROTEOLYTIC ACTIVATION OF PKCδ IS INVOLVED IN NEURONAL DEATH AFTER OGD IN MOUSE PRIMARY STRIATAL CULTURES

Striatal Ischemia

Ischemic stroke is a complex event and the region of the focal stroke can be recognized as either the core territory or the ischemic penumbra. The core is the center of the territory, characterized by blood flow deficits, low ATP or energy levels, ionic disruptions and metabolic failures, all contribute to the fast cell death progress. The ischemic penumbra, which is the peripheral region of the ischemic stroke, suffers milder insults, due to residual perfusion from the blood vessels (Lo, et al., 2003). However, as ischemic stroke is normally treated by restoration of oxygen to the brain in a normal medical procedure, a result of oxygen reperfusion (re-oxygenation) after ischemic insult, this can actually cause immediate cell death of originally surviving cells. This form of apoptotic cell death is termed "delayed ischemic cell death" (Mattson et al., 2000). The precise mechanism for this type of cell death is not fully understood. The cell death induced by cerebral ischemia is complex; this form of delayed cell death occurring after cerebral ischemia may result from at least three mechanisms: excitotoxicity and ionic imbalance, oxidative/nitrosative stress, and apoptotic-like cell death (Lo et al., 2003). Neurons have high metabolic rates and little energy is reserved and mitochondria serves as the main energy source in the brain. Consequently, disturbances of the mitochondrial function can lead to ATP depletion and release of apoptotic inducing factor (AIF) from the mitochondria (Panickar et al., 2005).

Necrotic and apoptotic cell death is another way of categorizing ischemic neuronal death. Necrosis is an acute cell death procedure occurring shortly after ischemic insult and in the core of the ischemia area, while apoptosis is a slowly progressing cell death procedure happening in the peri-infarct zone (Iijima, 2006). Recently, mitochondria are focused as the

regulators of neuronal death, due to their involvement in AIF release. Increased glutamate during ischemia opens the NMDA receptor, causing Ca^{++} abrupt release and resulting in neuronal death (Iijima, 2006). This Ca^{++} deregulation stresses the importance of Ca^{++} and Na^+ homeostasis for cell survival.   Sustained increase in intracellular Ca^{++} even after reperfusion leads to activation of Ca^{++}-dependant enzymes and initiation of apoptotic cascade. Disruption of the Ca^{++} buffering system, leads to Ca^{++} deregulation as a result of depolarization of the mitochondrial membrane and trigger Ca^{++} efflux from matrix to the cytoplasm, ultimately cell death (Kruman et al., 1999). The following studies highlight the importance of mitochondrial homeostasis in ionic homeostasis and initiation of apoptosis cascade.

Mitochondrial release of apoptotic factors is influenced by the Bcl-2 family, which includes Bax, Bak, Bad, Bim, Bid, as well as proteins that are anti-apoptotic, including Bcl-2, Bcl-xl (Daniel and Korsmeyer, 2004).   Bid is a proapoptotic protein that promotes cytochrome c release and activation of caspase-8 substrate, which then activate the cell death pathway, hence contributing to ischemic cell death. Furthermore, Bid cleavage was detected before caspase-3 cleavage, suggesting it is an upstream mediator. Consistent with its proapoptotic role, cell death was significantly reduced in Bid knockout mice (Plesnila et al., 2001). Taken together, studies suggest that Bid may serve as a critical mediator of cell death, via mitochondrial dysfunction.

Neuronal Subtype and Classification

Striatal neurons have been known to be susceptible to cerebral ischemia and loss of striatal GABAergic projection neurons is one of the hallmarks in transient ischemia. GABAergic neurons are very sensitive to ischemic effects in the striatum. GAD is a rate

limiting enzyme in GABA biosynthesis that has been used to express GABA-ergic neurons in the brain. Intrastriatal neurons consist of ~90% medium spiny projection neurons and ~10% interneurons (Marin et al., 2000). There are several subtypes in the GABAergic neuronal population, including the Ca^{++} binding protein positive neurons, calbindin, calretinin or parvalbumin, in the brain regions (Schwaller et al., 2002; Ulfig, 2002), where they function as Ca^{++} buffering system. In the adult striatum, the majority of the medium spiny projection neurons (75-82%) display calbindin immunoreactivity (Parent et al., 1996; Falk et al., 2006). Whether calbindin plays a protective role in the CNS insult is controversial and existing data are conflicting (Yenari et al., 2007). Intracellular Ca^{++} accumulation is known to potentiate ischemia induced injury in the brain (Kristian et al., 1998). Some studies have shown that Ca^{++} binding protein (CaBP) containing neuronal populations are better able to survive ischemic (Goodman et al., 1993) and excitotoxic insult (Mattson et al., 1991) than those that lack CaBP. While these studies suggest the beneficial role of the CaBP, others have suggested that the presence of CaBP has nothing to do with the resistance to CNS insults (Freund et al., 1992). In a study using overexpressing CaBP with viral vector, protects neurons from stroke insult (Yenari et al., 2007), supporting the protective role of CaBP.

Role of PKCδ in Cerebral Ischemia

As previously discussed, phosphorylation of PKCδ can regulate the proteolytic activation of the kinase during oxidative stress, which consequently influences the apoptotic cell death in dopaminergic neuronal cells (Kaul et al., 2005). PKCδ is shown to be an

oxidative sensitive kinase that can be activated by caspase-3 dependent proteolytic cleavage to induce apoptotic cell death in cell cultures of Parkinson's disease (Kaul et al., 2003; Kanthasamy et al., 2004). In this study, we recapitulated ischemic stress by using oxygen-glucose deprivation techniques in primary striatal neurons. The role of mitochondria mediated oxidative cell signaling event is discussed below.

Caspases are activated during cerebral ischemia (Krajewski et al., 1999; Plesnila et al., 2001) and ischemic cell death can be attenuated with caspase inhibitors (Le et al., 2002; Hara et al., 1997). Among the many caspases involved in cell death, caspase-3 is crucial in neuronal pathological conditions, including cerebral ischemia (Nicholson, 1999). Caspase-3 cleavage has been reported in many studies associated with oxygen/glucose deprivation, a well-characterized model for cerebral ischemia research (Krajewski et al., 1999; Shen et al., 2004). In the same study, they showed nicotinamide (vitamin B_3) may be acting through the mitochondrial pathway, blocking capase-3 cleavage and protecting cells from ischemic cell death. Another study showed blockage of caspase-3 with neurotrophins in cerebral ischemia injury, protected the neurons from apoptotic cell death (Kim et al., 2005). Likewise, in a caspase-3 knockout mice study, neuroprotection in cerebral ischemia-induced damage suggested caspase-3 as a central mediator in cerebral ischemic cell death (Le et al., 2002).

The mode of cell death correlates well with other studies observed during delayed cerebral reperfusion injury which contributes to irreversible brain damage seen after stroke (Lipton, 1999). Increase in PKCδ expression was noted in the penumbral area, suggesting a role for this isozyme in reperfusion injury (Phan et al., 2002). Bright et al showed that PKCδ selective inhibitor peptide δV1-1 protected against cerebral ischemic-reperfusion damage in both in vivo and in vitro models of cerebral ischemia.

Oxidative Stress in Cerebral Ischemia

Injury to the brain in cerebral ischemia is caused from the interruption of blood flow, and then a subsequent reoxygenation (ischemia/reperfusion). The brain is exceptionally sensitive to oxidative damage due to its high use of oxygen and glucose and enrichment of peroxidizable fatty acids and Fe ascorbate/ Fe pro-oxidants (Floyd and Carney, 1992). Cerebral ischemia involves several complex metabolic events, including the generation of nitrogen and oxygen free radicals. Increased production of free radicals in cerebral ischemia can arise from several mechanisms including glutamate stimulation of NMDA receptors (Lafon-Cazal et al., 1993), mytochondrial dysfunction (Braughler and Hall, 1989), activation of neuronal nitric oxide synthase (Lipton and Rosenberg, 1994), cyclooxigenase 2 (Iadecola et al., 1999), metabolism of free fatty acids released during ischemia (Schmidley, 1990) and others. These free radicals and other reactive oxygen species mediate much of the damage caused by transient cerebral ischemia, resulting in molecular pathological conditions such as damage to cellular membrane, proteins and nucleic acids and induction of apoptotic events. Major players include nitric oxide, which are generated by nitric oxide synthases (NOS), specifically NOS 1 in ischemic damaged neurons, and superoxide, which is activated through nitric oxide synthases, xanthine oxidase, leakage from the mitochondrial electron transport chain and other mechanisms (Love, 1999). The toxicity of nitric oxide, superoxide and peroxynitrite results in modifications of macro molecules, especially DNA, and induces apoptotic and necrotic cell death pathways. Reoxygenation during reperfusion provides oxygen as a substrate for numerous enzymatic oxidation reactions and for mitochondrial oxidative phosphorylation to produce adenosine triphosphate (Saito et al., 2005). Oxygen

free radical reactions and oxidative damage are held in check by antioxidant defense mechanisms. Superoxide dismutase (SOD) is an endogenous antioxidant that catalyses the dismutation of the superoxide anion radical. SOD plays an important role in the defense against free radical damage in reperfusion injury and helps in reducing the infarct size during ischemia and reperfusion (Kim et al., 2002; Zimmermann et al., 2004). However, when excessive amount of oxygen free radicals are produced or defense mechanisms are impaired, oxidative stress may result in aging, carcinogenesis and stroke (Floyd, 1990). Subtle changes, such as oxidative damage induced loss of glutamine synthetase activity, is a key event in stroke induced brain injury (Nakamura and Stadtman, 1984). Another determination of oxidative injury in cerebral ischemia is basal level of NAD^+, which is depleted in oxidative-damaged brain (Nicotera et al., 1997). Thus, oxidative stress dependent cell signaling events play a role in ischemia-induced brain damage.

Oxidative stress is caused by the imbalance between production of pro-oxidants and the antioxidant defenses. Dietary and supplemental natural antioxidants, such as vitamin C (Henry PT, Chandy MJ, 1998) and vitamin E (van der Worp H.B. et al., 1998), reduce infarct size and neurological impairment after permanent ischemia or ischemia followed by reperfusion in rats and primates. Vitamin E prevents apoptosis due to transient ischemia in stroke-prone spontaneously hypertensive rats (Tagami M. et al., 1999). In addition, genetic manipulation of intrinsic antioxidants and factors in the signaling pathways has provided substantial understanding of the mechanisms involved in cell death/survival signaling pathways and the role of oxygen radicals in ischemic cerebral injury (Saito et al., 2005).

In addition, there is increasing evidence that free radicals can act also by redox sensitive signal transduction pathways (Chan, 2001). Experimental studies suggests that free

radicals can induce cytochrome c release from mitochondria, which is an important step in the induction of apoptosis (Fujimura et al., 1999; Kim et al., 2000). Moreover, the contribution of oxidative stress to apoptotic cell death caused by cerebral ischemia is demonstrated by the efficacy of antioxidant treatment in attenuating caspase-3 activation, DNA fragmentation and lesion size in mice with cortical infarction (Kim et al., 2000). Taken together, appears that oxidative stress signaling events may be important in ischemia-induced neuronal damage.

Research interest

In the following two chapters, we will focus on investigations into cell death pathways in MA-induced cell death and OGD-induced cell death respectively. In chapter II, we will examine how MA induces autophagy and evaluate the role of autophagy in protein clearance and its association with the dysfunctional UPS. In addition, we will address the role of autophagy and determine whether it is a cytoprotective or cytotoxic mechanism. We will also look into the involvement of intrinsic pathway in MA-induced cell death and the role of PKCδ in cell signaling pathway.

In chapter III, we will focus on the role of PKCδ in OGD-induced cell death by evaluating of the effects of oxidative stress on the mitochondria and downstream cell death signaling events. Neuronal phenotype specificity studies will be studied to determine neuronal vulnerability to OGD-induced striatal neuronal injury.

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Chapter II: PKCδ is a key mediator of MA-induced cell death in N27 dopaminergic neuronal cells: Role for autophagy and UPS

A paper to be submitted to *Journal of Neurochemistry*

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ABSTRACT

Excessive oxidative stress is believed to play a pivotal role in methamphetamine (MA)-induced dopaminergic neuronal loss. However, the exact mechanism by which MA causes dopaminergic neuronal loss remains to be established. To investigate the effects of MA on oxidative stress-mediated cell signaling events, we exposed N27 dopaminergic neuronal cells to MA (1 and 2 mM) for a period of 3, 6, 12 or 24 h. MA treatment resulted in early induction of autophagy (3h), followed by proteasomal dysfunction, dissipation of mitochondrial membrane potential, caspase-3 activation PKCδ cleavage and delayed DNA fragmentation. These changes appear to be mediated via caspase-3-mediated proteolytic activation of PKCδ, as overexpression of cleavage resistant PKCδ mutant (CRM) effectively attenuated oxidative stress-mediated cell death signaling events-associated apoptotic cell death following MA treatment. Furthermore, inhibition of autophagy using 3-methyladenine (3-MA), a phosphatidylinositol 3-phosphate kinase inhibitor, not only altered the pattern of LC3 distribution but also accelerated MA-induced apoptotic cell death, suggesting a neuroprotective role for autophagy in MA-induced neurotoxicity. Taken together, these results suggest that MA-induced dopaminergic neurotoxicity is likely caused, at least in part, by pronounced proteolytic cleavage of PKCδ and associated oxidative cell signaling events. The pronounced accumulation of autophagic vacuoles may overwhelm the lysosomal clearance mechanism; hence resulting in neurodegeneration. Alternatively, the autophagic vacuoles may enable the segregation of toxic molecules from viable organelles, hence

limiting the spread of toxicity. Thus, therapeutic interventions aimed at improving the efficiency of protein clearance system may be effective in the treatment of neurological disorders including PD.

INTRODUCTION

Methamphetamine (MA) is a widely used recreational drug and the usage of the drug is confirmed to cause both medical and neurological effects. Chronic MA abuse leads to decrease in tyrosine hydroxylase (TH), depletion of dopamine (DA), loss of dopamine transporters and decrease in vesicular monoamine transporter proteins (Krosnova et al., 2005; Cadet and Bronnock, 1998; Larsen et al., 2002). All of these evidence lead to the conclusion that MA, a monoaminergic toxin, destroys dopaminergic neuronal terminals resulting in neurodegeneration (Krosnova et al., 2005). It has been suggested that the toxic effects of MA may be associated with oxidative stress. Many studies have shown MA induces elevation of reactive oxygen species (ROS), namely superoxide radicals and hydrogen peroxide, often result in pronounced DNA, lipid and protein damage (Lau et al., 2000; Cadet and Brannock, 1998).

Programmed cell death can be categorized into two distinct types, the classical apoptotic cell death (type I) and the autophagic cell death (type II) (Bursch et al., 2005; Shintani and Klionsky, 2004). Type II cell death, involved in the process of autophagy is is characterized by the double- membrane vesicles or multi- lamellar autophagic bodies (Maiuri et al., 2007). The formation of autophagic vacuoles is described in several neurodegenerative disease including Parkinson's disease and, Huntington's disease and Alzheimer's disease (Chu, 2006). Dopaminergic neurons are shown to be prone to

developing autophagic stress during injury (Chu, 2006). It has been reported that MA-induced degeneration of dopaminergic neurons involve induction of autophagy and elicit formation of multilamellar whorls (Larsen et al., 2002). These characteristics are believed to be possible attempts of the injured cells to eliminate damaged proteins and organelles induced by oxidative stress.

A characteristic feature of several neurodegenerative diseases is the dysfunction of ubiquitin-dependent proteasomal system (UPS) which is associated with protein aggregation (Strahlendorf et al., 2003; Rideout et al., 2004). Cellular accumulation of misfolded proteins is a feature in inclusion bodies-related diseases, such as Parkinson's disease, Alzheimer's disease, and Huntington's disease. Another major system of intracellular protein degradation, the autophagy-lysosomal system (ALS), is involved in removal of these ubiquitinated inclusions and dysfunctional organelles (Rideout et al., 2004). Impaired proteasomal system causes the induction of autophagy, which has been reported to be a compensatory mechanism believed to aid in the clearance of aggregated proteins (Komatsu et al., 2007). In some studies interplay is suggested between autophagy and ubiquitin-proteasomal system (Komatsu et al., 2006). However, the exact nature of this interaction remains obscure. Therefore, in our studies we focus on the mechanism behind MA-induced autophagy together with UPS in order to understand their contribution to MA-induced apoptotic cell death.

Inductive mechanisms associated with apoptosis include extrinsic or intrinsic pathways. The intrinsic pathway is described as initiated by mitochondrial release of proapoptotic factors proteins such as cytochrome c (Strahlendorf et al., 2003), this can be initiated by ROS, causing damage to the mitochondria resulting in release of mitochondrial cytochrome c into the cytosol (Jayanthi et al., 2004). It has been reported that MA-nduced

neurodegeneration is dependent on mitochondrial dysfunction both *in vivo* and *in vitro* (Krosnova et al., 2005; Jayanthi et al., 2004). Collectively, mitochondria mediated oxidative stress events are believed to play a crucial role in MA- induced neurodegeneration. Less is known about the mechanism of MA-induced autophagy and how it is regulated in mammalian dopaminergic neuronal cells.

In our studies, we evaluate another signaling pathway related to apoptotic cell death, involving in proteolytic cleavage of protein kinase C delta (PKCδ) by capase-3, shown previously by Kanthasamy et al (2006), explaining its crucial role in dopaminergic neuronal cell death (Latchoumycandane et al., 2005). Protein kinase C, a family of phospholipid-dependent serine/threonine protein kinases, plays a central role in signal transduction pathways and a wide variety of cellular functions, including cell proliferation, differentiation and cell death (Busa, 2003; Griner and Kazanietz, 2007). PKCδ is a target of apoptotic stimuli, is a novel member of the PKC family, containing a regulatory domain and catalytic domain. PKCδ is a substrate for caspase-3, which is activated by both caspase-8, -9 and cleaves PKCδ at the DMQD330N site, generating a 40kDa fragment. In addition it acts as a positive feedback caspase-3 activator (D'Costa and Denning, 2005; Busa, 2003; Ko et al., 2005). Previous studies have shown that DNA damage induces cytochrome c release from the mitochondria, subsequently activating the PKCδ-dependent caspase-3 mediated cell death signaling pathway (Yang et al., 2004; Basu 2003; Ko et al., 2005). Consistently, we have demonstrated the importance of caspase-3 mediated PKCδ in dopaminergic degeneration.

MATERIALS AND METHODS

Cell Culture Immortalized rat mesencephalic dopaminergic neuronal cells (N27) were grown in RPMI 1640 medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 50 units penicillin and 50µg/ml streptomycin. Cell cultures were maintained under 5% CO_2 at 37°C as previously described (Yang et al., 2004).

Cell Death Detection ELISA Plus Assay Kit (Roche Molecular Biochemicals, Indianapolis, IN) was used for the analysis of DNA fragmentation (Anantharam et al., 2002). Briefly, cells were spun down at 200g for 5 min, and washed once with PBS. Cells were then incubated with the lysis buffer, supplied from the kit, at room temperature. After 30 min of incubation, samples were centrifuged and 20µL of the supernatants were transferred to the streptavidin-coated 96-well plates (supplied from the kit), and 80µL of HRP-conjugated antibody cocktail in added to the samples. After 2 h of incubation at room temperature with mild shaking, unbound components were washed with the incubation buffer supplied. The nucleosomes retained by the antibody cocktail in the immunocomplex were quantified with ABTS as a HRP substrate. Measurements were done with SpectroMax 190 spectrophotometer (Molecular Devices) at 405nm and 490nm. The difference in OD absorbance values were calculated and used as the actual DNA fragmentation levels. Sample protein concentrations were detected with Bradford protein assay kit.

Brain Sample Preparations and Western Blot Analysis Western blot analysis was done as previously described (Sun et al., 2005). Brain samples were lysed on ice in lysis buffer, containing protease inhibitors and 1%Triton X-100. Samples were sonicated on ice for 15 seconds and lysates were centrifuged 100,000g for 50 min. Supernatants were collected for protein assay and equal amounts of proteins were separated with 10% SDS-PAGE and

transferred onto nitrocellulose membranes. Standard Western blot procedure was done for immunoblot with overnight incubation with anti-DAT (1:250; Millipore) at 4℃ and β-actin 1:5000 (Sigma, St. Louis, MO) to confirm equal protein loading. Membranes were washed several times and incubated with IR Dye 800-conjugated anti-rabbit IgG (1:5000) or Alexa Flour 680-conjugated anti-mouse IgG (1:10000; Molecular probes, Invitrogen) for 1 h at room temperature. Membranes were scanned using the Odyssey IR Imaging system (LICOR) and data were analyzed with Odyssey 2.0 software.

MDC Assay and Dual-Immunostaining   Monodansylcadaverine (MDC) is an autofluorescent drug which can be used as a marker for autophagolysosome. This MDC assay follows Daniela et al., 2001 with some modifications. Treated cells were incubated with 0.05mM MDC in PBS at 37℃ for 10 min. After incubation, cells were collected and lysed with 200μL of 10mM Tris-HCl, pH 8 containing 0.1% Triton X-100 for 30 min on a microtube shaker. Samples were then transferred to 96-well plates and measured with fluorescence photometry plate reader (excitation wavelength 380nm, emission filter 525nm). Cells were incubated with 0.2μM ethidium bromide directly in the 96-well plate and the DNA fluorescence was measured (excitation wavelength 530nm, emission filter 590nm). MDC activity was normalized to the DNA values for comparison.

For immunostaining, upon desired treatments time, cells plated on coverslips were incubated with 0.05mM MDC in PBS at 37℃ for 10 min. Samples were washed with PBS and general immunocytochemistry procedures are carried out. Briefly, cells are fixed with 4% paraformaldehyde and blocked with 5% donkey serum. Primary antibody, mouse anti-TH is incubated in 5% donkey serum at 4℃ overnight.   Second day, cells were washed with PBS

and incubated with donkey anti-mouse for 1 hour at room temperature. Coverslips are mounted on slides and examined under 60x objective with fluorescent microscope.

Transmission Electron Microscopy For transmission electron microscopy (TEM), cells grown on coverslips were fixed with 2% glutaraldehyde (w/v) and 2% paraformaldehyde (w/v) in 0.1M sodium cacodylate buffer, pH 7.2, for 48 h at 4℃. Samples were washed in buffer and then fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hour at room temperature. The samples were then dehydrated in a graded ethanol series, cleared with ultra pure acetone, infiltrated and embedded using a modified EPON epoxy resin (Embed 812; Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 h at 70℃. Thick and ultrathin sections were made using a Leica UC6 ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Ultrathin sections were collected onto copper grids and images were captured using a JEM 2100 200kV scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA).

Western Blot Analysis Treated cells were collected and lysed with RIPA buffer in ice and sonicated for 15 seconds on ice for each sample. Samples were centrifuged at 100,000 g for 40 min at 4℃. Supernatants were collected and protein assay was done for equal loading of proteins. For brain sample preparations and protein extractions from the samples please see experiment one for more detail. Samples were separated on 8-15% SDS-PAGE, depending on the molecular weight of the target protein and transferred onto nitrocellulose membranes. Standard western blot procedures were performed. In brief, membranes were blocked and incubated with primary antibodies overnight at 4℃. Primary antibodies used in the following experiments included anti-MAP-LC3-II (1:250; Abgent, San Diego CA), anti-Akt / anti-phospho-Akt, anti-mTOR / anti-phospho-mTOR (1:200; AbCam, Cambridge, MA), anti-

ubiquitin (1:2000; Dakocytomation), cytochrome c (1:500; Dakocytomation). β-actin (1:10,000) was used for equal protein detection and Cox-4 (1:1000) was detected for confirmation of mitochondria fractionation. Membranes were washed several times and incubated with IR Dye 800-conjugated anti-rabbit IgG (1:5000) or Alexa Flour 680-conjugated anti-mouse IgG (1:10000; Molecular probes, Invitrogen) for 1 h at room temperature. Membranes were scanned using the Odyssey IR Imaging system (LICOR) and data were analyzed with Odyssey 2.0 software.

Immunocytochemistry Dual immunostaining was done in both N27 cell cultures and primary cell cultures, procedures were very similar. Briefly, cells were fixed with 4% paraformaldehyde after the desired treatment time. Washing between steps were done with PBS, three times each. Samples were blocked with 5% serum (either donkey or goat), 0.4% BSA, 0.2% TRITON x-100 for 1 hour and primary antibodies were incubated in the same 5% serum solution overnight at 4℃. Primary antibodies used in the following series of experiments included goat anti-LC3-II (1:250; Santa Cruz), rabbit anti-ubiquitin (1:500; Dakocytomation), rabbit anti-LC3 (1:250), mouse anti-TH (1:1000), rabbit anti-MAP-LC3 (1:250; Santa Cruz), goat anti-cleaved caspase-3 (1:500; Santa Cruz) and anti-lamp2 (1:300; Invitrogen). Alexa Flour donkey anti-goat and donkey anti-rabbit or goat anti-rabbit and goat anti-mouse combinations were used for 1 h secondary antibody (1:2000) incubation. Hoechst staining was done for nucleus labeling and samples were then mounted on slides and viewed under the Nikon inverted fluorescent microscope (TE-2000U).

Primary Mesencephalic Culture Primary mesencephalic preparation procedures were followed as previously described (Yang et al., 2004). Ventral mesencephalon was dissected from E14~15 day old mouse embryos, cell dissociation was done by incubating the dissected

tissue in trypsin-EDTA (0.25%) for 30 min. Dissociated cells were plated on poly-D-lysine precoated glass cover slips in 24 well plates and incubated with 10% fetus bovine serum medium containing supplement B27, L-glutamine, penicillin, streptomycin and L-glutamate. Approximately 1 million cells were plated for each well. Cells were maintained in 5% CO_2 at 37°C humidified environment. 10μM cytosine-arabinoside was added on the next day to inhibit glial cell proliferation. Treatments were done 5~7 days post dissection.

Proteasomal Assay The assay was performed following Sun et al., 2006, with some modifications. Briefly, cells were resuspended in buffer containing 50mM Tris–HCl, 1mM EDTA and 10mM EGTA, supplementing with 10μM digitonin and incubating at 37°C for 20 min. After incubation, lysates were centrifuged at 16,000g for 10 min. Supernatants were collected and incubated with fluoregenic substrate, 75μM Suc-LLVY-AMC, for proteasomal activities. After 1 hour incubation, samples were detected with fluorescence plate reader with wavelengths for excitation 380nm and emission 460nm (Gemini Plate Reader, Molecular Devices Corporation). Enzymatic activity is expressed as fluorescence per micrograms of protein.

Subcellular fractionation Mitochondria isolation was conducted as described in Leo et al., 1998, with some modifications. Cells were resuspended in a homogenizing buffer containing 250mM sucrose, pH 7.5, 20mM HEPES, 10mM KCl, 1.5mM MgCl2, 1mM EDTA, 1mM EGTA and protease inhibitors on ice for 30 min. Further homogenizing was done with a Dounce homogenizer on ice. Samples are centrifuged at 1000g to separate unlysed cells and debris. Supernatant are centrifuged at 10,000g for 25 min, supernatants were obtained are cytosolic fraction and pellets are lysed in RIPA buffer for mitochondria fraction.

JC-1 Mitochondrial Depolarization Assay JC-1 (Molecular probes), a cationic lipophilic fluorescent dye to measure mitochondria membrane potential. JC-1 can enter the matrix of healthy mitochondria membrane and accumulating to express fluorescence red. However, JC-1 cannot enter and accumulate in unstructured mitochondria, resulting in the green fluorescence seen in the cytoplasm. The intensity of the red fluorescence in the cell can be an indicator of the membrane potential. Incubation of $2\mu g/mL$ of JC-1 was used for 20 min of incubation with the cells at 37°C and collected. Pellets were resuspended in $200\mu L$ of 10mM Tris-HCl, pH 8 containing 0.1% Triton X-100 for 30 min on a microtube shaker. Samples were then transferred to 96-well plates and measured with fluorescence photometry plate reader, red and green ratio is used for indicator of mitochondrial potential. Separate samples were prepared with cells grown on glass cover slips and treated with MA. Upon desired treatment time, cells were stained directly with JC-1 final concentration of $2\mu g/ml$ for 20 min and mounted on slides for imaging under the fluorescent microscope.

Kinase Assay The enzymatic activity of PKCδ is detected using immunoprecipitation kinase assay as described previously (Kitazawa et al., 2003). Treated cells were collected and lysed with lysis buffer containing 25mM HEPES, pH 7.5, 20mM β-glycerophosphate, 0.1mM sodium orthovanadate, 0.1%Triton x-100, 0.3mM NaCl, $1.5MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 10mM NaF, $4\mu g/ml$ aprotinin and $4\mu g/ml$ leupeptin. The cells lysates were centrifuged at 10,000g for 30 min, and supernatant was obtained for the cytosolic fraction. $500\mu g$ cytosolic fraction was immunoprecipitated with $2\mu g/ml$ PKCδ antibody. Immunoprecipitates were washed with $2\times$kinase buffer and resuspend in $20\mu L$ of this same buffer. PKCδ phosphorylation is initiated by adding $20\mu L$ of reaction buffer (0.4mg Histone H1, 50uL/mL phosphatidylserine, $4.1\mu M$ dioleolglycerol, and uCi of $[\gamma\text{-}32P]ATP$) to the

immunoprecipitates. After 10 min incubation at room temperature, samples were separated on 12% SDS-PAGE. Radioactive labeled histone H1 were detected using Phosphoimage system (Personal Molecular Imager, FX model, Bio-Rad) and analyze with Quantity One 4.2.0 software.

Caspase Enzymatic Activity Assay Caspase-9 and caspase-3 activity was measured using a specific fluorescent substrate, Ac-LEDH-AFC (caspase-9) and Ac-DEVD-AFC (caspase-3), as described previously (Kaul et al., 2003). Briefly, cells were lysed with lysis buffer (50mM Tris HCl, 1mM EDTA, and 10mM EGTA) containing 10mM digitonin for 30 min at 37℃. Lysates were centrifuged at 10,000g for 5 min and cell-free supernatants were collected. Supernatants were incubated with fluorogenic substrate Ac-LEDH-AFC or Ac-DEVD-AFC for 2h at 37℃ and fluorescence was measured with Gemini XS fluorescence plate reader (Molecular Devices) at excitation 400nm and emission at 505nm. The measured values were normalized to protein concentrations from the samples with Bradford protein assay (Bio-Rad, Hercules, CA).

Data Analysis and Statistics All data analysis was first performed with InStat 3.0 (GraphPad software, San Diego) and then graphed with Prism 4.0 software. One-way ANOVA was used for multiple comparisons and single comparisons were done using the Student's t-test. Student Newman Keuls was used for post analysis. *$p < 0.05$ was considered as significant difference.

RESULTS

MA induces apoptotic cell death in N27 dopaminergic cells

Previous studies have demonstrated the occurrence of MA-induced cell death in several cell lines (Seilden et al., 1987; Cadet et al., 1998; Krasnova et al., 2005). In the present study we investigated the optimal dose that is necessary to elicit an apoptotic response. To this end, we performed dose response cytotoxicity analysis. A dose-dependent decrease in cell viability (Fig. 1), as assessed by DNA fragmentation analysis, was observed in N27 cells exposed to increasing concentrations of MA. Following exposure to 0.1, 0.3, 1, 3 mM MA for 24 h, the cell viability was decreased by 25, 32, 47 and 57% respectively, as compared with control. An EC_{50} of 1.39 mM was deduced by nonlinear regression. In the subsequent set of experiments, we used the following concentrations of MA, namely 1 and 2 mM to determine its ability to elicit an autophagic and apoptotic response.

Activation of autophagy by MA

Careful examination of MA-treated cells under a light microscope revealed the time-dependent accumulation of microscopic vacuoles, which was evidenced as early as 3 h post treatment and peaked at 12-24 h post drug treatment (Fig. 2A). The number and size of the vacuoles increased in a time- and dose-dependent manner in MA-treated cells (Fig. 2A). However, vacuoles were hardly visible in control cells treated with phosphor-buffered saline (PBS). The dose-dependent effect of MA on the induction of autophagic vacuoles were further quantified by fluorescence microscopy. For this purpose, we used monodansyl cadaverine (MDC), a dye most frequently used for the quantification of acidic vacuoles. Exposure to 1 and 2 mM MA resulted in a 1.25- to 2- fold increase in MDC fluorescence

from 3 to 24 h (Fig. 2A), suggesting that MA induces up-regulation of autophagic vacuoles (AV) in dopaminergic neuronal cells. Based upon these results, dual staining for TH and AVs were performed in MA treated primary dopaminergic neurons to confirm and extend our findings in N27 cells (Fig. 2B). Treatment of primary midbrain mesencephalic neurons with 0.5 mM MA for 24 h resulted in neuritic shortening and punctate appearance of MDC positive structures in TH-positive neurons as compared with control cells where a diffuse distribution was evidenced. Furthermore, transmission electron microscopy (TEM) was utilized to obtain ultrastructural details of the AVs at 24 h post MA treatment. Majority of these vacuoles demonstrated close resemblance to AVs characterized by a double membrane structure enclosing partial digested organelles such as mitochondria and cytoplasmic contents. The magnitude of vesicular accumulation was much higher in MA –treated cells as compared with control cells. The following data indicate that MA induces a time-dependent increase in autophagic vacuoles in N27 dopaminergic cells.

MA induces LC-II accumulation

To further determine and quantify MA-induced autophagy in dopaminergic neurons, a common autophagy associated protein, light chain microtubule-associated protein (LC3-II), was determined by immunoblot analysis. LC3 has been shown to exist in two forms. LC3-I that resides in the cytoplasm is post translationally modified into membrane-bound LC3-II, that becomes an integral component of autophagosomes during induction of autophagy (Yang et al., 2005). Immunoblot analysis of proteins obtained from MA (1 and 2 mM) treated N27 cells revealed a time- and concentration- dependent increase in LC3-II levels (Fig. 3A). Weak bands corresponding to LC3LI were found in control cells.

Next, to determine the extent of LC3 redistribution mediated by MA, immunofluorescence microscopic examinations were performed at 24 h to trace the redistribution to autophagic compartments. In the control treated cells, LC3 exhibited diffuse green fluorescence in the cytoplasm, whereas in 2 mM MA-treated cells a characteristic punctuate pattern of distribution were evidenced, indicating the translocation to autophagosomal membranes. The number of MA-treated cells with punctate pattern of distribution further confirms MA-induced autophagic induction (Fig. 3B).

Primary mouse mesencephalic cultures were treated with 0.5 mM MA for 24 h using a dose that caused selective injury to dopaminergic neurons (Larsen et al., 2002). Double immunolabeling studies using TH and LC3 showed that MA elicited a granular pattern of staining in MA –treated primary mouse mesencephalic cultures.

MA treatment promotes dissipation of mitochondrial membrane potential (MMP) and release of mitochondrial cytochrome c release

In recent years, disruption of mitochondrial function has been shown to be a critical trigger in the induction of autophagy and the subsequent removal of damaged mitochondria (Elmore et al., 2001; Sansanwal et al., 2010). Furthermore, mitochondrial pathway has been shown to play a significant role in regulating MA-induced cell death (Krosnova et al., 2005; Jayanthi et al., 2004). To investigate the potential role of mitochondrial dysfunction in MA-induced neurodegeneration, we determined alteration of MMP and cytochrome c release in MA-treated N27 cells. Cells were incubated with JC-1, a lipophilic cationic dye that is routinely used to measure MMP. As shown in Fig. 4A, examination of vehicle treated control by fluorescence microscopy revealed bright orange punctate staining in the

cytoplasmic regions, consistent with the formation of JC-1 "J-aggregates" characteristic of actively respiring mitochondria. MA (2mM) produced a dramatic reduction in the intensity of orange red spots and predominance of green staining consistent with loss of MMP. Analysis of MMP by spectrometric pate reader analysis revealed a dose- and time- dependent decrease in MMP, evidenced as early as 2 h, with a ~50% reduction in MMP over a 12 h period post MA treatment (Fig. 4B). Furthermore, several lines of evidence have recently shown that exposure to dopaminergic neurotoxins result in altered MMP that is accompanied by release of proapoptotic factors including cytochrome c and smac/DIABLO in the cytosol (Kitazawa et al., 2001; Kaul et al., 2003) to initiate the apoptotic cascade. In the present study, treatment of N27 cells with increasing doses of MA (0.5 – 2 mM) resulted in increased levels of cytosolic cytochrome c as compared with control (Fig. 4C). In MA treatment, cytosolic cytochrome c levels by 400% and 1200% at 12 and 24 h respectively as compared with control.

MA-induces a dose- and time- dependent increase in caspase-3 activation

It is well known that cytosolic cytochrome c release is an upstream activator of multiple caspases, including caspase-9 and -3 (Kanthasamy et al., 2003; Latchoumycandane et al., 2005). Caspase-3 represents the ultimate cell death execution step in both neuronal and non-neuronal cells. In the present study, we examined the effect of MA on capase-3 activation in N27 cells. Treatment of N27 cells with MA resulted in time- and dose-dependent increase in caspase-3 activation as compared with control. MA treatment (1 and 2 mM) resulted in 250% and 400% increases at 12 h; 320% and 430% increases at 24 h, respectively as compared to control treated groups.

Modulation of PKCδ activation involved in MA-induced apoptosis and autophagy

Recently, activated caspase-3 was found to cleave PKCδ, yielding a 38-kDa regulatory fragment and a 41-kDa catalytic fragment that leads to persistent activation of PKCδ kinase activity. Exposure to MA (0.5 – 2mM) for 24 h led to a dramatic increase (175-200%) in the magnitude of catalytic and regulatory fragments, suggesting that MA-induces PKCδ activation (Fig. 6A). Similarly, a parallel increase in PKCδ kinase activity was evidenced (Fig. 6B). In fact, treatment of N27 cells for 24 h with 2 mM MA resulted in increased kinase activity as compared to control. To determine the exact contribution of PKCδ in MA-induced neurotoxicity, we overexpressed a kinase inactive dominant negative (DN) mutant (PKCδ^{K376R}) in N27 cells and subsequently exposed to MA. Exposure to 2mM MA resulted in a five-fold increase in DNA fragmentation in control cells expressing the empty vector. However, the response was attenuated in N27 cells stably expressing the PKCδ-DN mutant. Additionally, we tested the importance of proteolytic cleavage of PKCδ in MA-induced neurotoxicity by evaluating the effects of overexpression of cleavage resistant PKCδ kinase (PKCδ^{D327A}-CRM). N27 cells expressing Lac-Z was used as control. As shown in Fig 6D, MA-induced proteolytic cleavage of PKCδ was almost completely attenuated in PKCδ^{D327A}-CRM. Furthermore, 2mM MA-induced DNA fragmentation was completely abrogated in CRM cells, while three-fold increase in DNA fragmentation was observed in Lac-Z-expressing cells as compared with PBS treated control cells (Fig. 6E). Additionally, we determined whether MA-induces caspase-3 activation despite attenuation of apoptotic response. Spectrofluorometric plate reader avalysis of caspase-3 activity in MA-treated CRM cells showed that caspase-3 activation was significantly reduced (ns) in 1 and 2 mM MA-treated CRM cells at 6, 12 and 24 h respectively, as compared with empty vector

expressing N27 cells (Fig. 6F). To investigate the role of PKCδ in MA-induced autophagy, we studied the ultrastructural details in MA (2mM) –treated cells at 24 h using TEM. An increased accumulation of nascent or pre-autophagic vacuoles were evidenced in the cytoplasm of MA-treated CRM cells (Fig. 6G). Conversely, a large increase in AVs containing undigested cytoplasmic materials were observed in vector overexpressing N27 cells, underscoring the importance of PKCδ activation in both apoptotic cell death and autophagic processes.

MA induces loss of proteasomal function and promotes accumulation of ubiquitin-positive aggregates and colocalization with autophagic vacuoles

Several studies have shown the dysfunction of UPS may contributr to neuronal degeneration disease, such as Parkinson's disease (PD) and activation of autophagy (Strahlendorf et al., 2003; Rideout et al., 2004; Komatsu et al., 2006). In this set of experiments, the status of the UPS and the extent if cross talk with autophagy were evaluated. MA (1 and 2mM) decreased proteasomal activity in N27 cells in a dose- and time- dependent manner (Fig. 7A). Inhibition of proteasomal activity coincided with upregulation of autophagy suggesting that autophagy may serve as a compensatory response to replenish proteasomal dysfunction. Also, MA increased ubiquitination in a dose- and time- dependent manner (Fig. 7B). Moreover, ubiquitin-like immunoreactivity in MA (2mM)-treated cells accumulated as large aggregates that colocalized with LC3 (Fig. 7B). From these findings, we conclude that early proteasomal dysfunction triggers accumulation of ubiquitin-positive aggregates and presumably promote autophagy-mediated degradative machinery.

Defective clearance of autophagic vacuoles (AVs) in MA-treated N27 cells

An important function of autophagy is to facilitate protein disposal system via lysosomal clearance pathway. This is mediated by the fusion of autophagosomes and lysosomes during the later phase of autophagy. Dysfunction in the fusion process might overload the lysosomal clearance system, hence, resulting in accumulation of undigested cytoplasmic material and ultimately resulting in death. To test whether the LC3 labeled autophagosomes are efficiently transported to lysosomes, N27 and PKCδ-CRM cells were treated with MA (2mM), stained with LC3 and lysosome associated membrane protein (LAMP-2), lysosomal marker protein, and analyzed by confocal microscopy. Colocalization studies revealed that N27 cells exposed to MA (2mM), only a small portion of LC3-positive autophagosomes merged with LAMP-2-positive lysosomes whereas in PKCδ-CRM cells, a complete overlap of the ALS markers were evidenced, suggesting an efficient lysosomal clearance system in MA-treated PKCδ-CRM cells. The following studies suggest a putative negative regulatory effects of PKCδ on the ALS in MA-induced neurotoxicity in N27 cells.

Inhibition of autophagy increases the sensitivity of N27 cells to MA-induced apoptotic cell death

We investigated the possible link between autophagy and apoptotic cell death in MA-treated N27 cells. In the effort to determine the role of autophagy in MA-induced neuronal cell death, we used 3-methyladenine (3-MA), an autophagy inhibitor. Pretreatment with 3-MA (1mM) promoted MA-induced DNA fragmentation. As shown in Fig. 9A, 3-MA pretreatment results in 2.5-fold increase in MA-induced DNA fragmentation when compared with control treated cells. Taken together, these findings suggested that MA-induced

apoptotic cell death was potentiated by 3-MA and that the autophagy mediated by MA may be cytoprotective by nature.

MA-induced modulation of autophagy, UPS and PKCδ **in vivo**

To further evaluate the participation of autophagy, UPS and PKCδ in MA-induced striatal neurodegeneration *in vivo*, the present study assessed levels of LC3, ubiquitin, PKCδ in striatal tissues harvested from MA-treated rat (4 x 20mg/kg every 2 h). Consistent with our *in vitro* findings, the present data show that MA-induced upregulation of autophagy, ubiquitin and PKCδ cleavage at 24 h post drug treatment (Fig. 10). To further examine whether MA-induced delayed upregulation of autophagosomal, ubiquitin and PKCδ markers, the current study assessed the cumulative accumulation of the above mentioned markers at 1 week after MA treatment. The results showed that MA-induced autophagy, ubiquitin accumulation and PKCδ cleavage in the striatum although the magnitude of upregulation of markers were not significantly different from 24 h MA-treated striatal tissue.

DISCUSSION

This study demonstrates the occurrence of apoptotic cell death as evidenced by an increase in DNA fragmentation levels in an *in vitro* N27 dopaminergic cell culture model of dopaminergic neurodegeneration following MA treatment. Furthermore, along with cell death a dramatic increase in the accumulation of autophagosomes in the cytoplasm were evidenced in MA-treated cells. Interestingly, the upregulation of autophagosomes also positively correlated with increased levels of ubiquitinated proteins which suggest a putative crosstalk between the two major protein degradation systems, namely ubiquitin-proteasomal

system (UPS) and autophagy-lysosomal system (ALS). In this context, our studies revealed that inhibition of autophagic activity enhances MA-induced apoptotic cell death which suggests a cytoprotective role for autophagy, at least in N27 dopaminergic neuronal cells exposed to MA. Furthermore, PKCδ-mediated event appears to play a critical role in MA-induced neurodegeneration.

Recent studies from our lab showed that PKCδ, a member of novel PKCs, plays a proapoptotic role in various cell types (Kanthasamy et al, 2003). We also showed that PKCδ is an oxidative stress kinase in response to demise stresses (Kanthasamy et al., 2003; Kaul et al., 2005). Oxidative stress and apoptotic in cell death have been implicated in several neurodegenerative diseases, including Parkinson's disease (PD). Recently Krosnova et al., 2001 demonstrated that MA -induced neurotoxicity is also associated with the production of hydroxyl and superoxide radicals which highlights the importance of oxidative stress mechanisms in dopaminergic neurodegeneration. Previously, we showed that oxidative stress in dopaminergic neuronal models persistently activates PKCδ by proteolysis via caspase-3 cleavage, which yields a 41-kDa catalytically active fragment and a 38-kda regulatory fragment (Anantharam et al., 2002). In addition, the proteolytic activation of PKCδ was found to contribute to apoptotic cell death in cell cultures models of PD (Kaul et al., 2003; Yang et al., 2004). Likewise, we observed that caspase-3 dependent PKCδ cleavage was associated with cell death in MA-treated dopaminergic neuronal cells. Interestingly, concurrent LC3-II accumulation and activation of PKCδ was observed in MA-treated cells which may imply that PKCδ might exert regulatory effects on autophagic induction. A recent model proposed by Chen et al., 2009, provides evidence that PKCδ/JNK1 signaling mechanism mediates a disassociation of Bcl-2 and Beclin 1, hence

resulting in autophagy. However, as hypoxic stress sustains, proteolytic activation of PKCδ and caspase-3, the protection afforded by autophagy is compromised; and hence commits the cell to undergo apoptosis (Chen et al., 2009). At the present time, the molecular mechanisms underlying PKCδ and autophagy cross talk is unclear, it warrants further investigation.

Despite the critical contribution of caspase-3 dependent proteolytic cleavage of PKCδ in MA-induced dopaminergic neurodegeneration, the signaling pathways that regulate PKCδ activation remains obscure. The events upstream of caspase-3 activation in MA-induced apoptotic cell death involve via a deregulation of mitochondrial signaling cascade that promote cytochrome c release. Mitochondrion plays an important role in metabolism and cell survival/ death decisions. In addition, neurons are especially dependent upon normal mitochondrial function to support the cells' high energy demands. This is reflected in neurodegenerative diseases including - Huntington's, Parkinson's and Alzheimer's disease - whereby mitochondrial dysfunction and disruption of mitochondrial dynamics have been implicated in the disease pathology (Casarrino and Bennett, 1999). The mitochondria are major sites of oxidative phosphorylation in the cell. These respiration activities produce reactive oxygen species (ROS) which can promote cytotoxicity and cell death (Kim et al., 2007). As a major source of ROS, mitochondria are especially prone to ROS damage, inducing mitochondrial permeability transition caused by opening of the pores in mitochondrial inner membrane. Such depolarization of mitochondrial membrane potential leads to the release of apoptotic factors and therefore, serves as a prelude to apoptotic cell death. The current JC-1 results reflect early mitochondrial membrane depolarization at 2-3 h of MA treatment (Fig. 4), which triggers the mitochondria to release apoptotic factors, resulting in caspase-3 activation and PKCδ cleavage. In addition, ultrastructural evidence of

mitochondrial disruption/dysfunction is presented in TEM images (Fig. 6G), namely, damaged mitochondria showing mitochondrial swelling and loss of cristae that is enclosed within double membrane vesicles, inductive of mitophagy in 2mM MA-treated N27 cells at 24 h. Mitochondrial quality control is maintained through mitophagy. Mitophagy is a process by which mitochondrial turnover is carried out though autophagic sequestration and delivery to lysosomes for hydrolytic degradation. This timely elimination of aged and dysfunctional mitochondria are essential in cell survival in terms protection of cells from harm caused by disordered mitochondrial metabolism and prevention of proapoptotic protein release. Emerging evidence suggest that this process is not random but a selective process (Chen and Chang, 2009). Recently Narendra et al., 2008 demonstrated that Parkin is one of the proteins suspected to act as a sensor for the trigger of mitophagy, thereby facilitating the degradation of effected organelles. Together, these studies support the idea that modulation of mitochondria depolarization may be a suitable therapeutic target to minimize MA-induced cell death.

Parallel to the autophagy-lysosomal system (ALS), is the ubiquitin-proteasomal system (UPS). Majority of the intracellular proteins and misfolded proteins are degraded by the UPS, while some long-lived proteins and damaged organelles are degraded by ALS. We hypothesize that UPS dysfunction may contribute to neurotoxicity and result in cell death. In support of this hypothesis, we demonstrate the accumulation of ubiquitinated proteins in MA-induced cell death at 12 h, which is preceded by downregulation of proteasome activity as early as 3 h following MA treatment (Fig. 7). Interestingly, cell death is attenuated in CRM (caspase-3 cleavage inhibited cells) in association with dramatic reduction in ubiquitin positive protein aggregates. The modulatory effects of PKCδ-CRM

cells on clearance of ubiquitinated proteins together with restoration of proteasomal activity to control levels suggests that PKCδ activation in N27 cells may contribute to the impairment of UPS. Recent studies suggest that proteasomal inhibition facilitates the induction of autophagy, suggesting that the two mechanisms are functionally coupled (Ding et al., 2007; Kouroku et al., 2007). UPS is important for degradation of misfolded proteins exported from endoplasmic reticulum (ER), when UPS is suppressed and misfolded proteins build up. However, when proteasomal function is deregulated, autophagy is activated to compensate for the reduced proteasomal function. Although the two systems target different proteins, while UPS degrades short-lived proteins and ALS degrades long-lived proteins, both serve a similar purpose of degrading proteins for energy reutilization. In our model, both systems are evaluated in terms of their activation under MA-induced mitochondrial stress environments. Accumulation of ubiquitinated proteins in MA-treated cells and down regulation of both UPS and autophagy in MA-treated N27 cells (Fig. 7C), may suggest that disruption of protein degradation machinery may be a causative factor in MA-induced neurodegeneration.

In conclusion, the present study shows that MA-induced autophagy and apoptosis in N27 cells is associated with UPS dysfunction and impairment of mitochondrial function. Furthermore, this process is regulated by proteolytic cleavage of PKCδ, and is involved in the induction of mitochondrial proapoptotic cell signaling events. The beneficial effect of improved autophagy coupled with improved lysosomal clearance may be useful in limiting MA-induced dopaminergic neurotoxicity.

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FIGURE 1.

EC$_{50}$ = 1.392mM
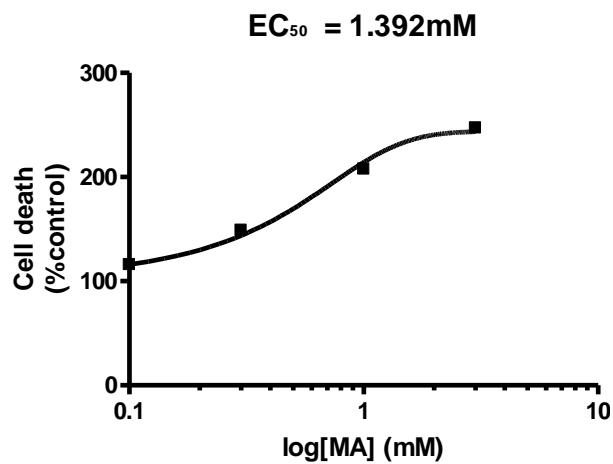
Figure 1. **Methamphetamine (MA) induced neurotoxicity in N27 mesencephalic dopaminergic neuronal cells.** DNA fragmentation assay was performed in N27 dopaminergic cells treated with 0.1 to 3mM MA for 24 h to measure cell death. Data represents results from six individual measurements.

FIGURE 2.
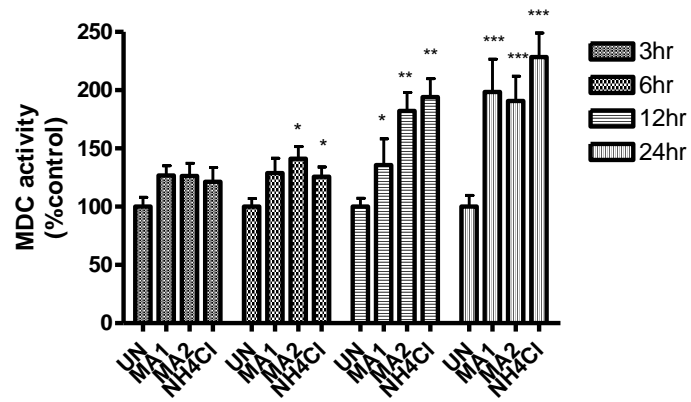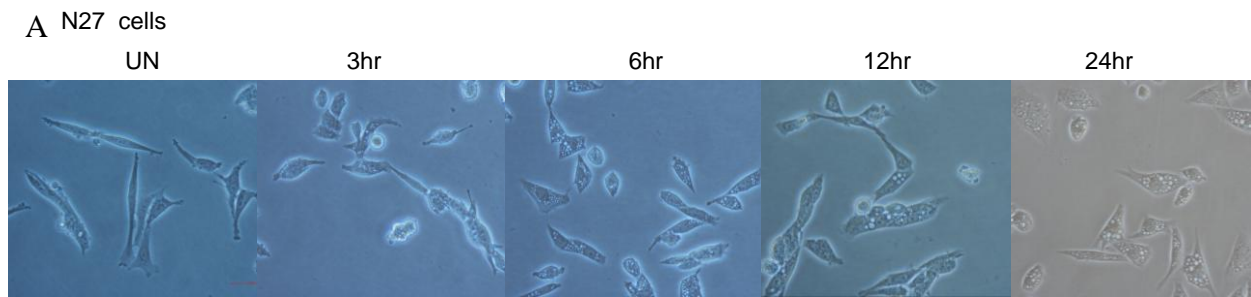
A N27 cells

B
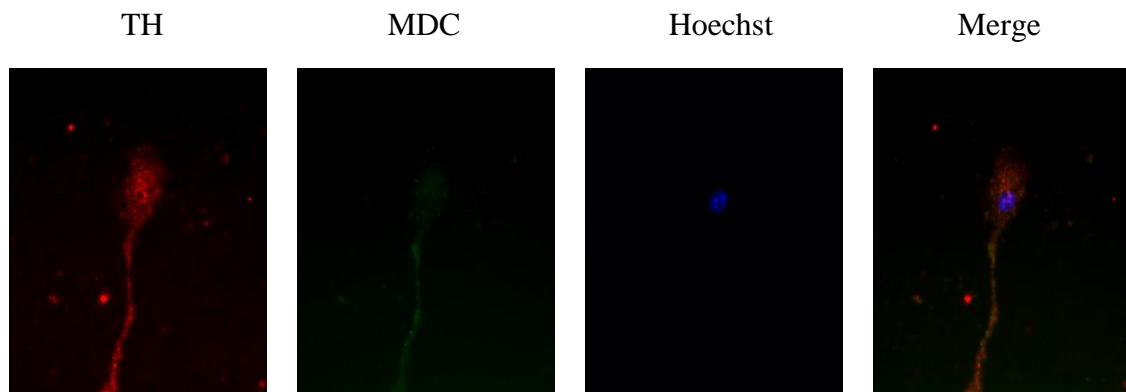
Control

MA0.5mM (24 h)
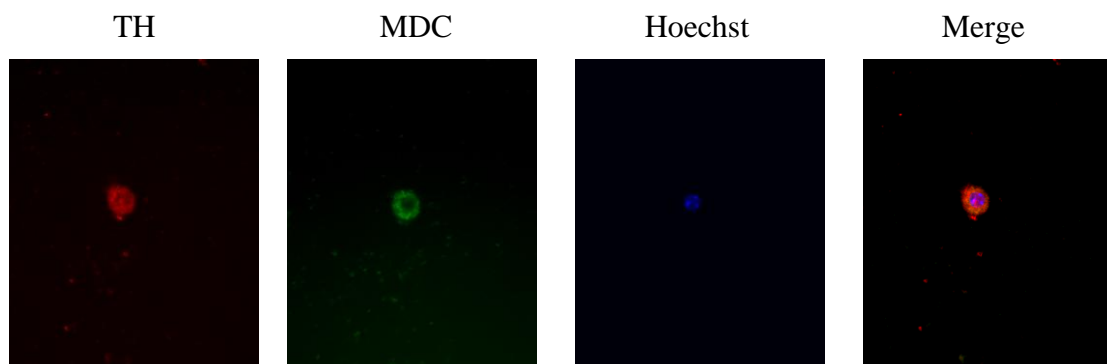
C   N27 cells- untreated (control)
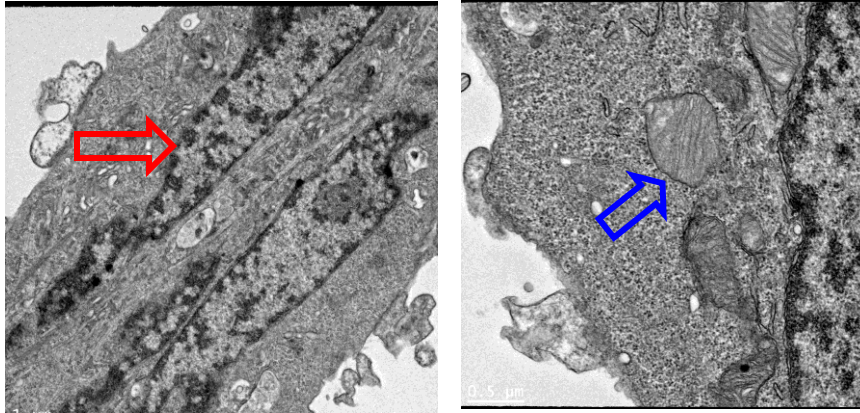
N27 cells- 2mM MA (24 h)
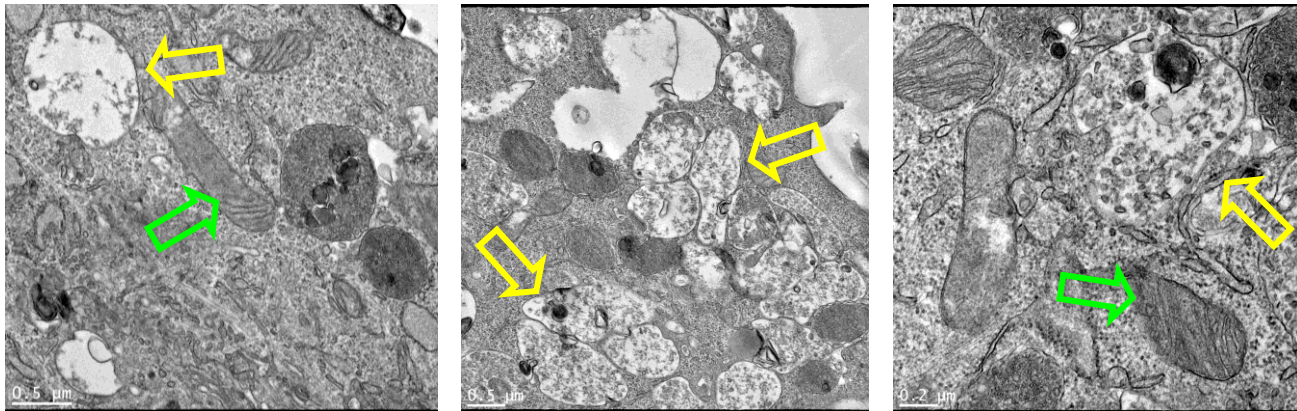
Figure 2. **MA induces autophagic vacuoles (AV) in N27 dopaminergic neurons.** A, N27 cells were treated for 3, 6,12 and 24 h with 1mM and 2mM MA and induction of AVs was observed under 40x microscopic objective. MDC staining was used for AV quantification and experiment details are depicted in methods section. NH_4Cl was used for positive control. MDC values represent mean +/- S.E.M. from at least six individual measurements. Asterisks (*p<0.05, **p<0.01, p***<0.001) indicate significant difference compared with control neurons. B, Dual immunostaining was done in MA (0.5mM)-treated primary striatal neurons
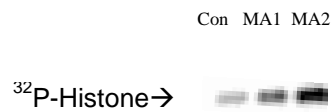
at 24 h for MDC and TH detection and compared with control neurons. Samples were observed with a Nikon TC2000 fluorescent microscope under the 60x oil objective. C, TEM studies were done to examine 2mM MA-treated N27 cells at a higher magnification. Red arrow identifies the healthy nucleus of an untreated N27 cell; blue arrow points to several healthy and functioning mitochondria, characterized by the intact mitochondrial cristae. The rigid borders of the cell represent processes spreading out from a healthy cell. In the MA(2mM)-treated cell, yellow arrows point to several AVs in the cytoplasm, some filled with particles and organelle-like structures. The border of this treated cell is less rigid and more rounded.

FIGURE 3.

A

B

C

Control

| TH | LC3 | Hoechst | Merge |

MA0.5mM (24 h)

| TH | LC3 | Hoechst | Merge |
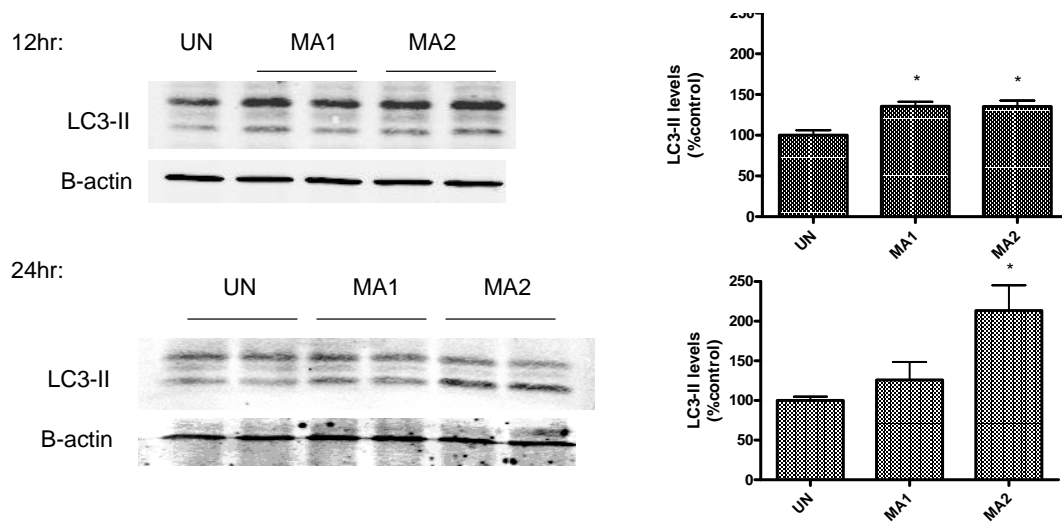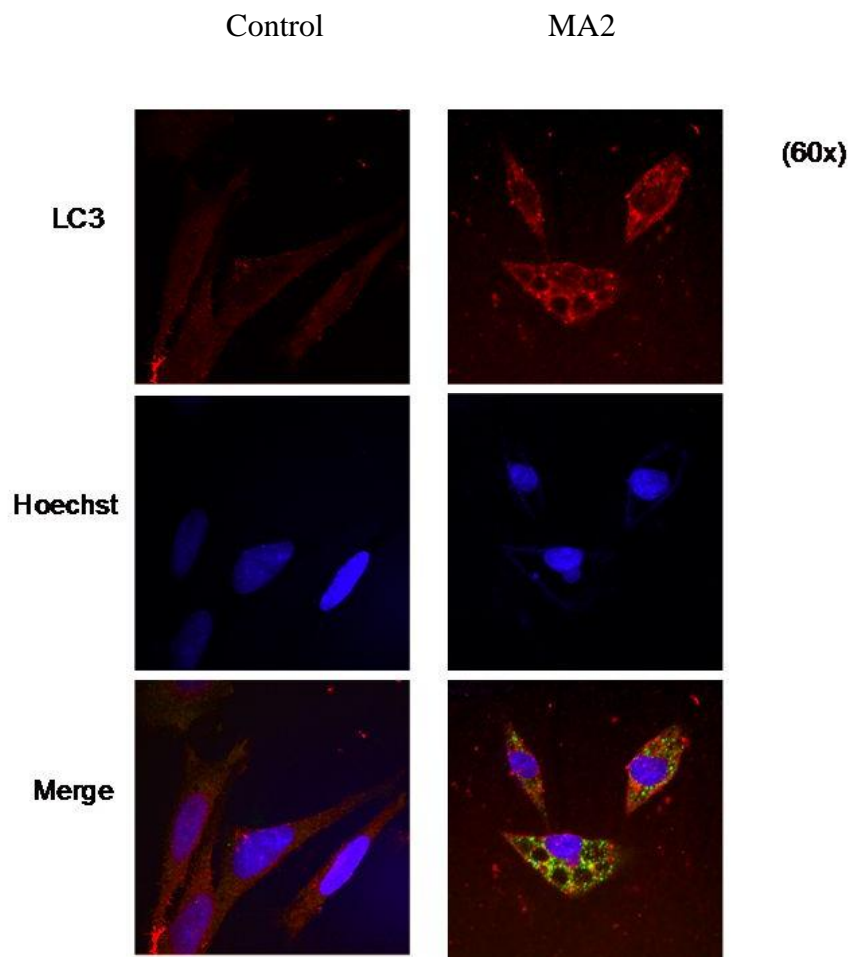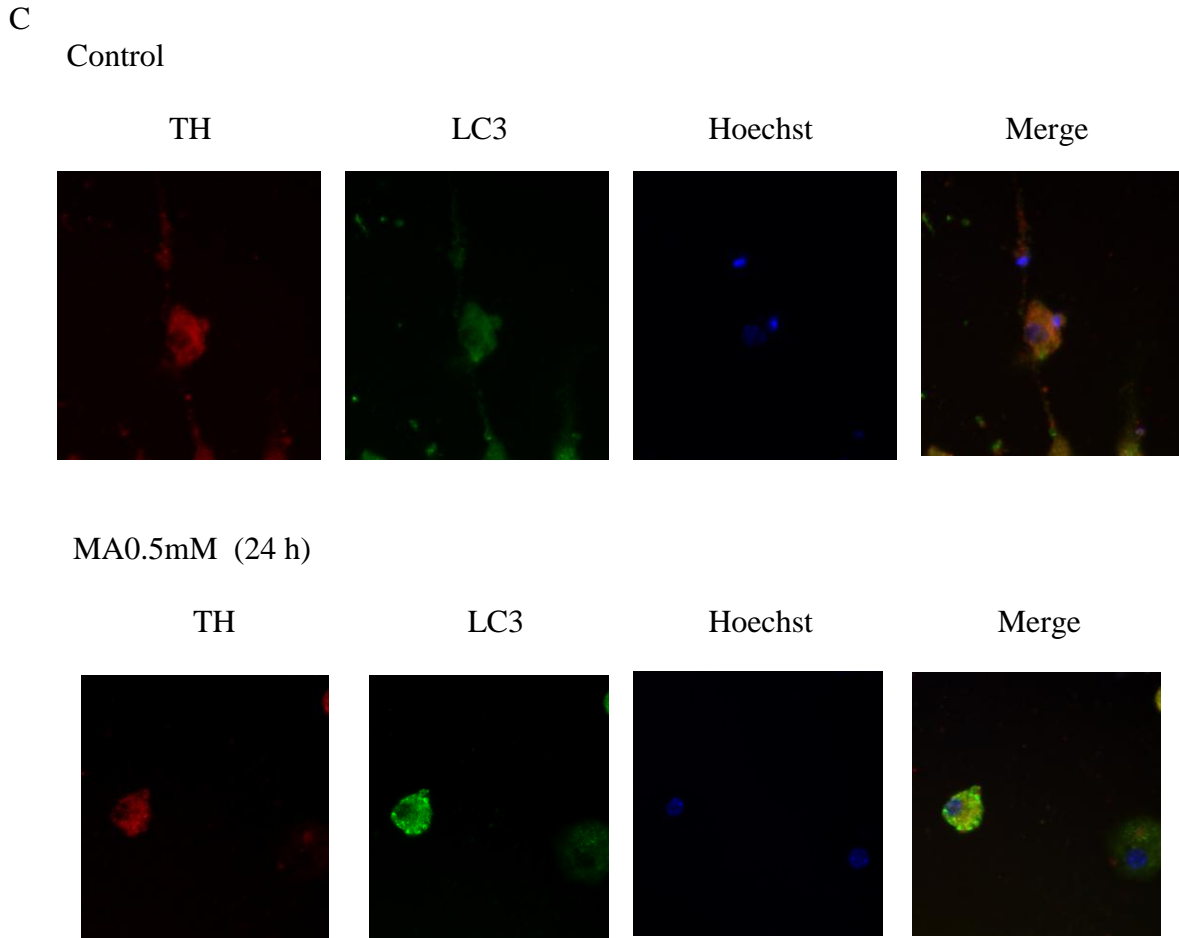
Figure 3. **LC-II is associated with the formation of autophagy in MA-treated neurons**. A, Immunoblot analysis was performed to detect for LC3-II expression in MA (1 and 2 mM) -treated N27 cells at 12 and 24 h and compared to control neurons. Procedures are detailed in the methods section. Blots show LC3-I (18kDa) and LC3-II (16kDa) bands. β-actin was probed for equal protein loading. B, Immunocytochemistry staining for LC3 was performed in MA (2mM)-treated N27 cells at 24 h and compared with control neurons. C, Dual immunostaining was done in 0.5mM MA-treated primary mesencephalic neurons 24 h. TH-positive neurons were detected along with LC3-II and compared to control cells. In both experiments, cells were viewed under a Nikon TE2000 fluorescence microscope with 60x magnification.

FIGURE 4.

A — Control, MA2-1hr, MA2-2hr (60x); rows: red, green, combine

B

(a) 3 h:

(b) 12 h:

C

12hrs in cytosolic fraction				
Treatment (mM)	Control	MA 0.5	MA1	MA2
Cytochrome C				
B-actin				

24hrs in cytosolic fraction				
Treatment (mM)	Control	MA 0.5	MA1	MA2
Cytochrome C				
B-actin				
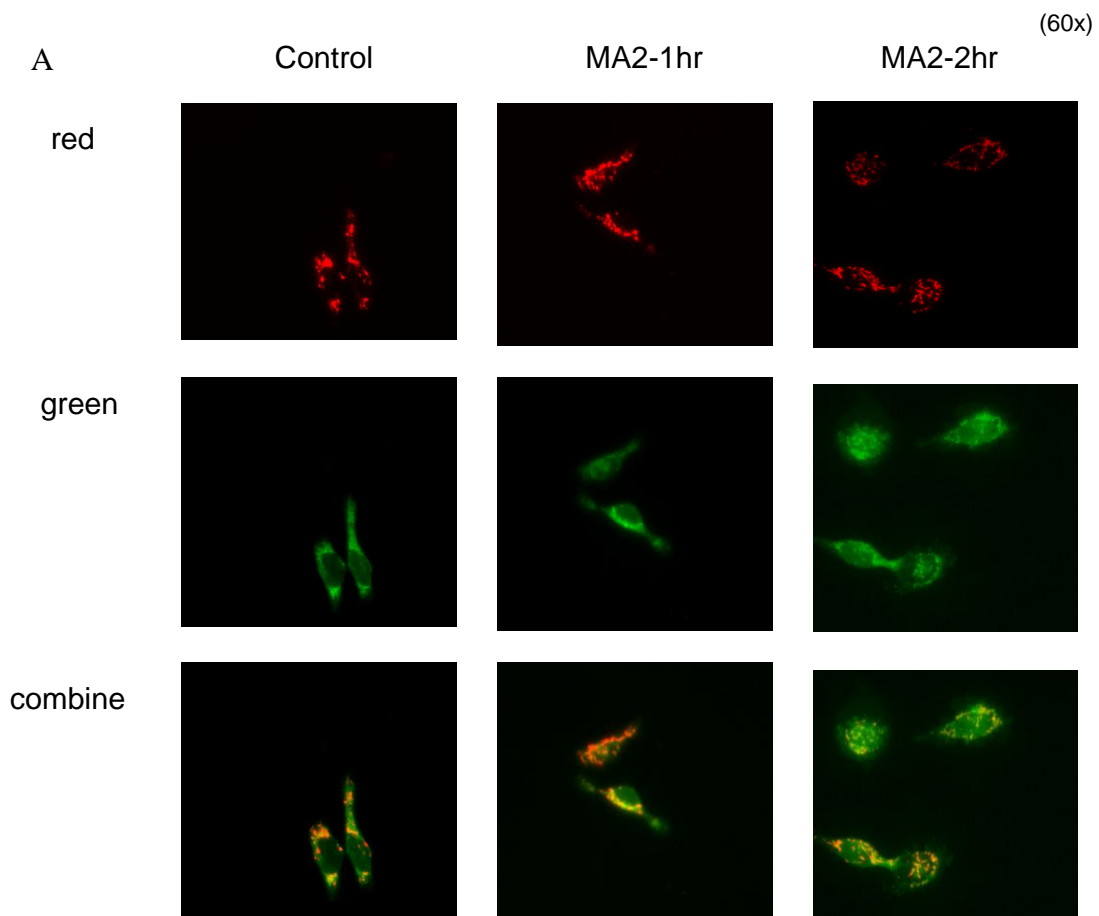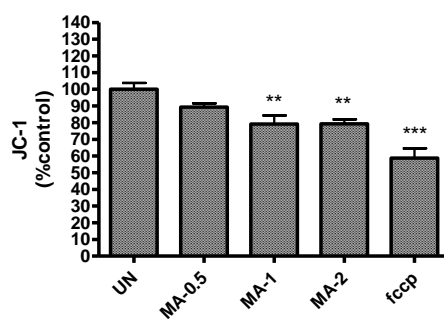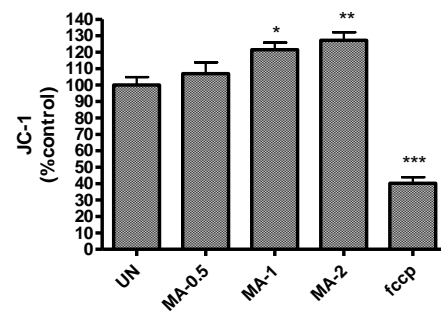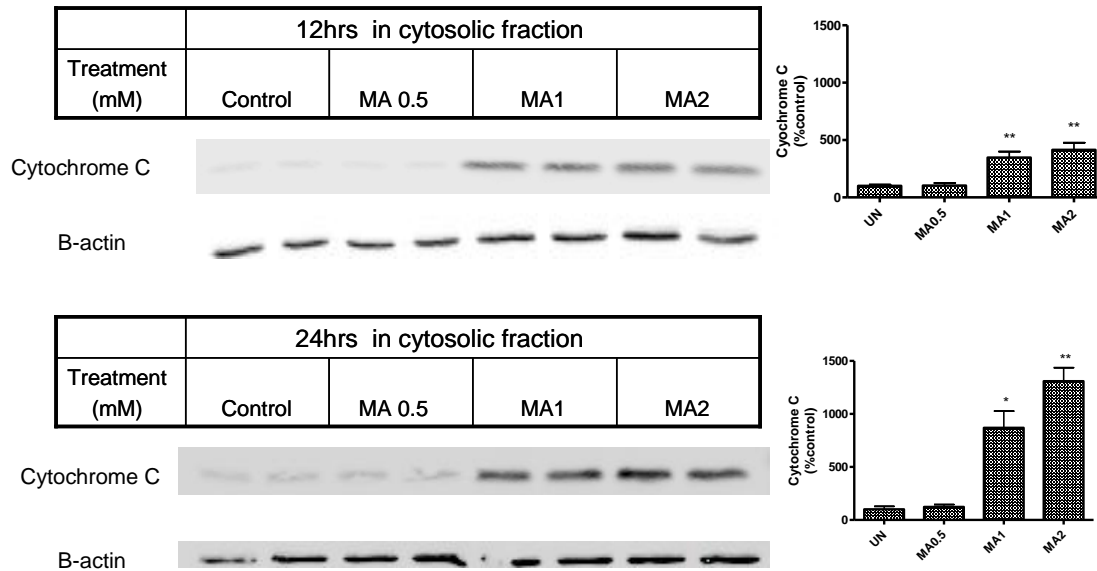
Figure 4. **Mitochondrial pathway is involved in MA-induced apoptotic cell death in N27 cells.** A, JC-1 staining, which detects MMP activity, was performed in 2mM MA-treated N27 cells at 1 and 2 h, and compared to control cells. Procedures are described in methods section. Images were immediately taken with a Nikon TC200 fluorescence microscope under 60x objective. B, JC-1 staining was done in 1 and 2 mM MA-treated N27 cells and fluorescence activity was measured at 3 and 12 h, and compared to control neurons. 40μM CCCP induces mitochondrial membrane depolarization and was used for positive control. Results represent mean +/- S.E.M. of six individual measurements. Asterisks (*p<0.05, **p<0.01 and ***p<0.001) indicate significant difference compared with control group. C, Immunoblot analysis was performed for detection of cytochrome c (15kDa) release into cytosolic fraction in 0.5, 1 and 2 mM MA-treated N27 cells at 12 and 24 h. Cox-4 antibody was used for labeling mitochondrial fraction and β-actin was probed for equal protein loading. Duplicates were shown for each treatment. Asterisks (*p<0.05, **p<0.01) indicate significant difference compared with control group.

FIGURE 5.
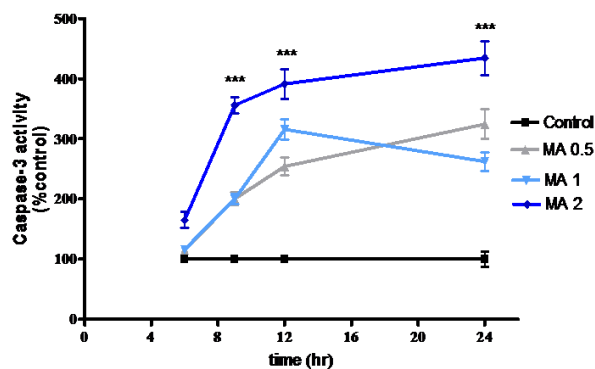
Figure 5. **MA induces caspase-3 activity in N27 neuronal cells.** Caspase-3 activity was detected in 0.5, 1 and 2mM MA-treated N27 cells at 6, 12 and 24 h. Experimental procedures are detailed in the methods section. Data represent mean +/- S.E.M. of six individual measurements. Asterisks (***p<0.001) indicate significant difference compared to control neurons.

FIGURE 6.

A

B

^{32}P-Histone→

C

D   CRM cells

UN   MA1   MA2

←cleaved PKCd

B-actin

UN   MA1   MA2

←cleaved PKCd

B-actin
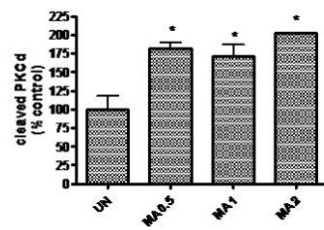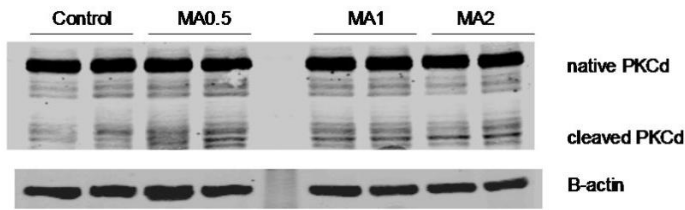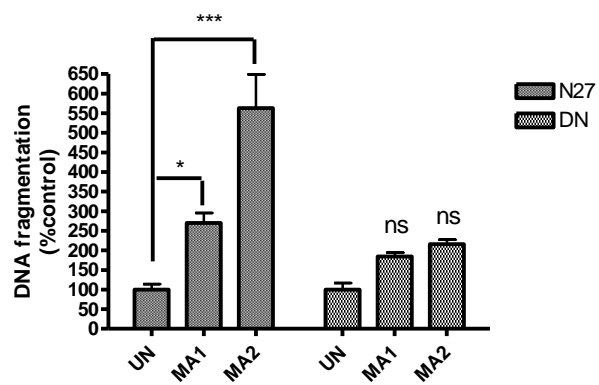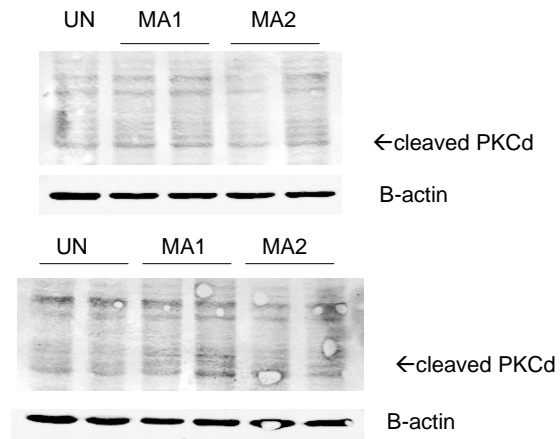
E
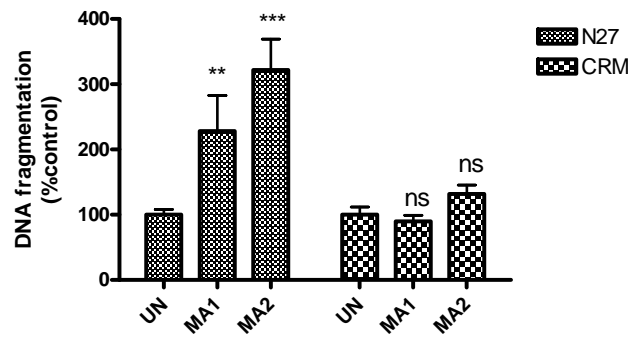
F
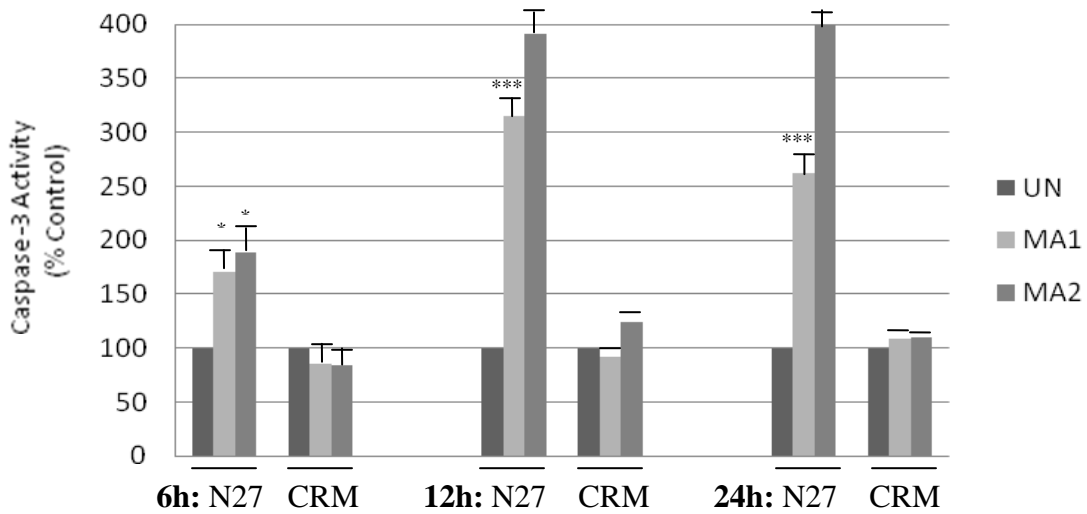
G

(a)   N27 cells- untreated (control)
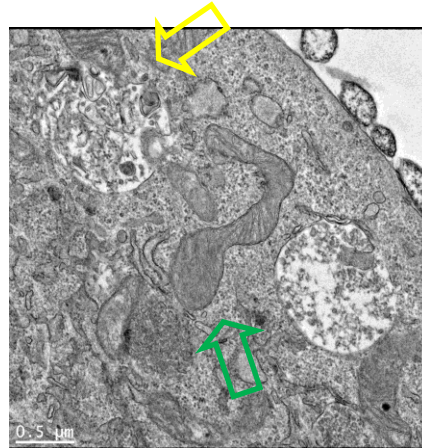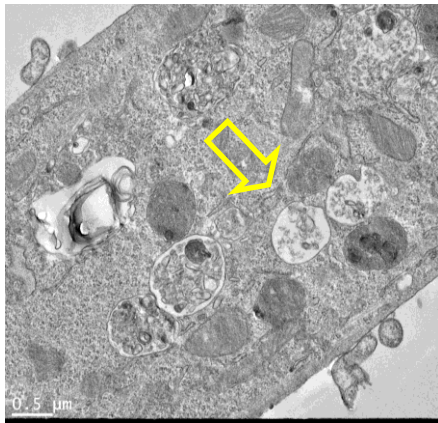
N27 cells- 2mM MA (24 h)

(b) CRM cells- untreated (control)
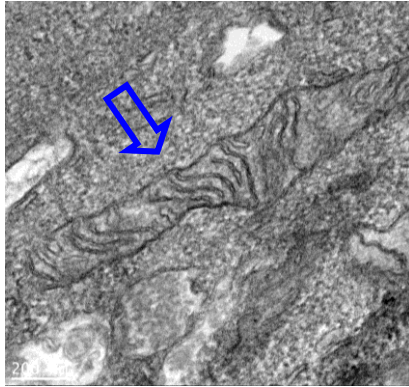
CRM cells- 2mM MA (24 h)
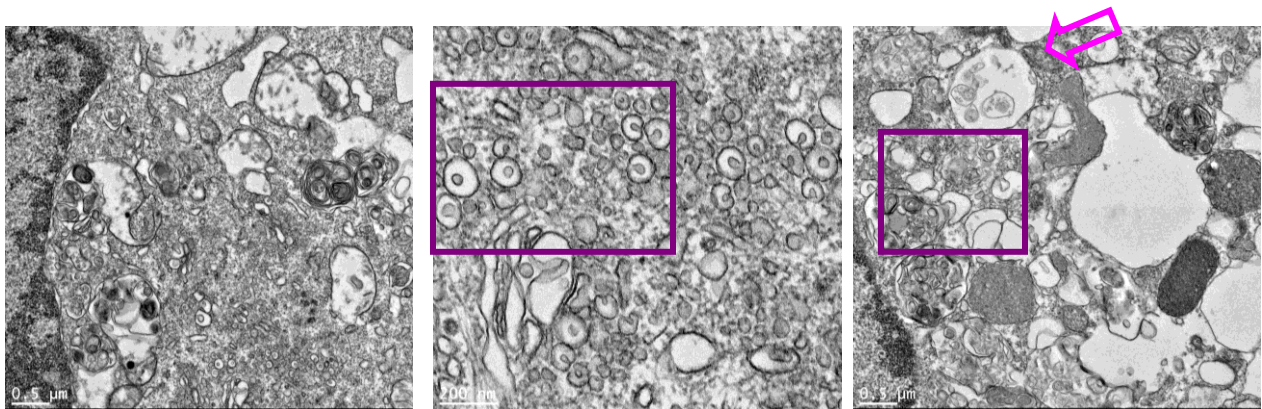
Figure 6. **PKCδ is a potential regulator in MA-induced cell death.** A, Immunoblot studies were performed in MA (0.5, 1 and 2 mM) -treated N27 cells at 24 h and compared to control neurons. Cleaved PKCδ (41 kDa) was detected and β-actin was reprobed for equal protein loading. Duplicate bands are shown. B, PKCδ activity was measured through kinase assay in MA (1 and 2 mM) -treated N27 cells at 24 h and compared to control sample. Phosphorylated histone bands are shown. C, DNA fragmentation activity was detected for cell death determination in MA (1 and 2 mM) -treated dominant negative (DN) N27 kinase activity inactivated cells (PKCδ [K376R]) at 24 h. Values represent mean +/- S.E.M. from at

least six individual measurements. Asterisks (*p<0.05 and p***<0.001) indicate significant difference compared with control group and ns represents non-significant compared to control. D, E, F, Confirmation of PKCδ activity, DNA fragmentation and caspase-3 activity was detected in MA (1 and 2mM) -treated caspase-3 cleavage resistant mutant PKCδ[D327A] (PKCδ-CRM) cells. PKCδ activity was determined via western blot detection of cleaved PKCδ (41 kDa) in PKCδ-CRM cells (D) and compared to results in N27 cells (A). β-actin is reprobed for equal protein loading. DNA fragmentation activity was detected in MA-treated PKCδ-CRM cells and compared with N27 cells (E). Caspase-3 activity was detected in MA - treated PKCδ-CRM at 6, 12 and 24 h and compared to N27 cells. Data represent mean +/- S.E.M. of six individual measurements. Asterisks (*p<0.05, **p<0.01 and p***<0.001) indicate significant difference compared with control group and ns represents non-significant compared to control. G, TEM studies show cellular morphology at a higher magnification in 2mM MA-treated N27 (a) and PKCδ-CRM (b) cells compared to control cells. Red arrow shows a healthy nucleus in N27-untreated cell; green arrow identify a swollen and disruptured mitochondrion revealing loss of cristae structure in MA-treated cell; yellow arrow shows AVs filled with disorganized and undigested organelles. Whereas in MA-treated PKCδ-CRM cells, numerous cup shaped structures representing preautophagosomes (purple boxes) are distributed in the cytoplasm. Middle pictures shows higher magnification of the cup shaped structures. AVs in the cytoplasm of the MA-treated PKCδ-CRM cells (pink arrows), reveals less disorganized particles compared those in N27 cells.

FIGURE 7.
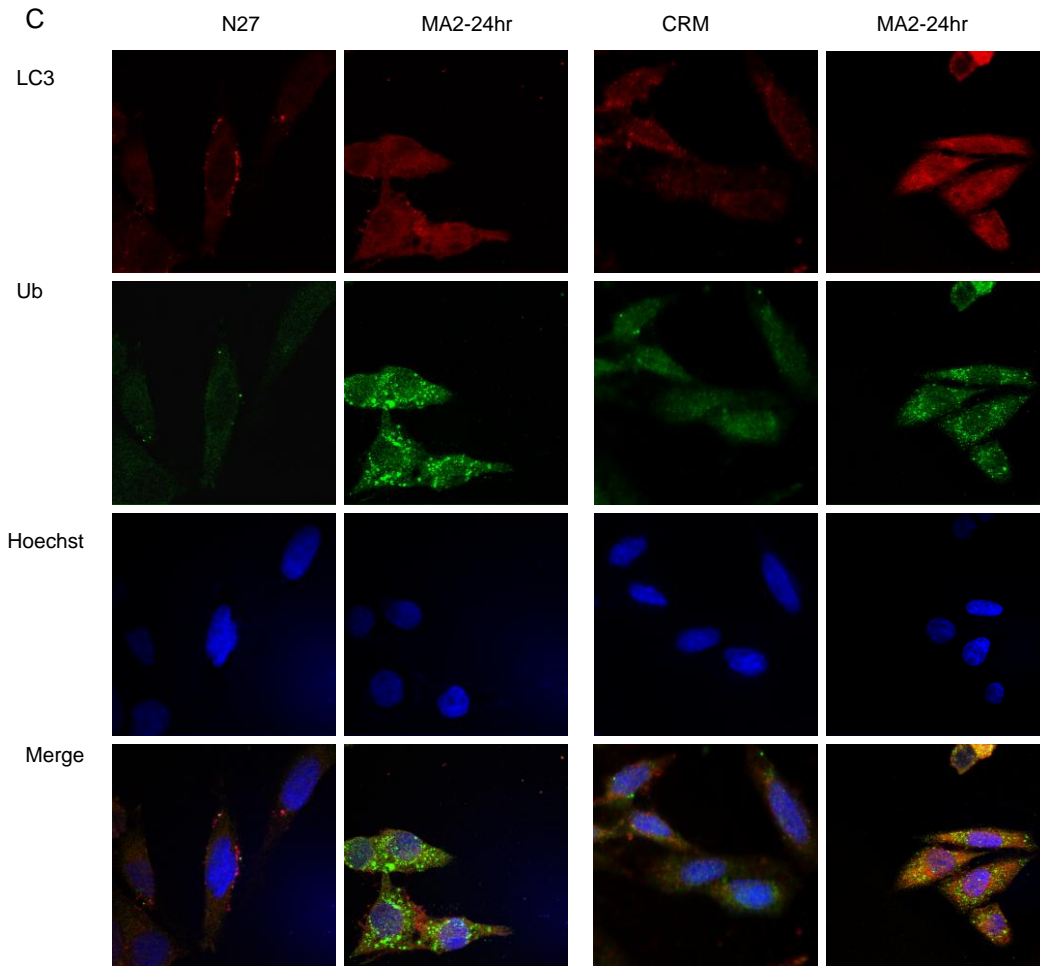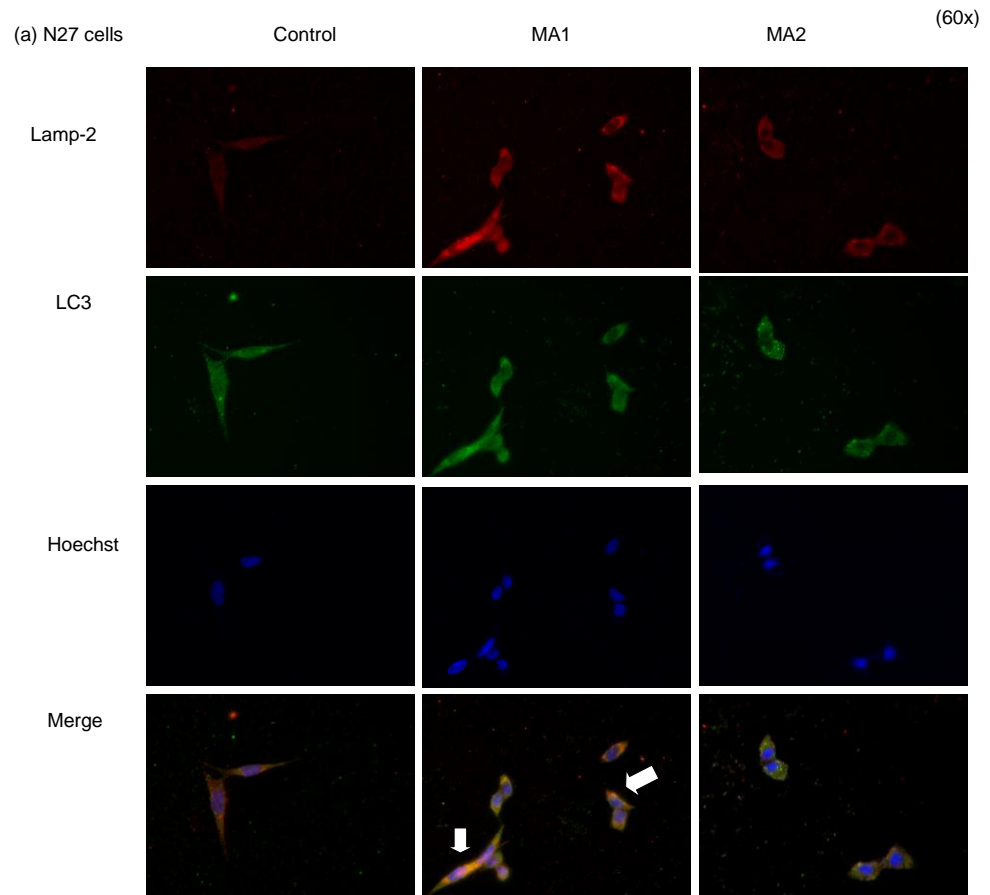
A   (a) N27 cells
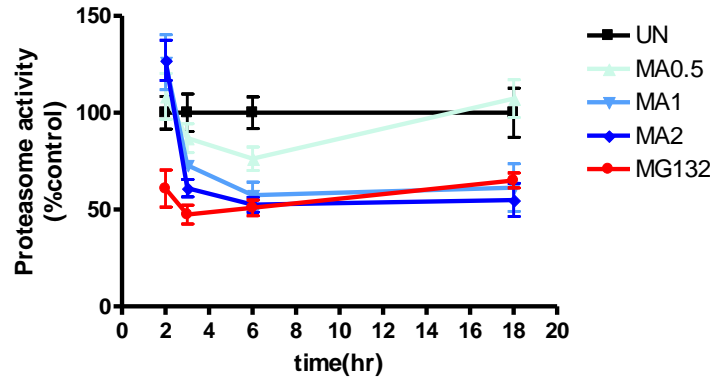
(b) PKCδ-CRM cells
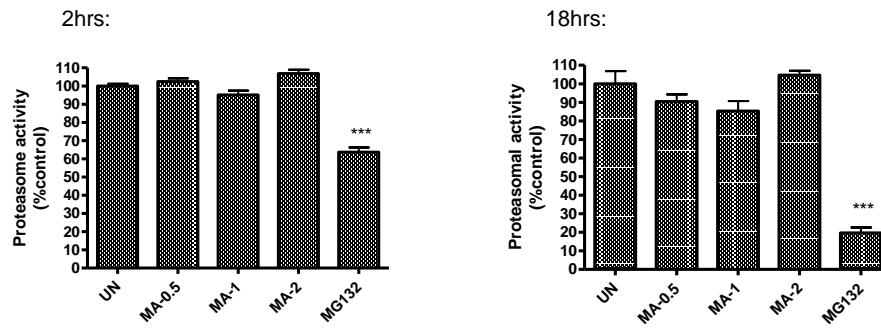
B
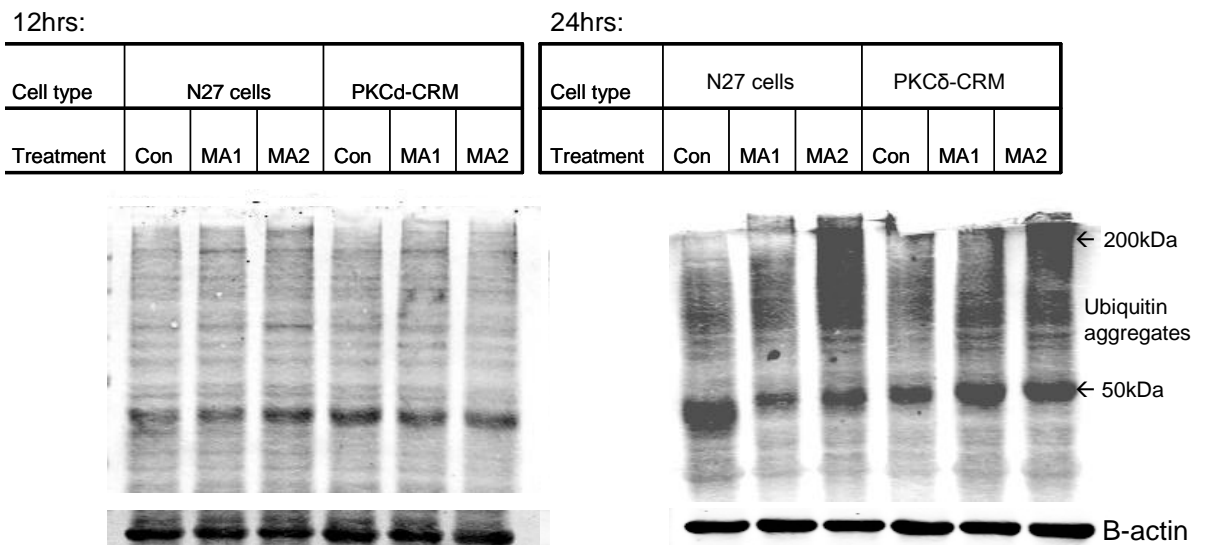
Figure 7. **MA induces accumulation of ubiquitin-conjugated proteins and downregulates proteasomal activity in MA-treated N27.** A, Proteasome activity was detected in 1 and 2 mM MA-treated N27 cells (a) and PKCδ-CRM cells (b) at 2 to 18 h. 5μM MG-132 was used for positive control to inhibit proteasomal activity. Results represent mean +/- S.E.M. of six individual measurements. Asterisks (***$p<0.001$) indicate significant difference compared to control group. B, Immunoblot analysis was performed to detect for ubiquitin aggregated proteins (250~50 kDa) in MA (1 and 2 mM) -treated N27 and PKCδ-CRM cells at 12 and 24 h, and compared to control group. C, Dual immunostaining was performed to detect for LC3 and ubiquitin in 2mM MA-treated N27 cells and compared

with PKCδ-CRM cells at 24 h.   Neurons were viewed under 60x objective with a Nikon

TC2000 fluorescence microscope as described in methods sections.
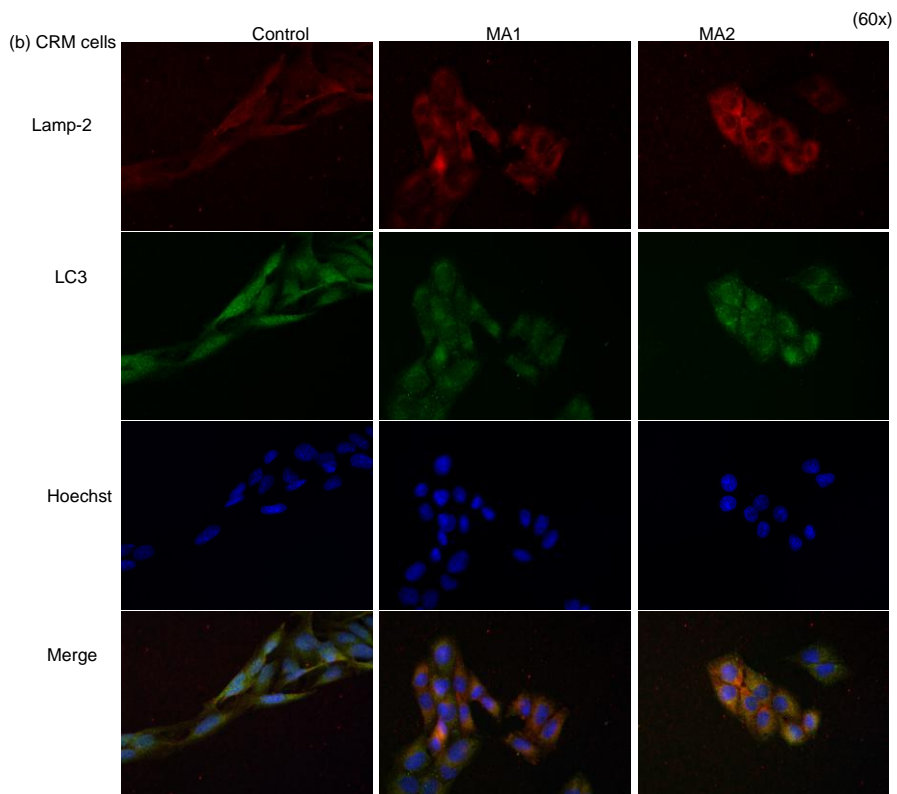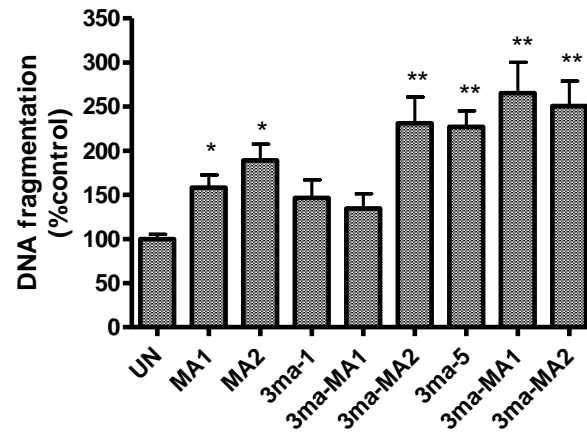
FIGURE 8.

Figure 8. **Dysfunctional autolysosomal fusion results in MA-induced apoptosis.**

Dual immunolabeling detected for LAMP-2 and LC3 in 1mM and 2mM MA-treated N27 cells (a) and PKCδ-CRM cells (b) to reveal the relationship between lysosome and autophagy respectively. Results were compared between N27 and PKCδ-CRM cells. Cells were viewed under 60x with a Nikon TC2000 fluorescence microscope as described in methods section.
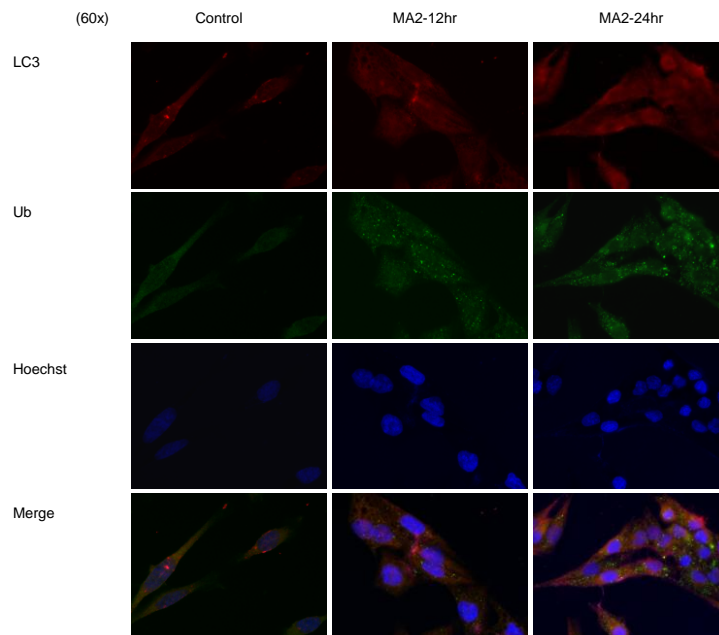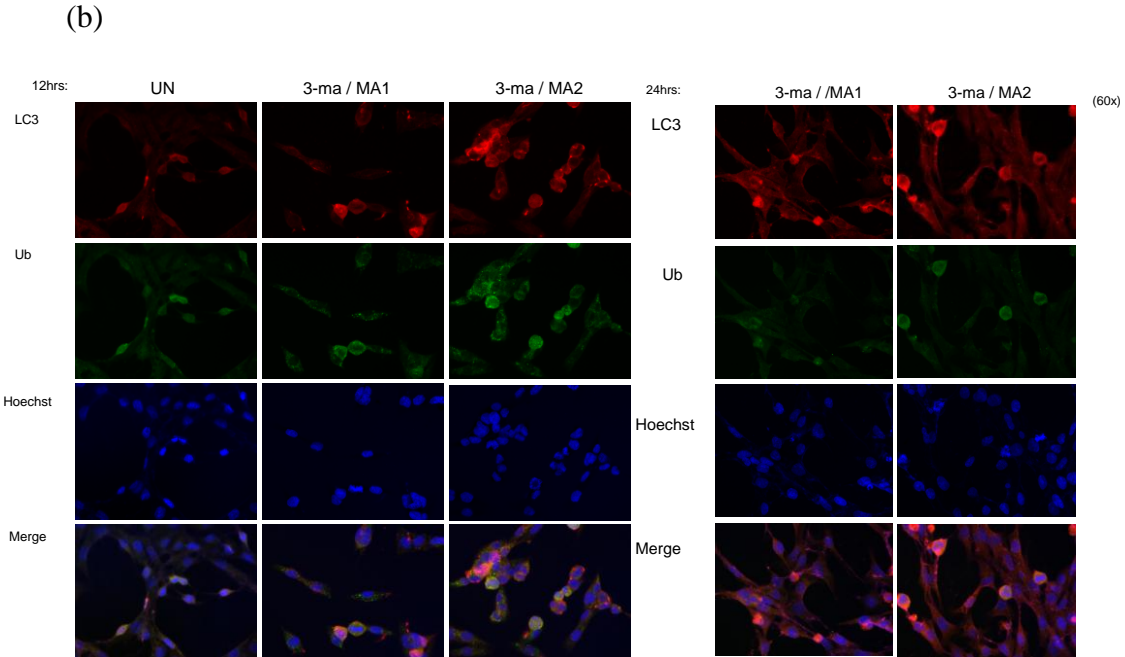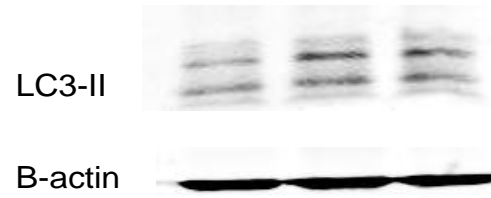
FIGURE 9.

A

B

(a)

(b)

Figure 9. **DNA fragmentation levels increase in 3-methyladenine (3-MA) pretreated N27 cells.** A, DNA fragmentation assay is performed to reveal cell death levels in 3-MA (1 or 5mM) -pretreated N27 cells followed by MA (1and 2 mM) treatment for 24 h. Values represent mean+/- S.E.M. from six individual measurements. Asterisks (*p<0.05, **p<0.01) indicate significant difference compared to control neurons. B, Dual immunolabeling detected LC3 and ubiquitin in 3-MA (1mM) -pretreated N27 cells followed by MA (1 or 2 mM) treatment at 12 and 24 h (b) compared to MA-treated only group (a). Cells were viewed under 60x oil objective with a Nikon TC2000 fluorescence microscope.
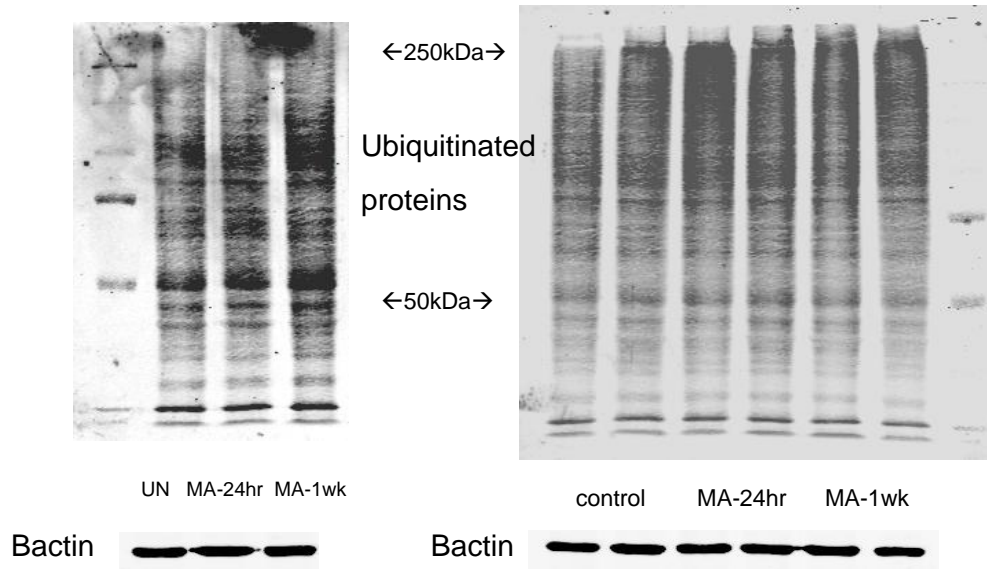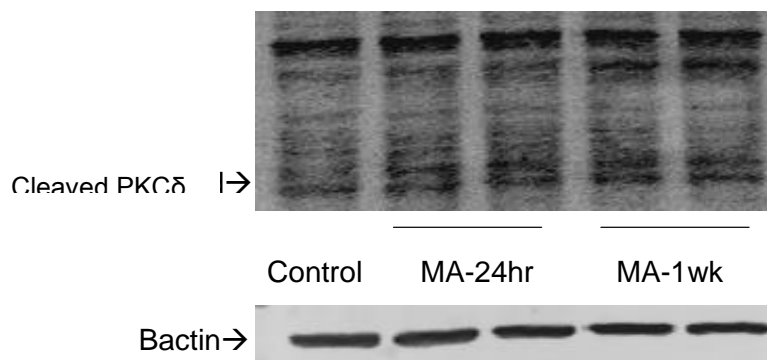
FIGURE 10.

A

Treatment	UN	MA	MA
time		24hr	1wk

LC3-II

B-actin

B

Bactin   Bactin

C

Cleaved PKCδ |→

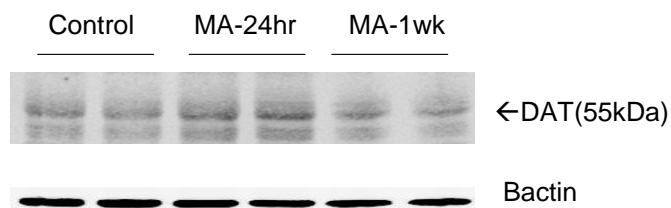Control   MA-24hr   MA-1wk

Bactin→

D

Figure 10. **MA induces autophagic cell death *in vivo* studies.** Western blot analyses were done in MA-treated rodent brain samples, treated with MA (4x20mg/kg) for either 24 h or 1week. A, LC3-II (16 kDa); B, Ubiquitinated aggregation (250~50 kDa); C, Cleaved PKCδ (41 kDa); D, DAT (55kDa) protein expression was detected in MA-treated tissue and compared with the control group as described in the method section. β-actin was probed for equal protein loading.

Chapter III: Proteolytic activation of PKCδ is involved in neuronal death after OGD in mouse primary striatal cultures

A paper to be submitted to *Journal of Neurochemistry*

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ABSTRACT

Stroke is the third leading cause of morbidity and mortality in the US, yet effective therapeutic interventions for the treatment of ischemia-induced brain damage are limited. The majority of the oxidative stress-related neurodegenerative studies in ischemic research have focused on either cortical or hippocampal regions; however, the cellular mechanisms underlying ischemic damage to the striatum, a dopamine enriched brain region that is highly susceptible to oxidative damage, remains poorly understood. Although the role of oxidative stress in apoptotic cell death is well characterized, the downstream executioner events that lead to DNA fragmentation following caspase-3 activation are currently not well established. In the present study, we have identified that PKCδ, a member of the novel PKC isoform family and an oxidative stress kinase, is highly expressed in striatal neurons. Primary mouse striatal neurons were subjected to oxygen-glucose deprivation (OGD) for 2 h and then incubated in normoxic conditions for 12 and 24 h. OGD-induced a significant increase in caspase-3 activity in striatal neurons within 12 h, followed by proteolytic cleavage of PKCδ, leading to persistent activity of PKCδ. This was accompanied by a persistent increase in PKCδ kinase activity, which was effectively blocked in genetically PKCδ knockdown mice (PKCδ-KO). Importantly, PKCδ-KO primary striatal neurons conferred resistance to OGD-induced neuronal injury. Together, these results demonstrate that caspase-3-dependent proteolytic activation of PKCδ is a master regulator of cell death in striatal neurons during ischemia-induced neuronal injury.

INTRODUCTION

Stroke occurs when the oxygen supply to the brain is interrupted by blockage or rupture of the blood vessels. The lack of oxygen to the brain causes brain cells to die and results in symptoms such as paralysis, speech impairment, inability to understand and other damages to the brain. Stroke kills nearly 150,000 people in the United States alone annually and is the leading cause of adult disability. Despite recent medical and surgical advances, e.g. antiplatelet agents, anticoagulant therapy, carotid endarterectomy, bypass surgery and endovascular treatment, the general approach to preventing acute ischemic brain damage resulting in irreversible cerebral infarction remains inadequate. This study focuses on mechanisms related to ischemic insult-induced delayed neuronal death, and also to identify certain types of neurons that are especially vulnerable to ischemic stress.

The striatum ischemia has been known for its susceptibility to cerebral ischemia in animal models of cerebral ischemia (Yang et al., 1998). Research has focused on finding the cause of such vulnerability to ischemia in the striatal neurons. Several studies concluded that the innervations of NMDA receptors in these neurons plays a role in accumulation of glutamate and increases the susceptibility of stroke damage in striatal ischemia (Yang et al., 1998; Ghibri et al., 1994). Another widely discussed receptor is the GABA receptors that have been shown to mediate striatal ischemia (Centonze et al., 2001). Large aspiny neurons are the only non-GABAergic neurons in the striatum. After transient cerebral ischemia, large aspiny neurons survive while medium aspiny neurons die. Previous studies have shown that differential changes in the intrinsic membrane properties and excitatory synaptic transmission play a role in this selective vulnerability. However, the role of inhibitory synaptic transmission in this selective vulnerability is still unknown. A growing body of literature

suggests that PKCδ is involved in apoptotic pathway observed in delayed cerebral reperfusion injury and other damage after stroke (Lipton et al., 1999). Evidence such as increased PKCδ mRNA and protein levels are reported in the cortex and hippocampus after ischemia insult (Miettinen et al., 1996; Koponen et al., 2000). Bright et al., showed the involvement of PKCδ in ischemic and reperfusion damage produced in the hippocampus region (Bright et al., 2004). They used Δv1-1, a specific PKCδ inhibitor, to demonstrate reduced cellular injury and cerebral tissue damage in both in vivo transient focal ischemia model and in vitro hippocampal slice culture model respectively. The present study provides evidence for the involvement of PKCδ in striatal cell death using OGD model of ischemia. It has helped us to provide an explanation for the selective vulnerability of striatum to OGD –induced apoptotic cell death.

MATERIALS AND METHODS

Primary Striatal Neuronal Culture Tissue was dissected from the striatum region of E17 PKCδ +/+ and PKCδ -/- mice embryos and digested with trypsin-EDTA for 30 min by incubation 37℃ water bath. Dissociated cells were counted and plated on poly-D-lysine (1mg/ml) precoated cover glass at a density of 1.6 million cells per well in 6-well plates or 0.5 million cells per well in 24-well plates. Primary neuronal culture medium contained B27 supplement, L-glutamine (500μM), L-glutamate (25μM), penicillin/streptomycin (100μg/ml). Cells were maintained at a humidified environment containing 5% CO_2 at 37℃. Cytosine arabinoside (10 μM) were treated for 12 h to reduce glial cell proliferation two days after surgery. Experiments were carried out 5~7 days post surgery.

Oxygen-Glucose Deprivation (OGD) Procedures Following procedures were carried out 5~7 days post surgery. Medium of the plated primary neurons were replaced with Ringer's

solution (116.4 mM NaCl, 5.4mM KCl, 1.8mM $CaCl_2$, 0.8mM $MgSO_4$, 2.6mM NaH_2PO_4, 26.2 mM $NaHCO_3$, 5.6mM glucose, and 20.1mM HEPES) for the normoxic control group; prepurged with N_2 and glucose excluded Ringer's solution was used for the OGD treatment group. The OGD treatment group was placed in a Billups-Rothenberg modulator incubator chamber (Billups Rothenberg, Del Mar, CA) and purged with N_2 at 37℃ for 2 h. After OGD treatment, original primary neuron culture medium was replaced for both treated and control groups and re-oxygenated for either 6, 12 or 24 h under original cell culture environment, incubating at 37℃ in a humidified atmosphere containing 5% CO2.

DNA Fragmentation Cell Death Detection Primary neurons were plated in 24 well plates and after OGD treatment, cells were collected and Cell Detecion ELISA Plus assay kit (Roche Diagnostics) was performed according to the manufacturer assay instructions and also described previously (Kaul et al., 2003). OGD-treated cells were washed with PBS and cell pellets were then resuspended with the lysis buffer from the assay kit. The lysates were spun down at 200g for 10 min after 30 min incubation in lysis buffer and 20μL of the supernatant was incubated for 2 h with the mixture of HRP-conjugated antibody. After washing away the unbound components, final reaction product was measured colorimetrically with 2,2'-azino-di-[3-ethylbenz-thiazoline sulfonate] as a HRP substrate using SpectorMax 190 Spectrometer (Molecular Devices) at 405nm and 490nm. The difference of absorbance between OD405 and OD490 was used to evaluate the actual DNA fragmentation levels. Values were normalized to protein concentrations obtained through Bradford (Bio-Rad, Hercules, CA) protein assay.

Lactate Dehydrogenase (LDH) Assay After desired re-oxygenation time of the OGD-treated cells, supernatants were collected from each well including the control group and

centrifugation was done to obtain cell-free extracellular supernatant. Incubation of the supernatant with the appropriate reagent mixture was prepared according to the instructions from the Cytotoxicity Detection Kit (Roche Diagnostics) at room temperature for 30 min. Intensity of color change was measured at 490nm proportional to LDH activity.

Live-Dead Cell Staining Kit - Propidium iodide (PI) Staining Cell viability was detected with Live-dead cell staining kit (BioVision, Mountain View, CA). Primary neurons were grown in 24 well plates and after 2 h of OGD treatments, cells were re-oxygenated for 12 or 24 h. Medium was removed and incubation buffer containing 2.5μg/mL PI, provided by the manufacturer, was incubated with the cells for 20 min at 37℃. Samples are then mounted onto glass slides and immediately observed under excitation 488nm and emission of 615nm under Nikon inverted fluorescence microscope (model TE-2000U, Nikon, Tokyo, Japan). Images are captured with SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI) and analyzed with MetaMorph 5.7 analysis software (Universal Imaging). Live and dead cells were counted and statistically analyzed with Graphpad 3.0 software.

Subcellular Fractionation and Western Blot Analysis Mitochondrial and cytoplasmic fractions were isolated from cell lysates using differential centrifugation as described in Qin et al., 2004. The crude mitochondrial suspension was further purified using a sucrose gradient (Sun et al., 2007). 15~30μg of proteins were loaded in each lane and resolved in 10~15% SDS-PAGE. After separation, proteins were transferred onto nitrocellulose membranes, and non-specific binding sites were blocked with blocking buffer (Bio-Rad, Hercules, CA). Primary antibodies were incubated in blocking buffer with the following dilutions, including anti-PKCδ (1:1500; Santa Cruz Biotechnology Inc., Santa Cruz, CA), anti-cytochrome c (1:500; BD Biosciences Pharminogen, San Jose, CA), Cox-IV (1:1000;

Invitrogen, Carlsbad, CA) and β-actin (1:5000; Sigma, St. Louis, MO) was incubated to confirm equal protein loading. After overnight incubation of primary antibodies at 4℃, membranes were washed several times and incubated with IR Dye 800-conjugated anti-rabbit IgG (1:5000) or Alexa Flour 680-conjugated anti-mouse IgG (1:10000; Molecular probes, Invitrogen) for 1 h at room temperature. Membranes were scanned using the Odyssey IR Imaging system (LICOR) and data were analyzed with Odyssey 2.0 software.

MitoTracker Staining and Immunocytochemistry MitoTracker Red CMXRos (Invitrogen, Carlsbad, CA) was used for mitochondrial targeted staining. Staining procedures were carried out according to the manufacturer's protocol. In brief, cells were incubated with 0.5μM of MitoTracker Red CMXRos at 37℃ for 20 min, and then fixed with 4% paraformaldehyde. Dual immunostaining with primary antibodies including goat anti-caspase-3 (1:250; Santa Cruz Biotechnology) or goat anti-cytochrome c (1:500; Santa-Cruz) was diluted in 5% donkey blocking serum and incubated with samples overnight at 4℃. Alexa Flour 488-conjugated donkey anti-goat IgG was used as secondary antibody diluted in PBS and incubated with the cells for 1hour. Finally, nucleus staining with Hoechst 33342 was incubated for 1min at a final concentration of 10μg/ml before mounting onto slides. Images were taken with the fluorescent microscope using the 60x objective.

Western Blot Analysis Treated cells were collected and lysed with RIPA buffer in ice and sonicated for 15 seconds on ice for each sample. Samples were centrifuged at 100,000 g for 40 min at 4℃. Supernatants were collected and protein assay was done for equal loading of proteins. Western blot analysis was done as previously described (Sun et al., 2005). Samples were sonicated on ice for 15 seconds and lysates were centrifuged 100,000g for 50 min. Supernatants were collected for protein assay and equal amounts of proteins were

separated with 10% SDS-PAGE and transferred onto nitrocellulose membrane. Standard Western blot procedure was done for immunoblot with overnight incubation with anti- PKCδ (1:1500; Santa Cruz) at 4°C and β-actin (1:5000; Sigma, St. Louis, MO) to confirm equal protein loading. Membranes were washed several times and incubated with IR Dye 800-conjugated anti-rabbit IgG (1:5000) or Alexa Flour 680-conjugated anti-mouse IgG (1:10000; Molecular probes, Invitrogen) for 1 h at room temperature. Membranes were scanned using the Odyssey IR Imaging system (LICOR) and data were analyzed with Odyssey 2.0 software.

Hoechst Staining Hoechst 33342 is a specific staining for the nucleus, incubating for 1min at a final concentration of 10μg/ml before mounting onto slides. Apoptotic cells are represented by the small and shrunken nuclei in contrast to the healthy large nuclei. Apoptotic and healthy cells are counted and statistically analyzed. Five microscopic views under 10x magnification are chosen and counted from each sample, numbers are calculated and graphed.

TUNEL Staining and Immunocytochemistry Primary striatal neurons were plated on poly-D-lysine pre-coated cover glass in 24 well plates and 2 h of OGD was performed. After 24 h of re-oxygenation, fixation was done with 4% paraformaldehyde. Samples were incubated with primary antibodies, rabbit anti-PKCδ (1:1000; Santa Cruz Biotechnology) or rabbit anti-GAD67 (1:250; Santa Cruz Biotechnology), in 5% donkey blocking serum overnight at 4°C. Secondary antibody, Alexa Flour 488-conjugated donkey anti-rabbit IgG (1:2000; Molecular Probes, Eugene, OR) was used for incubation in PBS for 1 hour. Terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL) staining was done according to the instructions provided by the manufacturer (Roche Diagnostics). In brief, after completion of secondary antibody, cells grown on cover glass

were incubated with the staining mixture from the kit at 37℃ for 60 min in the dark. Finally, nuclei staining was done with Hoechst 33342, incubating for 1min at a final concentration of 10μg/ml before mounting onto slides.

Caspase Enzymatic Activity Assay Caspase-9 and caspase-3 activity was measured using a specific fluorescent substrate, Ac-LEDH-AFC (caspase-9) and Ac-DEVD-AFC (caspase-3), as described previously (Kaul et al., 2003). Briefly, cells were lysed with lysis buffer (50mM Tris HCl, 1mM EDTA, and 10mM EGTA) containing 10mM digitonin for 30 min at 37℃. Lysates were centrifuged at 10,000g for 5 min and cell-free supernatants were collected. Supernatants were incubated with fluorogenic substrate Ac-LEDH-AFC or Ac-DEVD-AFC for 2 h at 37℃ and fluorescence was measured with Gemini XS fluorescence plate reader (Molecular Devices) at excitation 400nm and emission at 505nm. The measured values were normalized to protein concentrations from the samples with Bradford protein assay (Bio-rad, Hercules, CA).

Mitocasp Dual Staining Dual staining of mitochondria and caspase3/7 was done with a commercial kit, MitoCasp (Cell Technology Inc., Mountain View, CA). Primary neurons were objected to 2 h OGD and re-oxygenated for 24 h. Following to the manufacturer's instructions, Mitochondria Membrane Potential Dye and Caspase detection reagent mixture was added to the cells and incubated at 37℃ for 60 min. After incubation, samples were washed with PBS and images were captured immediately under Nikon inverted fluorescence microscope (model TE-2000U, Nikon, Tokyo, Japan); captured with SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were analyzed with MetaMorph 5.7 analysis software (Universal Imaging). Caspase detection was done under excitation

wavelength 488nm, emission wavelength 515-530nm. Mitochondria membrane potential detection was done under excitation wavelength 549nm and emission 574nm.

Immunocytochemistry Primary striatal neurons were plated on poly-D-lysine pre-coated cover glass in 24 well plates after 2 h of OGD procedure. Upon re-oxygenation for 24 h, cells were fixed with 4% paraformaldehyde. Following fixation, samples were blocked with blocking buffer containing 5% goat/donkey serum, 0.4% BSA, 0.2% Triton X-100 for 1 h at room temperature. Dual immunostaining is done with primary antibodies including rabbit anti-GAD67 (1:250; Santa Cruz Biotechnology) and mouse anti-PKCδ (1:1000; Santa Cruz Biotechnology). Primary antibodies were incubated overnight at 4℃ in 5% goat serum blocking buffer. Secondary antibodies, including Alexa Flour 488-conjugated goat anti-rabbit IgG or Alexa Flour 555-conjugated goat anti-mouse IgG (1:2000; Molecular Probes, Eugene, OR), were diluted in PBS for 1 hour incubation at room temperature. PBS was used for washing in between steps. Nuclei were stained with Hoechst 33342 at a final concentration of 10μg/ml for 1 min. Finally, cells were washed with PBS and mounted with anti-fade mounting medium. Slides were viewed under Nikon inverted fluorescence microscope (model TE-2000U; Nikon, Tokyo, Japan); captured with SPOT digital camera (Diagnostic Instruments, Inc., Sterling Heights, MI). Images were analyzed with MetaMorph 5.7 analysis software (Universal Imaging).

Data Analysis and Statistics All data analysis was first performed with InStat 3.0 (GraphPad software, San Diego) and then graphed with Prism 4.0 software. One-way ANOVA was used for multiple comparisons and single comparisons were done using the Student's t-test. Student Newman Keuls was used for post analysis. $p < 0.05$ was considered as significant difference.

RESULTS

OGD induces neuronal death in primary striatal neurons

To investigate the vulnerability of primary striatal neurons to neuronal injury following ischemia, we utilized a commonly used *in vitro* model of OGD. Previous work has demonstrated that exposure of murine cortical culture to 2 h of OGD leads to delayed neuronal death and is mediated via caspase-3 signaling pathway (Namura et al., 1998; Gill et al., 2002); however, studies in striatal neurons are still lacking. Hence, to characterize the time course of OGD-induced neuronal injury in striatal neurons, we exposed wild type striatal cultures to 2 h of OGD or normaxic conditions (NML) and cell death was assessed at 12 and 24 h following reperfusion. OGD treatments resulted in 50, 40 and 100% increase in neuronal loss at 6, 12, 24 h respectively, as assessed by a cell death oligonucleosome enzyme-linked immunoabsorbant assay (ELISA) (Fig. 1A). Furthermore, the neuronal death at 12 and 24 h of reoxygenation after exposure to OGD for 2 h was investigated using PI uptake and LDH release studies, routinely used methods for studying cell death (please see methods and materials section for details). We found only a few PI-positive cells in control cells exposed to normaxic conditions while in cells exposed to OGD and reoxygenation for 12 and 24 h respectively, an average increase in fluorescence of 30 and 35% respectively ($p < 0.001$) were evidenced (Fig. 1B). The difference between 12 and 24 h is not very significant ($p < 0.05$). In a comparable study using LDH release as an endpoint, release of LDH into the medium following 2 h of OGD increased with increasing period of reoxygenation. Reoxygenation for 6, 12, 24 h resulted in an increase of 55, 35 and 50% respectively as compared to control cells exposed to normaxic conditions ($p < 0.001$). Taken

together, these results demonstrate that cultured mouse striatal neurons are vulnerable to OGD-induced neuronal injury.

OGD induces dissipation of MMP and mitochondrial release of cytochrome c in striatal neurons

In the next set of experiments, we studied the status of mitochondrial function preceding and during OGD-induced striatal neuronal degeneration. To achieve this, measurements of MMP and mitochondrially released factors, such as cytochrome c, were performed. MitoTracker red, a mitochondrial stain that is actively taken up by live mitochondria, was used to study the impact of OGD on striatal neuronal MMP. Following exposure of striatal neurons to 2 h of OGD and 12 h of reperfusion of neurons in normaxic conditions were stained with MitoTracker red and the MMP changes were visualized by fluorescence microscopy. Results showed decreased intensity of staining in OGD-treated cells (Fig 2A), suggesting membrane depolarization. However, in control cells the intensity of staining was more pronounced suggestive of normal respiring mitochondria. Mitochondrial membrane depolarization leads to release of apoptotic factors including AIF and cytochrome c. Subsequently, we performed dual labeling studies to determine mitochondrial translocation of cytochrome c. At 12 h of reoxygenation after OGD, striatal neurons displayed a decrease in MitoTracker uptake accompanied by a diffuse distribution of cytochrome c, whereas a punctate distribution of cytochrome c immunostaining were evidenced in cells exposed to normaxic conditions, suggestive of mitochondrial release of cytochrome c (Fig 2B). To further confirm the mitochondrial release of cytochrome c in striatal neurons following OGD exposure, immunoblot analysis was performed. As shown in

Fig 2C, cytochrome c levels in the cytosol is increased in OGD-treated cells as compared with control cells exposed to normoxic conditions. Taken together, our studies suggest that OGD-induced striatal cell death is mediated by loss of MMP and associated release of cytochrome c.

OGD induces caspase-9 and -3 activation in striatal neurons

Mitochondrial release of cytochrome c is a well-established upstream activator of caspase-9 and -3 (Kojima, 1998; Tafani et al., 2000). Caspase-3 plays a central role in the execution of apoptotic cell death in both neuronal and non-neuronal cells. In the present study, we evaluated the effect of OGD on caspase activation in striatal neurons. As shown in Fig. 3A, caspase-9 activity increased 30% at 12 h post OGD ($p<0.05$). However, csapse-9 activity was absent in striatal neurons derived from PKCδ-KO mice. Next, we measured the activity of caspase-3, as effector caspase. Similar to caspase-9 activation, exposure of striatal neurons to 2 h OGD followed by reperfusion for 12 and 24 h respectively resulted in a 1.5 and 3 -fold increase in caspase-3 activity (Fig 3A). This elevation was also absent in striatal neurons derived from PKCδ-KO mice. To confirm the mitochondrial involvement of caspase-3 activation, we performed immunofluorescence studies to determine whether depolarized mitochondria colocalized with caspase-3. As shown in Fig. 3B and C, we found marginal activation of caspase-3 in control neurons; however, in OGD-treated neurons, a pronounced increase in caspase-3 staining. Together, the data indicate that caspase-9 activation precedes caspase-3 activation and may be partly mediated via mitochondrial dysfunction during OGD-mediated cell death of striatal neurons.

OGD triggers proteolytic processing of PKCδ in striatal neurons

PKCδ is a critical target of caspase-3 and a downstream protein implicated in oxidative stress-induced neuronal loss (Kaul et al., 2005; Kanthasamy et al., 2003). Recently, we and others have demonstrated that PKCδ may be an endogenous substrate for caspase-3 (Raval et al., 2005; Latchoumycandane et al., 2005). Activated caspase-3 cleaves PKCδ to a 38-kDa regulatory and 41-kDa catalytic fragment to enable persistent increase in kinase activity. As shown in Fig. 4A in control cultures (NML), the levels of cleaved PKCδ (34 and 44 kDa) fragments were low. OGD induced a marked increase in cleaved PKCδ in striatal neurons, evidenced at 12 and 24 h as compared to control culture. The data suggests that PKCδ might be critically involved in OGD-induced cell death of primary striatal neurons.

PKCδ deficiency confers resistance to OGD-induced striatal neuronal injury

To test the hypothesis that PKCδ not only mediates but also accompanies neuronal cell death during cerebral ischemia, we studies the effects of OGD on wildtype (WT) and PKCδ-KO cultured striatal neurons. As shown in Fig. 5A, 24 h after OGD, WT cells display condensed and brightly stained nuclei, consistent with apoptotic mode of cell death, whereas in cells cultured from PKCδ-KO mice, this type of staining pattern was rather sparse. Cell counting showed 180% increase in the number of condensed nuclei as compared with WT cells that were exposed to nomoxic conditions. However, the corresponding increase in condensed nuclei in PKCδ-KO neurons was only 50%, thus constituting a 130% decrease in cell death following OGD in PKCδ-KO cells. In a comparative study using LDH analysis, cultured neurons from PKCδ-KO mice were resistant to OGD-induced LDH release (Fig. 5B). In fact, a complete reversal of OGD-induced LDH release was observed in striatal neurons

cultured from PKCδ-KO mice as compared to WT animals. To confirm that PKCδ activation occurs in dying neurons, we studied the extent of localization of PKCδ to terminal deoxynucleotidyl transferase-mediated biotin-dUTP nick-end labeling (TUNEL)-positive neurons by immunofluorescence studies. PKCδ deficient neurons exhibited decreased TUNEL-positive staining and colocalization with PKCδ immunopositive cells in contrast to WT neurons (Fig. 5C) exposed to OGD. The data is consistent with the model in which PKCδ is a central mediator of OGD-induced cell death.

PKCδ expression in GAD67-posotive neurons and increased staining for TUNEL following OGD

To investigate whether PKCδ may act as a deleterious factor in the demise of GABAergic neurons following OGD, we first asked whether PKCδ is expressed by GABAergic neurons in culture. Fig. 6A depicts dual immunolabeling for PKCδ and GAD67, GABAergic neuronal marker. As demonstrated in the merged images, GABAergic neurons in culture express PKCδ. In normoxic control, a moderate level of PKCδ staining was evidenced, however, in OGD-treated neurons, an intense PKCδ staining was evidenced suggesting an upregulation of PKCδ in GAD67-positive neurons. In an effort to determine the neurodegenerative capacity of GAD67- positive neurons, we performed TUNEL staining to assess the extent of apoptotic nuclei. As shown in Fig. 6B, control striatal neurons showed weak TUNEL staining in GAD67-positive neurons, whereas in neurons exposed to OGD for 2 h and 24 h reperfusion, several GAD67-positive cells stained positively for TUNEL. The increase in TUNLE-positive staining in GAD67 immunoreactive neurons suggests that GABAergic neurons are vulnerable to OGD-induced striatal injury. Collectively, these data

suggest that PKCδ might be a critical mediator in the demise of GABAergic neurons following exposure of striatal culture to OGD.

DISCUSSION

Majority of the studies in ischemic research have focused on either cortical or hippocampal regions; however, the cellular mechanisms underlying increased vulnerability of the striatum, a dopamine enriched brain region to ischemia-induced brain damage remains poorly understood. In our study, cerebral ischemia is modeled by exposing primary striatal neurons to OGD environment. This model of oxidative stress is believed to mimic ischemic situations occurring in the brain following cerebral ischemia (Ravel et al., 2005). Multiple complementary methods including DNA fragmentation, LDH activity and live-and-dead cell kit have been used to demonstrate striatal ischemic cell death (Fig. 1). The critical role of mitochondria in regulating cerebral ischemia has been demonstrated through various chemical inhibitory studies, utilizing lidoflazine and nortriptyline, specifically targeted for the protection of mitochondria from ischemic damage caused by stroke (Rosenthal et al., 1987; Zhang et al., 2008). A major proapoptotic event that follows mitochondrial stress is cytochrome c release, which is a critical step in the evaluation of cell death after cerebral ischemic insult (Fujimura et al., 1998; Saguwara et al., 1999). A downstream signaling event is the activation of caspase-3, an effector protease that plays the execution role in apoptotic cell death, including cerebral ischemia-induced injury (Manabat et al., 2003). Furthermore, other studies have shown that genetic knockdown of caspase-3 results in neuroprotection in ischemic insult (Le et al., 2002). Our findings in the present study are indeed consistent with existing literature, aimed at studying the importance of mitochondrial events in cerebral

ischemia. Similarly, we have observed similar form of oxidative stress-induced cell death events in our studies, including loss of MMP followed by cytochrome c release (Fig. 2) and caspase-3 activation in primary striatal neurons subjected to OGD treatment (Fig. 3).

In the present study, we have shown PKCδ-positive neurons in primary striatal culture and their colocalization with TUNEL staining in 24 h post OGD-treated neurons (Fig. 6). We have also provided evidence that PKCδ is activated via proteolytic cleavage (Fig. 4), suggesting the mediatory role of PKCδ in OGD-induced neuronal death. Our findings are consistent with other studies demonstrating the central role of PKCδ in hippocampal neuronal demise (Raval et al., 2005). Likewise, studies from our lab demonstrated that H_2O_2 induced oxidative stress resulted in cell death in N27 dopaminergic cells and was proven to occur via mitochondria mediated oxidative stress (Kaul et al., 2005). In recent years, our lab has repeatedly demonstrated the importance of mitochondrial cell death pathways involving PKCδ proteolytic cleavage in mediating dopaminergic neuronal degeneration in N27 dopaminergic cells exposed to diverse Parkinsonian toxins (Kaul et al., 2003; Yang et al., 2004). Taken together, our results suggest that proteolytic activation of PKCδ may be a master regulator in ischemia-induced brain damage evidenced.

PKCδ is a member of the novel PKC family that can be activated by various forms of cellular stimuli through several mechanisms. Some of these modes of activation include membrane translocation (Joseloff et al., 2002; Sun et al., 2000), tyrosine phosphorylation (Kaul et al., 2005; Denning et al., 1996), proteolytic cleavage by activated enzymes (Kanthasamy et al., 2003; Reland et al, 1999; Denning et al., 2002) and more recently, a nuclear localization signal has been identified in the C-terminal region of PKCδ (DeVries et al., 2002) that suggests a possibility of nuclear targeting by the catalytically activated PKCδ.

It appears that different modes of PKCδ activation are highly dependent on the type of cell line, the stimulus and the expression characteristic of PKCδ. In our ischemic model, we demonstrated activation of caspase-3 induced proteolytic cleavage of PKCδ in primary striatal neurons. As of now the role of PKCδ in cerebral ischemia research have focused on cortical ischemia or hippocampal ischemia (Bright et al., 2004; Koponen et al., 2000), our study provides novel evidence for the participation of PKCδ in striatal ischemia and data on PKCδ activation via caspase-3 dependent mechanism (Fig. 4). The apoptotic role of PKCδ is also confirmed in PKCδ knockdown studies, where cell death is attenuated in striatal neurons derived from PKCδ-KO mice (Fig. 5).

Further, in depth studies revealed subtype specific vulnerability of GABAergic neurons to ischemic insults, colocalizing with TUNEL (Fig. 6B). The colocalization of PKCδ within GABAergic neurons shown in Fig. 6A suggests that the increased vulnerability of GABAergic neurons may be partly explained by PKCδ mediated regulation of ischemic cell death pathway. Taken together, these results suggest that caspase-3 dependent proteolytic activation of PKCδ is a key regulatory event that serves as a trigger in the demise of GABAergic neuronal phenotypes during ischemic insult to the striatum. Further studies are warranted to study the striatal neuronal subtype specific vulnerability to ischemia-induced brain damage. This may lead to the development of novel therapeutic strategies in the treatment of stroke.

In conclusion, our study presents data on the regulatory role of caspase-dependent PKCδ activation in striatal ischemia induced by OGD methods. Dysfunction of the mitochondria is also shown via classical characteristic events such as depolarization of mitochondrial membrane potential and release of proapoptotic factors namely cytochrome c.

Close examination of striatal neuronal vulnerability to OGD revealed that GABAergic neurons, which are PKCδ positive, are particularly vulnerable to reperfusion-associated neuronal injury. The resistance of PKCδ-deficient neurons to OGD-induced apoptotic cell death and associated attenuation of mitochondria-dependent cell death signaling events highlight the critical role of PKCδ in mitochondria-mediated cell death signaling events in primary striatal cultures.

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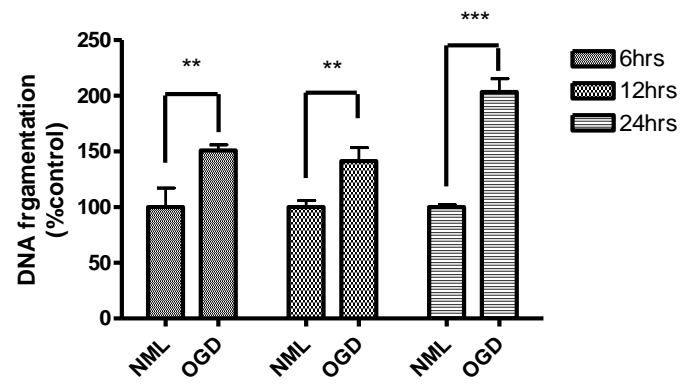
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FIGURE 1.

A

B

NML-12hr (10x)

Live	dead	merge

OGD-12hr (10x) Live   dead   merge

NML-24hr (10x)

Live	dead	merge

OGD-24hr (10x)

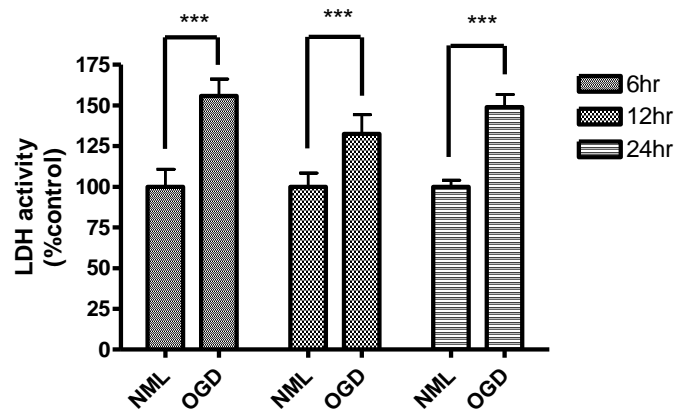| Live	dead	merge
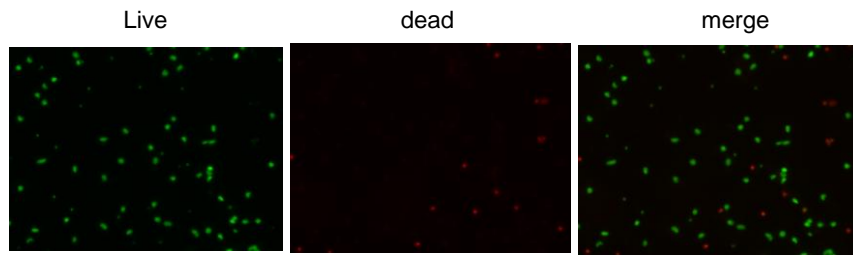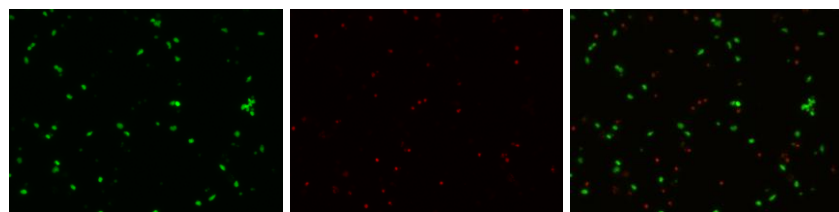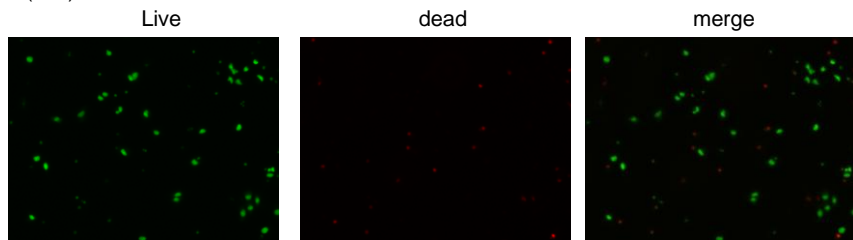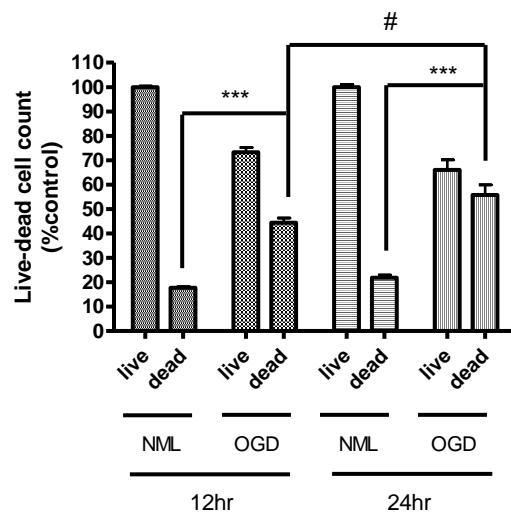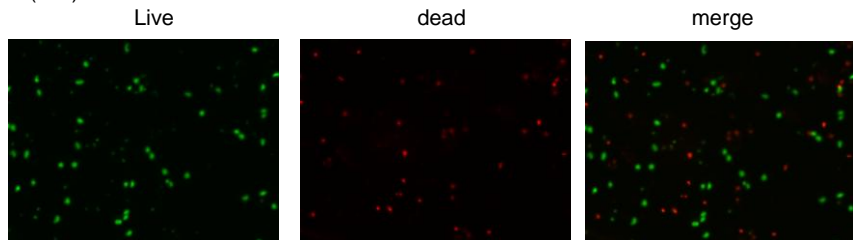
C

Figure 1. **OGD-induced striatal cell death.** A, DNA fragmentation analysis was done at 6, 12 and 24 h post OGD treatment in primary striatal neurons and compared to cells in normoxic conditions (NML). Data represents results from six individual measurements and are expressed as mean+/-S.E.M. Asterisks (**p<0.01, ***p<0.001) indicate significant difference compared to control group (NML). B, Live-dead cell staining (PI) revealed immunostaining of healthy and apoptotic shown in green and red fluorescence respectively. Primary striatal neurons were treated with OGD and PI staining was done 12 and 24 h post OGD. Cells were counted from 5 microscopic views under 10x magnification for each sample and numbers are calculated and graphed. Asterisks (***p<0.001) indicate significant difference compared to control group (NML), and pound signs (#p<0.05) indicate significant differences in 12 and 24 h. C, LDH assay revealed cell death levels in 6, 12 and 24 h post OGD-treated primary neurons. Data represents results from six individual measurements and are expressed as mean+/-S.E.M. Asterisks (***p<0.001) indicate significant difference compared to control group (NML).

FIGURE 2.

A

B

C

Figure 2. **OGD-induced mitochondrial impairment involves dissipation of membrane potential and mitochondrial release of cytochrome c.** A, MMP was shown with MitoTracker red staining in 12 h post OGD-treated primary neurons and compared with cells under normaxic conditions (NML). Neurons were fixed, stained and viewed under a Nikon TE2000 fluorescence microscope at 10x magnification. B, Dual immunostaining with MitoTracker and cytochrome c was done in 12 h post OGD-treated primary neurons. Colocalization of mitochondria (red) and cytochrome c (green) was observed and images were taken under 60x magnification under a Nikon TE2000 fluorescence microscope. C, Western blot analysis was used to detect cytochrome c (15kDa) in both cytosolic and mitochondrial compartments in 12 h post OGD-treated primary neurons. Cox-IV (17kDa) was detected as a marker for mitochondria, determining the cellular fractionation procedure. β-actin was detected for equal protein loading.

FIGURE 3.

A

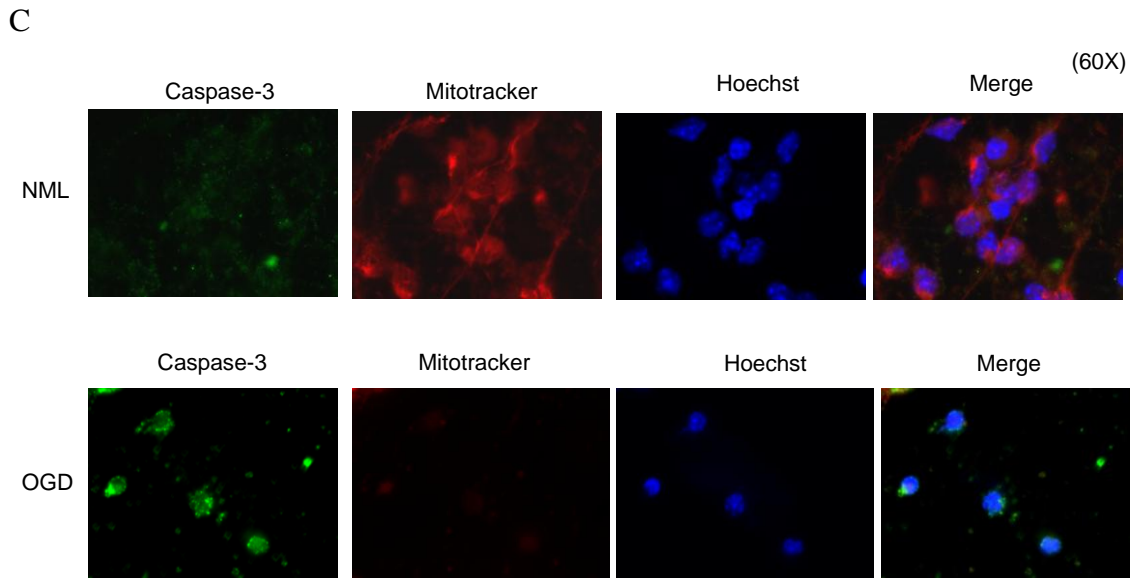Caspase-3 actitvity at 12 and 24 hrs post OGD

B

C
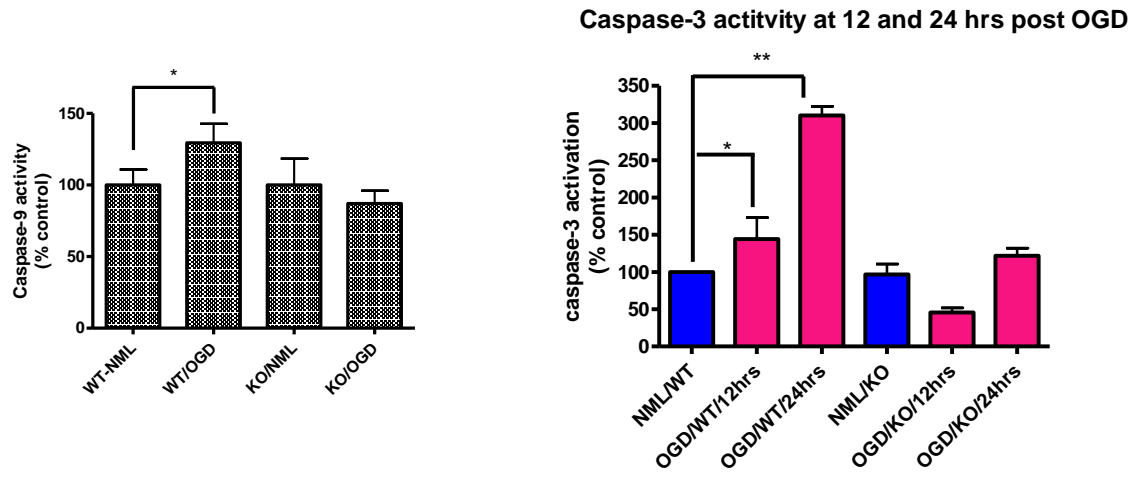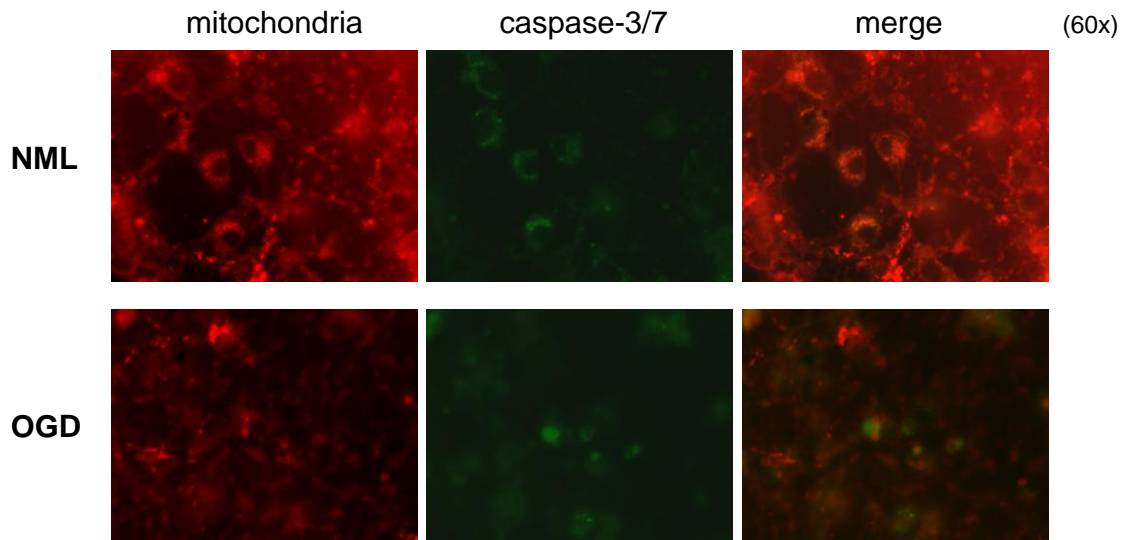
Figure 3. **Mitochondria regulates ischemic cell death in PKCδ-WT cells via caspase-3 mediated PKCδ pathway.** A, Caspase-9 and -3 activity was detected in both OGD-treated PKCδ-WT and -KO primary neurons, detailed procedures are described in the methods section. Caspase-9 activity was detected in 12 h post OGD-treated neurons and caspase-3 activity was detected in 12 and 24 h post OGD-treated neurons. Data represents results from six individual measurements and are expressed as mean+/-S.E.M. Asterisks (*p<0.05, **p<0.01) indicate significant difference compared with control group (NML). B, MitoCasp, a dual staining that detects mitochondria (red) and caspase-3/7 (green), was used for immunostaining in 24 h post OGD-treated PKCδ-WT primary striatal neurons. C, Dual immunostaining methods detected for Mitotracker (red) and caspase-3 (green) in 12 h post OGD-treated PKCδ-WT primary neurons. All images were viewed and taken under a Nikon TE2000 fluorescence microscope under 60x magnification.

FIGURE 4.

A

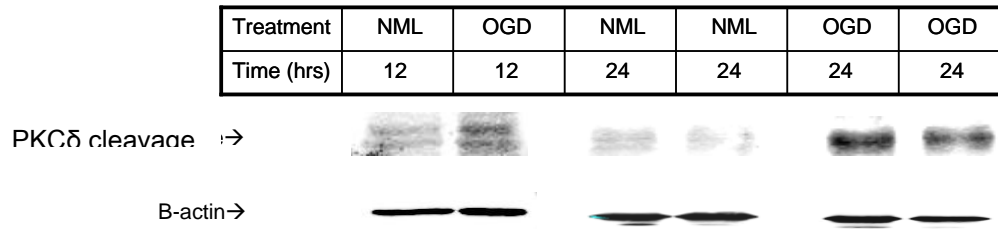| Treatment	NML	OGD	NML	NML	OGD	OGD
Time (hrs)	12	12	24	24	24	24

PKCδ cleavage →

B-actin→

Figure 4. **OGD triggers proteolytic cleavage of PKCδ in striatal neurons.** Western blot studies detected for cleaved PKCδ (41 kDa) in samples derived from 12 and 24 h post OGD-treated primary neurons and compared to control group (NML). β-actin (43 kDa) was reprobed for equal protein loading. Please see methods section for detailed procedure.
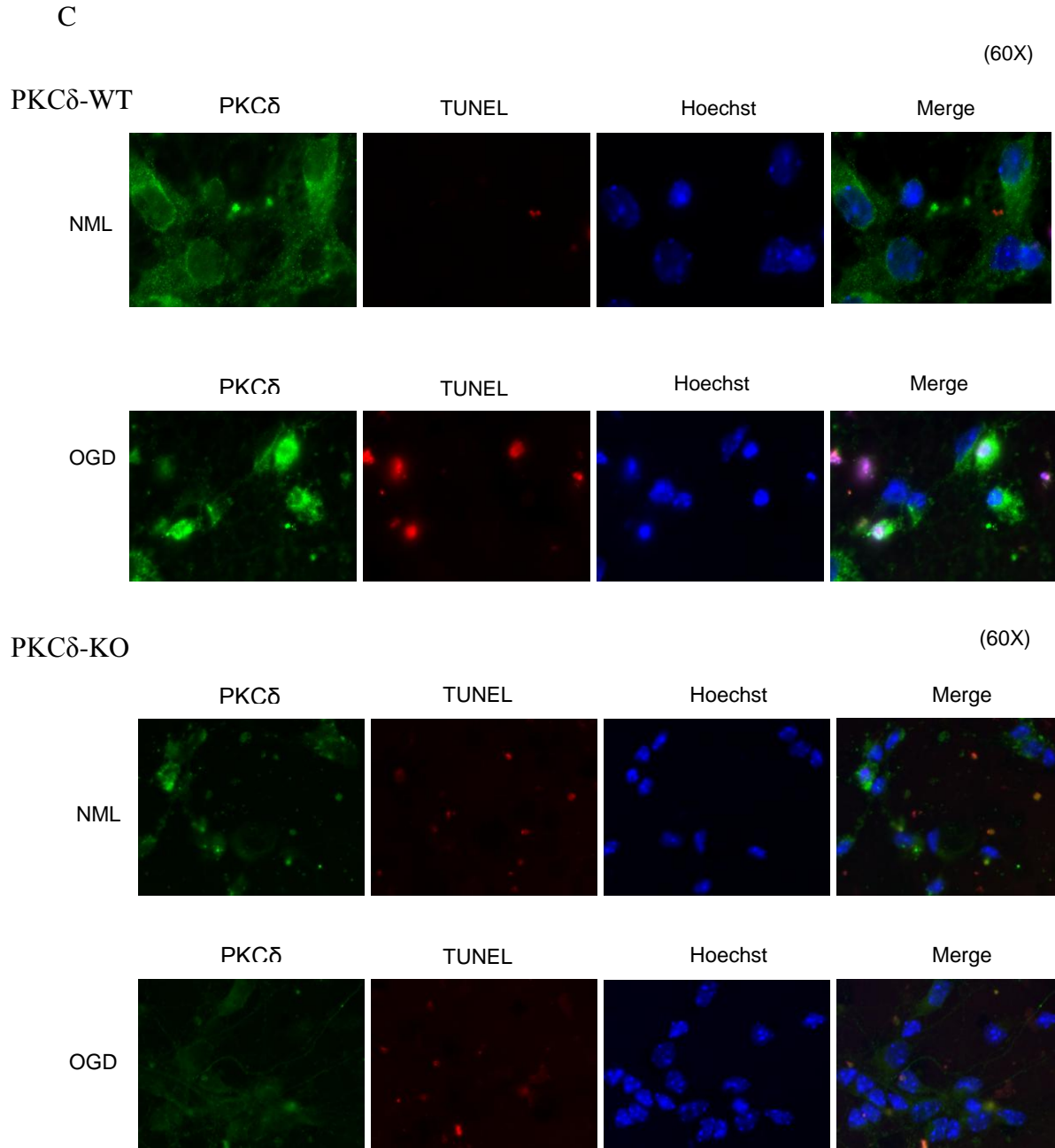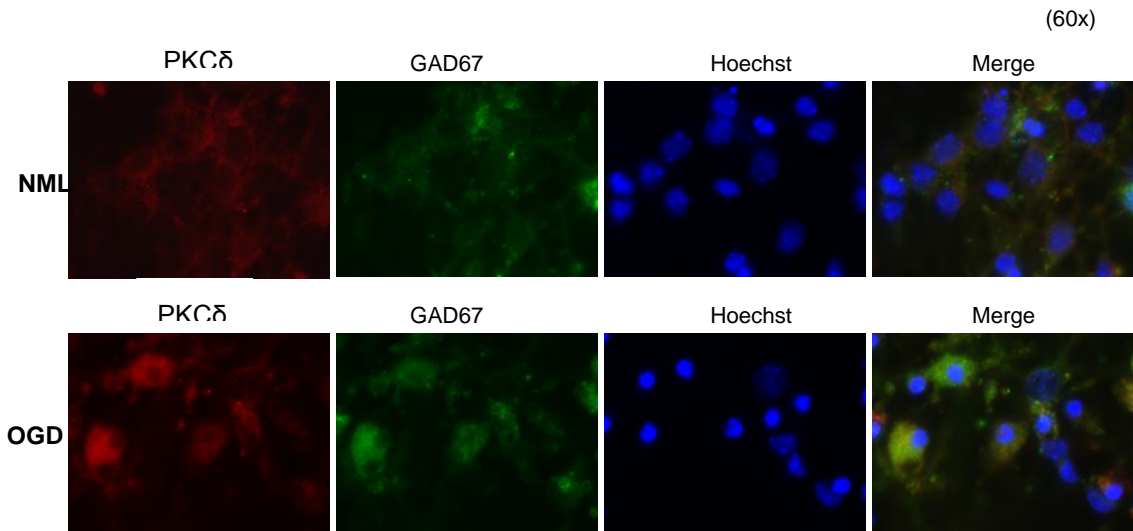
FIGURE 5.

A

(10x)

B

C

(60X)

PKCδ-WT
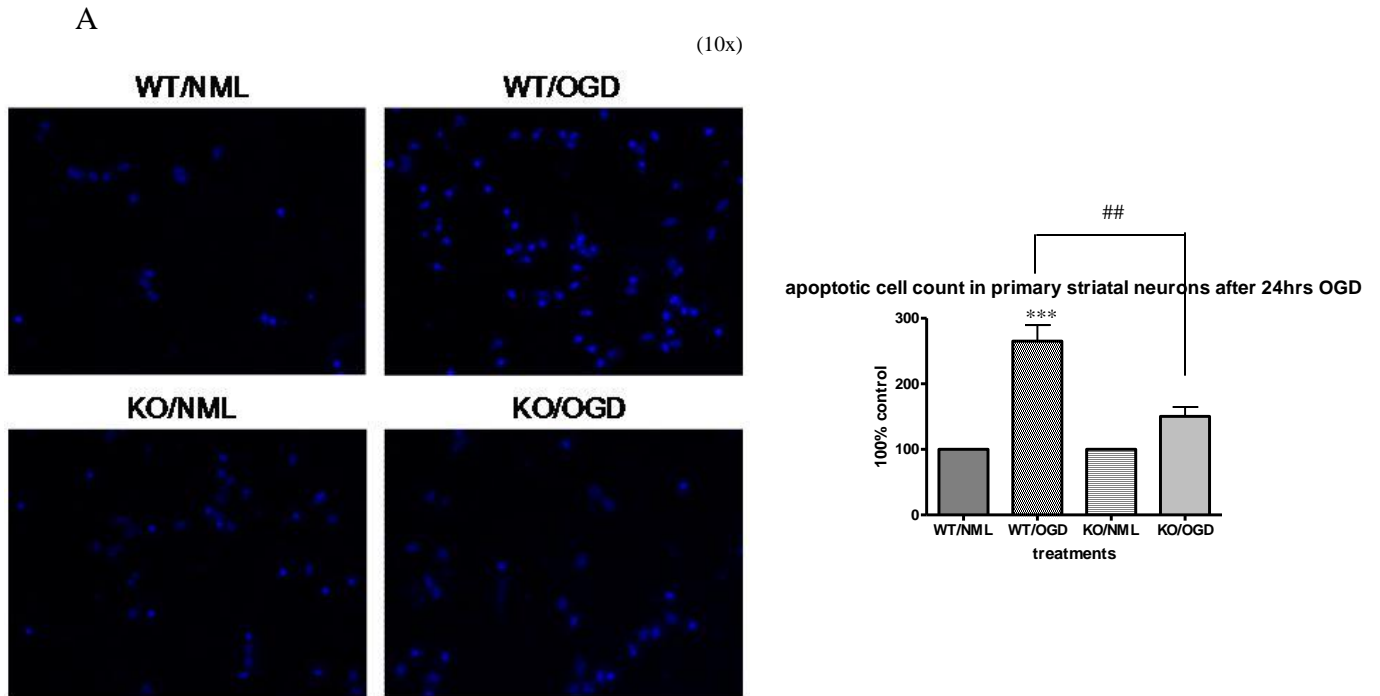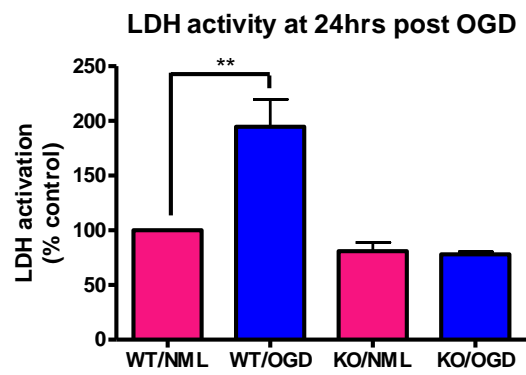
Figure 5. **Ischemic cell death is attenuated in primary neurons from PKCδ-knockout (KO) mice compared to PKCδ-wildtype (WT) mice.** A, Both PKCδ-WT and -KO primary neurons were stained with Hoechst 3324 for nuclei morphology studies at 24 h post OGD treatment. Apoptotic cells and healthy cells are distinguished and counted with a Nikon TE2000 under 10x magnification. Asterisks (***p<0.001) indicate significant difference

compared with control group (NML) and pound sign (##p<0.01) indicate significant differences between OGD-treated PKCδ-WT and -KO neurons. B, LDH activity was assessed in 24 h post OGD-treated PKCδ -WT and -KO neurons. Medium was collected for detection, details are described in methods section. Data represents results from six individual measurements and are expressed as mean+/-S.E.M. Asterisks (**p<0.01) indicate difference compared with control group (NML). C, Dual immunostaining method was used to detect for PKCδ and TUNEL 24 h post OGD-treated PKCδ-WT and –KO neurons and compared to cells under normoxic conditions (NML). Samples were viewed and pictures were taken with a Nikon TE2000 fluorescence microscope under 60x magnification.
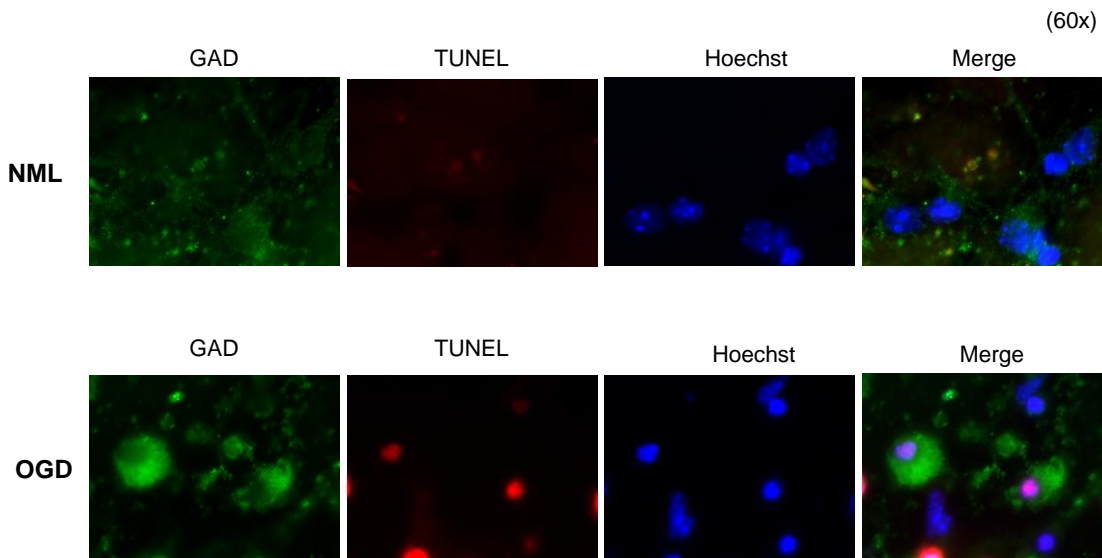
FIGURE 6.

A

B

Figure 6. **Striatal GABAergic neurons are immune-positive for PKCδ and colocalize with TUNEL.** A, Dual immunostaining methods is used to detected PKCδ-positive neurons (red) and GABAergic (GAD67)-positive neurons (green) in 12 h post OGD-treated cells. B, Dual immunostaining also revealed GABAergic (GAD67)-positive neurons colocalize with

TUNEL in 12 h post OGD-treated cells compared to control cells (NML). Samples were viewed and pictures were taken with a Nikon TE2000 fluorescence microscope under 60x magnification.

Chapter VI: General Conclusions

GENERAL CONCLUSIONS

The major goal of my project is to understand the cellular mechanisms underlying dopaminergic and striatal neurodegeneration. Although oxidative stress and apoptosis are considered key mediators during delayed ischemic cell death and MA-induced neurotoxicity process, the downstream executioner events that lead to DNA fragmentation subsequent to caspase-3 activation are currently not well established. Furthermore, the contribution of autophagy in the mechanism of neurodegeneration is poorly understood. Based on our experimental findings, together with existing literature, we propose that local microenvironment, type of stressor, magnitude of induction of the cell death pathway might be key determinants in the implementation of specific type of cell death pathways. We propose the following model for the neuronal cell death mechanisms induced by MA and OGD in dopaminergic and striatal neurons, respectively:

Pathway 1:

PKCδ was activated via proteolytic cleavage in both models. Furthermore, the regulatory role of PKCδ in cell death was demonstrated by cytoprotection in MA-treated CRM cells. Moreover, inhibition of PKCδ activation resulted in significantly reduced caspase-3 activity and associated apoptotic cell death; hence underscoring the central role of PKCδ in mediating apoptotic cell death in response to MA.

The important role of mitochondria in mediating cell death pathway was discovered by experiments monitoring MMP during MA treatments and the dissipation of MMP that resulted in mitochondrial release of cytochrome c release and activation of the downstream

cell death signaling events. Our findings emphasize the importance of intrinsic pathway in mediating the demise of dopaminergic neurons following exposure to MA. A well-maintained protein clearance system is essential for cellular wellbeing. In the context, the presence of elevated levels of ubiquitinated proteins has been considered to be an important hallmark of neurodegenerative disease. In fact, MA induces massive accumulation of ubiquitin-positive intracellular bodies within dopaminergic neurons. Defective UPS function could result from oxidative stress when oxidatively damaged proteins overwhelm the degradation capacity of proteasome. This is consistent with our findings showing downregulation of proteasomal activity and accumulation of ubiquitinated proteins associated with MA-induced cell death. Furthermore, emerging evidence indicate that impaired proteasomal system can cause the induction of autophagy, which is considered to be a compensatory mechanism believed to aid in the clearance of aggregated proteins. However, MA-induced autoaphgy is less investigated and less is known regarding the association between UPS and autophagy in neurotoxicity models. Our findings point to a putative cross talk between UPS and autophagy in MA-induced cell death, by showing colocalization of ubiquitin and LC3 in MA-treated neurons. The role of autophagy in cell death is a strongly debated issue and remains controversial. Our results suggest autophagy may play a neuroprotective role in MA-induced cell death to minimize the magnitude of neurotoxicity (Scheme 1- Pathway 1). However, we propose that the neuroprotective intentions of autophagy may eventually be overwhelmed by increasing proapoptotice signals or maybe due to dysfunction of the ALS, shifting the autophagy-apoptosis balance toward apoptosis.

Pathway 2:

OGD leads to mitochondrial dysfunction, hence causing the induction of mitochondria –
mediated deleterious intrinsic cell signaling events resulting in the release of proapoptotic
factors including cytochrome c subsequently leading to the induction of downstream caspase-
3 dependent signaling events that facilitate the loss of GAD67-positive neuron via proteolytic
activation of PKCδ.

To summarize, in both MA and OGD experimental models of neurodegeneration,
impairments in mitochondrial function facilitate proteolytic cleavage of PKCδ, thereby
resulting in its sustained activation for protracted periods of time ensuring the successful
dismantling of neurons via apoptotic cell death, despite a futile attempt made by autophagy
to protect the dying neurons as in the case of MA-induced dopaminergic neuronal injury.
Hence, the therapeutic use of antioxidants may protect neurons from further insults
associated with age related pathogenic changes and acute insult that ultimately result in
neuronal demise. In addition, agents that cause a moderate enhancement in autophagy may
be beneficial in the treatment of neurodegenerative diseases including PD and cerebral
ischemia.

Scheme 1
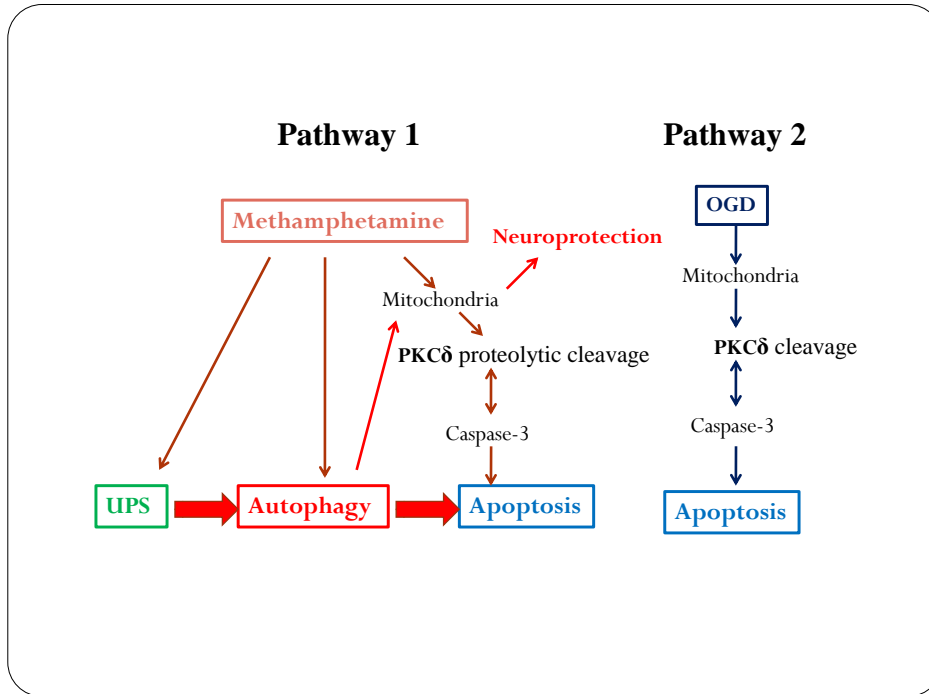
Scheme 1. Diverse pathways participate in cell death mechanisms. Mitochondria appear to play a central role in the induction of several of the cell signaling pathways as depicted. In this context, PKCδ acts as a master regulator in the regulation of diverse cell signaling mechanisms. Normal cellular function is governed by a balance between homeostatic and deleterious factors. Once the neuron is overwhelmed by a particular level of stress response, the cell is committed to apoptotic cell death irrespective of the induction of the cytoprotective response, namely autophagic response.

ACKNOWLEDGEMENTS

I would like to first sincerely thank my major professor Dr. Arthi Kanthasamy, who has given me the opportunity to learn from her and conduct research in her lab. I have learned the professionalism required in conducting intensive life science research. I am grateful for the assistantship grants she has provided me with to enable my three years of studies/work under her supervision, and even more, her timely efforts in giving me constructive advice. During these years, I have learnt experimental techniques, experimental design and improved on scientific writing skills. In addition, I would also like to thank my committee members, Drs. Walter Hsu and Aunumantha Kanthasamy, for their professional guidance and for being the highly respected role models they have set.

Below I would also like to thank the people that have given me the support and motivation for completing my master studies. Dr. Anantharam Vellareddy has been of great help around the lab and it was also a pleasure to work alongside with Dr. Qinglin Li. Special thanks to previous graduated lab members, Drs. Danghui Zhang and Faneng Sun, and current lab members Chunjuan Song, Huajun Jin, Hari Swaminathan and others for their kind help with completing my research. I would also like to mention the supporting staff of Biomedical Sciences department, Linda Erickson and Kim Adams, who were helpful in many ways.

Finally, I am very grateful of my parents and family who has been supportive of me in both ups and downs of life.