CHAPTER III
LITERATURE REVIEW

3.1 Methamphetamine

Methamphetamine (METH) is a psychostimulant and a common drug of abuse in Thailand and worldwide. Although it is difficult to draw the actual number of drug abusers, the United Nations Office on Drugs and Crime (UNODC) estimated that 16-51 million people between the age of 15 and 64 used amphetamine-type stimulants drugs at least once (Sinchai, Plasen et al. 2011). It is the cause of national problem in Thailand as well, at which 72% of patients at Thanyalak Institute for Drug Abuse (TIDA) were METH abusers (Kulsudjarit 2004).

METH is a central nervous system (CNS) stimulant that can be synthetically prepared by catalytic hydrogenation of ephedrine or pseudoephedrine. METH is similar in structure to other CNS stimulants such as amphetamine and methcathinone, which can also be produced from ephedrine and pseudoephedrine by chemical reduction. Thus, METH is a synthetic stimulant, as opposed to drugs obtained from natural resources, such as cannabinoids (derived from *Cannabis sativa*) and cocaine (derived from *Erythroxylon coca*).

METH affects the biochemical mechanisms responsible for regulation of heart rate, body temperature, blood pressure, appetite, attention, mood and responses associated with alertness and alarm conditions. The acute physical effects of the drug closely resemble to the physiological and psychological effects of an epinephrine-provoked fight-or-flight response, including increased heart rate and blood pressure, vasoconstriction, bronchodilation, and hyperglycemia. As a result, long term usage of this drug can cause a variety of mental disorders, including anxiety, confusion, and hallucination (Thrash, Thiruchelvan et al. 2009).

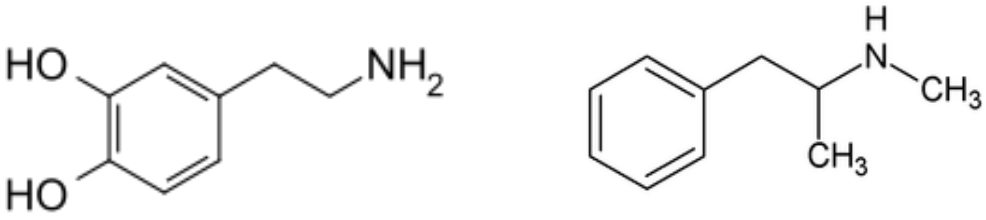
METH has been known for many years to induce neurotoxicity, which may cause long-lasting changes in the central dopaminergic pathway. Several lines of evidence suggest that loss of the nigrostriatal dopaminergic neurons and subsequent dopamine depletion in the striatum is associated with METH exposure. In humans,

examination of *post-mortem* brain tissue from METH abusers revealed a significant decrease in striatal dopamine and dopamine transporter density (Wilson, Kalasinsky et al. 1996). Administration of METH also caused neurotoxicity in both rodents and nonhuman primates (Zhang, Kitaichi et al. 2006). It has been further shown that repeated administration of METH reduces dopamine transporter binding affinity and, in extreme cases, causes apoptosis (Davidson, Gow et al. 2001; Cadet, Jayanthi et al. 2003; Kita, Wagner et al. 2003). Toxicological studies on the brain tissue of human METH users have shown long-term and possibly irreversible damage to dopaminergic neurons and loss of striatal dopamine transporters even after three years of abstinence from the drug (Cadet, Jayanthi et al. 2003). Researchers have found dopaminergic toxicity in mice after a single dose of METH (25 mg/kg) or after multiple doses of METH varying from 2.5 to 10 mg/kg given at two-hours interval (Chan, Di Monte et al. 1994). After such doses of METH, long term decrease in nigrostriatal dopamine similar to that seen in Parkinson's disease was observed.

Further studies have demonstrated a link between METH intoxication with some molecular steps that operate during neurodegenerative disorders of dopaminergic neurons. Evidence shows that oxidative damage by METH impairs ubiquitination and degradation of proteins by proteasomes. This may aid in the aggregation of α-synuclein, which is the main protein that forms eosinophilic inclusions known as Lewy bodies, a pathological hallmark of Parkinson's disease. In keeping with this evidence, and similar to neurodegeneration, METH administration is known to produce ubiquitin positive neuronal inclusions within nigral dopaminergic cells *in vitro* and in animal models. These neuropathologic findings were further confirmed in humans thus posing a strong bridge between METH intoxication and neurodegenerative disorders in humans (Pasquali, Lazzeri et al. 2008; Thrash, Thiruchelvan et al. 2009).

3.1.1 Mechanism of action of METH

The possible mechanism underlying METH induced extensive dopaminergic cell death is that METH triggers an excessive release of DA, increasing ROS, and thus, inducing mitochondrial disruption and apoptotic cell death. Excessive release of DA by METH is based on several molecular targets (such as the dopamine transporter [DAT], the vesicular monoamine transporter-2 [VMAT-2], and monoamine oxidase [MAO]), which are involved in the uptake, storage, and release of dopamine. METH is a substrate for these molecules, a feature predictably due to its similar chemical structure to dopamine (Figure 3.1). METH causes the DAT and VMAT-2 to reverse their direction flow. It displaces dopamine in the vesicles, releasing DA into the cytoplasm and from the cytoplasm to the synapse leading to cytoplasmic and synaptic aggregation of dopamine. METH also indirectly prevents the monoamine reuptake of these neurotransmitters, causing them to remain in the synaptic cleft for a prolonged period. In addition, the blockade of monoamine oxidase (MAO) type A by METH occludes its physiological metabolism and leaves cytosolic DA prone to self-oxidization, forming DA-quinones and cysteinyl aggregates, resulting in an increase in ROS formation, mitochondrial malfunction, apoptosis and nerve terminal destruction (Larsen, Fon et al. 2002; Pasquali, Lazzeri et al. 2008; Thrash, Thiruchelvan et al. 2009; Sinchai, Plasen et al. 2011).



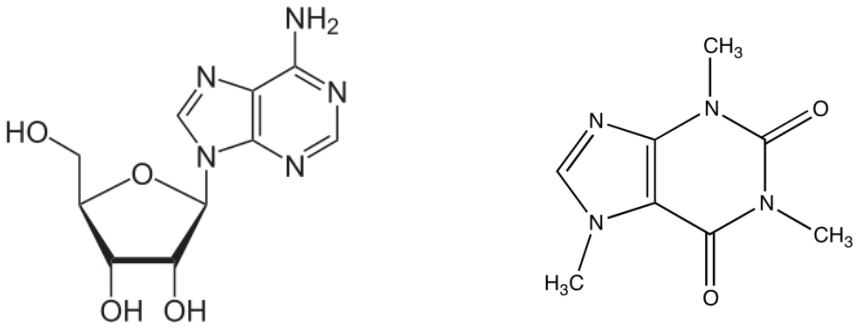
Dopamine **Methamphetamine**

Figure 3.1 Structural diagram of Dopamine and Methamphetamine

3.2 Caffeine

Caffeine, white crystalline xanthine alkaloid that acts as a stimulant drug, is the most widely used psychoactive substance in the world. Caffeine is found in varying quantities in the seeds, leaves, and fruit of some plants. It is most commonly consumed by humans in infusions extracted from the seed of the coffee plant and the leaves of the tea bush, as well as from various foods and drinks containing products derived from the kola nut. It is also an ingredient in various over-the-counter drugs (OTCs) including headache, cold, allergy, pain relief, and alerting drugs.

Caffeine produces behavioral effects that are similar to those of typical psychomotor stimulant drugs that are known to be dopaminergically mediated (e.g., amphetamine and cocaine). The most notable behavioral effects of caffeine occur after low to moderate doses (50–300 mg) and are increased alertness, energy and ability to concentrate. Moderate caffeine consumption leads very rarely to health risks. Higher doses of caffeine rather induce negative effects such as anxiety, restlessness, insomnia and tachychardia, these effects being seen primarily in a small portion of caffeine-sensitive individuals (Nehlig 1999).



Adenosine **Caffeine**

Figure 3.2 Structural diagram of Adenosine and Caffeine (Wikipedia).

The popularity of caffeine as a psychoactive drug is due to its stimulant properties. Effects of caffeine are mediated by dopaminergic mechanisms which vital for the regulation of motor behavior. The primary mechanism of caffeine enhancing dopaminergic activity is due to the blockade of adenosine receptors, adenosine A_1 and A_{2A}, in the brain as caffeine molecule is structurally similar to adenosine (figure 3.2). The pattern of distribution of adenosine A_1 and A_{2A} receptors in the brain differs

strikingly. The A_1 receptor has a widespread distribution. The highest levels are found in the hippocampus, cerebral and cerebellar cortex, and certain thalamic nuclei, while only moderate levels are found in caudate-putamen and nucleus accumbens (Lorist and Tops 2003). At the cellular level, the majority of adenosine A_1 receptors are located on presynaptic nerve terminals, where they mediate the inhibition exerted by adenosine on the release of neurotransmitters, including glutamate, dopamine and acetylcholine. The inhibitory control on neurotransmission exerted by adenosine, via A_1 receptors, is thought to account for the positive effect produced by caffeine on arousal, vigilance and attention. In contrast to the rather ubiquitous distribution of A1 receptors, the expression of adenosine A_{2A} receptors in the brain is limited to regions heavily innervated by dopamine-containing fibers, such as the striatum and the olfactory tubercle (Fisone, Borgkvist et al. 2004). A_{2A} receptors are coexpressed with dopamine D_2 receptors in the striatal neuronal cells (Nehlig 1999). Several lines of evidence showed the antagonistic interaction between adenosine A_{2A} and dopamine D_2 receptors. At the plasma membrane level, stimulation of A_{2A} receptors results in decreased affinity of the dopamine D_2 receptor for agonists. At the cytoplasm level, A_{2A} receptors are opposite regulating physiological response to D_2 receptors. All together, by antagonizing the negative modulatory effects of adenosine receptors on dopamine receptors, caffeine leads to the inhibition and blockage of adenosine A_{2A} receptors, leading to a potentiation of dopaminergic neurotransmission, produced its psychomotor stimulant effect.

Caffeine is the world's most widely consumed psychoactive drug, but, unlike many other psychoactive substances, it is both legal and unregulated in nearly all parts of the world. However, sometime it was considered as a potential drug of abuse. Caffeine was concocted to amphetamine-type stimulants tablets such as Ya-Ba to increase their weight and volume and to enhance the stimulating effect of METH. There are many studies show the potentiating effect of METH by caffeine. Kuribara (1994) found enhancement of amphetamine stimulation effect by caffeine in mice (Kuribara 1994). Simola et al. (2006) showed that caffeine pretreatment potentiated the motor effects of amphetamine in both intact and 6-hydroxydopamine-lesioned rats (Simola, Tronci et al. 2006). In addition to potentiating stimulant effect, the neurotoxicity of this combination is also studied. Sinchai et al. (2011) demonstrated

that caffeine potentiates methamphetamine-induced toxicity both *in vitro* and *in vivo*. Combination treatment of METH and CAF at individually non-toxic concentrations significantly decreased viability of human neuroblastoma SK-N-SH cells and increased the mortality rate of rats (Sinchai, Plasen et al. 2011). The results indicate that caffeine potentiates the toxic effects of methamphetamine, possibly via a mechanism involving an increase in dopamine release and excess ROS generation. However, more studies regarding the mechanism of caffeine potentiating effect of METH is needed to be done.

3.3 Autophagy

Autophagy or self-eating process is an intracellular degradation process responsible for the clearance of most long-lived proteins and organelles in which cytoplasmic components are delivered into lysosomes for degradation. This system has been implicated in various physiological processes including protein and organelle turnover, stress response, cellular differentiation, programmed cell death, and also in some pathological conditions.

Three different autophagic pathways are known: 1) macroautophagy, 2) microautophagy, and 3) chaperone mediated autophagy (CMA). In macroautophagy, a double-membraned structure (called a phagophore) elongates to engulf a portion of cytoplasm, and then fuses to form a vesicle called autophagosome. Autophagosome fuses with lysosome, thereby forming autolysosome, where the cytosolic contents are degraded by lysosomal hydrolase (Figure 3.3). In microautophagy, a portion of cytoplasm is directly engulfed into lysosomes by invagination of the lysosomal membrane. CMA involves the selective transport of cytosolic proteins that contain a pentapeptide motif related to KFERQ across the lysosomal membrane via the chaperone hsc70 and the lysosomal membrane receptor LAMP-2A (Garcia-Arencibia, Hochfeld et al. 2010). Since macroautophagy is the most studied type of autophagy and is found to be involved in pathogenesis of diseases, therefore this type of autophagy will be studied in this experiment and hereafter macroautophagy will be referred to as autophagy.

3.3.1 Signaling pathways regulating mammalian autophagy

Autophagosome formation is regulated by many signals that fall into two broad categories: mammalian target of rapamycin (mTOR)-dependent and mTOR-independent.

3.3.1.1 mTOR-dependent signaling

The mammalian target of rapamycin (mTOR) kinase is a master negative regulator of autophagy (Levine and Klionsky 2004) (Figure 4). mTOR is a central sensor of energy status, growth factors and nutrient signals, and can be inhibited by drugs such as rapamycin (Rubinsztein, Gestwicki et al. 2007). Cells can monitor and respond to impaired nutrients or energy via a number of mechanisms, including essential amino acid detection via Rag (Sancak, Peterson et al. 2008; Sancak, Bar-Peled et al. 2010) and activation of AMP-activated protein kinase (AMPK) in response to elevated AMP to ATP ratios (Meijer and Codogno 2006). Both of these pathways decrease mTOR activity in response to depleted nutrients. Insulin and growth factor signals also feed in to the mTOR pathway via insulin receptors, which ultimately signal to activate mTORC1 (Klionsky and Emr 2000). mTORC1 controls autophagy by direct interaction with the ULK1-Atg13-FIP200 complex. Under nutrient-rich conditions, mTOR suppresses autophagy through direct interaction with the ULK1–Atg13–FIP200 complex and mediates phosphorylation-dependent inhibition of the kinase activity of Atg13 and ULK1 (Mizushima, Kuma et al. 2003)

3.3.1.2 mTOR-independent signaling

There are a number of mTOR-independent signals that signal to the autophagy pathway. The first evidence for the existence of mTOR-independent regulation of mammalian autophagy comes from studies showing that autophagy is negatively regulated by intracellular inositol and inositol 1,4,5-trisphosphate (IP_3) levels. Inhibition of inositol monophosphatase (IMPase) reduces free inositol and IP_3 levels, which leads to an upregulation of autophagy (Garcia-Arencibia, Hochfeld et al. 2010; Metcalf, Garcia-Arencibia et al. 2010).

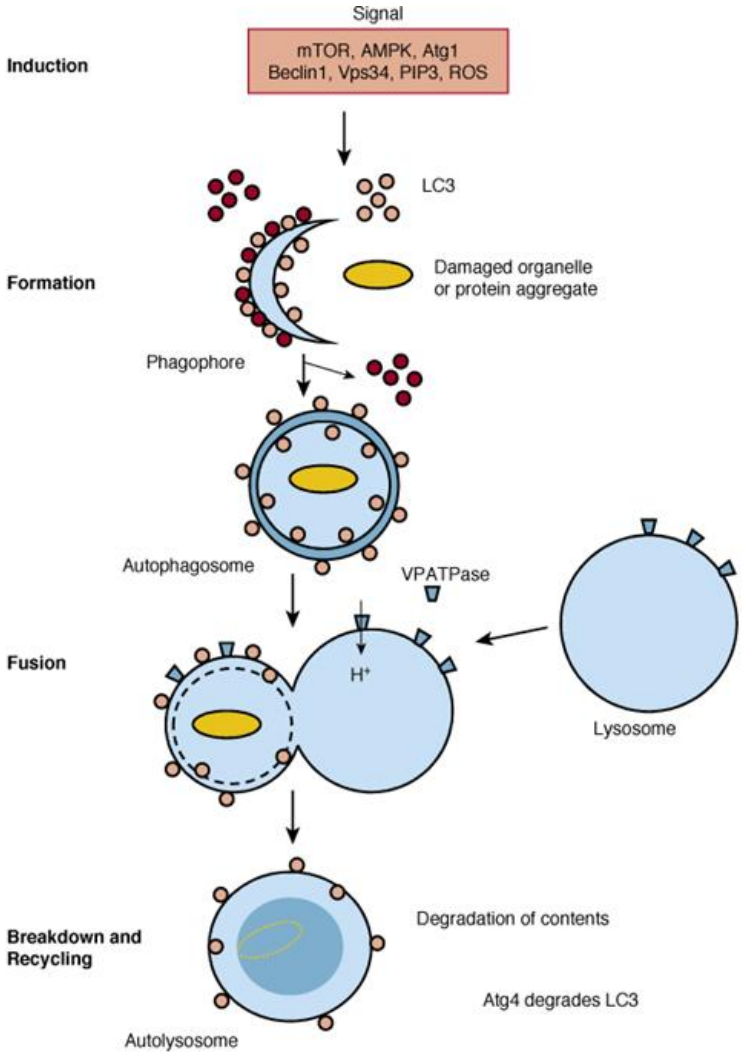


Figure 3.3 Model for autophagy (modified from Gottlieb, 2010)

Model for autophagy and autophagic cell death. Under starvation conditions or other stresses, autophagy is induced. The autophagic induction process starts with an isolation membrane (phagophore). The origins of the membranes are still not fully understood, and there may be multiple sources. Membrane could be generated de novo or from existing compartments such as ER, mitochondria, and the plasma membrane. Phagophore engulfs cytoplasmic materials including protein aggregates and organelles. This is followed by vesicle nucleation, vesicle elongation and retrieval, and formation of the characteristic double-membraned autophagosomes. These autophagosomes fuse with lysosomes to form autolysosomes. The enclosed cytoplasmic materials and the inner membrane are degraded by the acidic proteases in the autolysosomes. The most widely used marker to study autophagy is LC3 (microtubule-associated protein 1 light chain 3, a mammalian homologue of yeast Atg8), as it is the only known protein that specifically associates with autophagosomes and not with other vesicular structures. During autophagy, the cytosolic form of LC3 (LC3-I) is lipidate (conjugated with the lipid phosphatidylethanolamine (PE)) to generate LC3-II lipidated and translocates to autophagosome membranes (Chen, Azad et al. 2010; Garcia-Arencibia, Hochfeld et al. 2010).

Beclin-1 is a key regulator of autophagy pathways through its ability to form a number of different complexes. Autophagy can be inhibited by the binding of the apoptosis-related proteins Bcl-2 or Bcl-XL to Beclin 1. Starvation induces Jun N-terminal kinase 1 (Jnk1) activity, which phosphorylates Bcl-2, thereby disrupting the interaction between Beclin 1 and Bcl-2 to induce autophagy. This mechanism might also account for the upregulation of autophagy. On the other hand, when Beclin-1 incorporated into the class III PI3-K complex, which comprises Vps34, Vps15 and Atg14, Beclin-1 is stimulatory and targets to pre-autophagosome structures (Garcia-Arencibia, Hochfeld et al. 2010; Metcalf, Garcia-Arencibia et al. 2010).

Autophagy is also modulated by ROS. Generally, ROS is harmful to the cell. However, they also have physiological function including cell signaling. Starvation (a potent inducer of autophagy) increases levels of ROS in a PI3K-dependent manner. One way in which ROS may be acting to regulate autophagy is by the modulation of the action of Atg4 on Atg8/LC3. Atg4-mediated delipidation of LC3-II on the cytosolic surface of autolysosomes, allows it to be recycled. Atg4 is inactive and unable to cleave Atg8 from membranes when in its oxidized state. It is therefore possible that under oxidative conditions Atg4 is oxidized and inactive, which allows Atg8 to lipidate and thus initiates autophagy, while reduced Atg4 form is active and favors Atg8 delipidation (Garcia-Arencibia, Hochfeld et al. 2010).

3.3.2 Autophagy contributing to cell survival or cell death?

Basal autophagy plays an important role in cellular homeostasis, by degrading of long-lived proteins, protein aggregates and organelles. Autophagy contributes to intracellular quality control by clearing the misfolded protein to maintain the normal functional protein in cell. In addition, autophagy can also be induced by various physiological and pathological situations, such as, nutrient starvation, or pathogen infection. Under nutrient starvation, the autophagic process breaks down cytoplasmic materials into amino acids and fatty acids that can be used for protein synthesis or can be oxidized by the mitochondrial electron transport chain (mETC) to produce ATP for cell survival. Autophagy is induced upon infection, in which pathogen is localized inside the autophagic vacuoles. This indicates that

autophagy acts as a host defense mechanism in infected cells (Eskelinen and Saftig 2009; Garcia-Arencibia, Hochfeld et al. 2010).

Autophagy is critical to life. It can contribute to cell survival and also cell death. Limited self-eating can provide cells with metabolic substrates to meet their energetic demands under stressful conditions or favor the selective elimination of damaged organelles as mentioned. On the other hand, autophagy dysregulation, either impairment or over activation can trigger and mediate programmed cell death. Defects in the autophagy machinery have been implicated in the pathogenesis of various diseases, such as, neurodegenerative disease, cancer, and infectious diseases. The activation of the autophagic pathway beyond a certain threshold can directly promote cell death, by causing the collapse of cellular functions as a result of cellular atrophy (Rami 2009).

Considering all of the available data, It is believed that autophagy has a beneficial effect of protecting against neurodegeneration; however, how autophagy can prevent neurodegeneration is not completely understood. One hypothesis is that autophagy eliminates protein aggregates or inclusion bodies, possibly in a direct manner (Castino, Lazzeri et al. 2008).

3.3.3 Detection of autophagy

The study of autophagy generally involved detection of an autophagic process and assessment of the functional output. Since autophagy is characterized by the formation of double-membranced autophagosomes, the verification of autophagy is mainly based on the detection of autophagosomes (Chen, Azad et al. 2010). Various methods have been developed for detecting autophagy including the detection of processed LC3II by western blot analysis. LC3 (microtubule-associated protein 1 light chain 3, a mammalian homologue of yeast Atg8) is the only known protein that specifically associates with autophagosomes and not with other vesicular structures. During autophagy, the cytosolic form of LC3 (LC3-I) is lipidate (conjugated with the lipid phosphatidylethanolamine) to generate LC3-II lipidated and translocates to autophagosome membranes. Thus LC3II can be used as a marker of an autophagosome. However an autophagosome is not always represent autophagic flux

which is a dynamic process of autophagy indicating the cellular autophagic activity. Autophagosome is an intermediate structure in a dynamic pathway, the number of autophagosomes observed at any specific time point is a function of the balance between the rate of their generation and the rate of their conversion into autolysosomes (Mizushima, Yoshimori et al. 2010). Thus when autophagic flux is measured, an additional control is required. A lysosomal inhibitor has to be used to reflect the dynamic nature of autophagy. Lysosomotropic reagents such as ammonium chloride, chloroquine, or bafilomycin A1, which inhibit acidification inside the lysosome or inhibit autophagosome-lysosome fusion blocked the degradation of LC3-II, resulting in the accumulation of LC3-II (Tanida, Minematsu-Ikeguchi et al. 2005). The differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors represent the amount of LC3 that is delivered to lysosomes for degradation (i.e., autophagic flux). In the condition that autophagic flux is increased, the differences in the amount of LC3-II between samples in the presence and absence of lysosomal inhibitors are higher than in the stagnation of clearance condition.

3.4 Apoptosis

Apoptosis is a form of cell death in which cell committing suicide. It is a type of programmed cell death characterized by DNA fragmentation, nuclear condensation, blebbing membrane, and cell shrinkage. Apopotosis is vital important for multicellular survival in development process, balance cell proliferation to maintain a constant organ size, and maintain normal cellular function by eliminate damaged or infected cells (Cooper and Hausman 2009).

3.4.1 Feature of apoptosis

Various morphological changes occurred during apoptosis. Early in apoptosis, the cells round up, losing contact with their neighbors. In the nucleus, chromosomal DNA is fragmented, chromatin condenses, and the nucleus becomes break up to small pieces. The plasma membrane is blebbing, and finally the cell is

breaked into a various size of compact membrane-enclosed structures of cellular content, called 'apoptotic bodies' (figure 3.4). The apoptotic bodies are recognized and phagocytosed by macrophages , thus are removed from the tissue. This form of cell death is different from the necrotic mode of cell-death, in which lyse, releaseing their content into the cell's environment results in damage of surrounding cells and inflammatory response (Leist and Jaattela 2001). However, necrosis can also be the final result in situations where there is too much apoptosis occurring for phagocytotic cells to cope with, especially in cell culture, where professional phagocytotic cells are usually lacking (Lawen 2003).

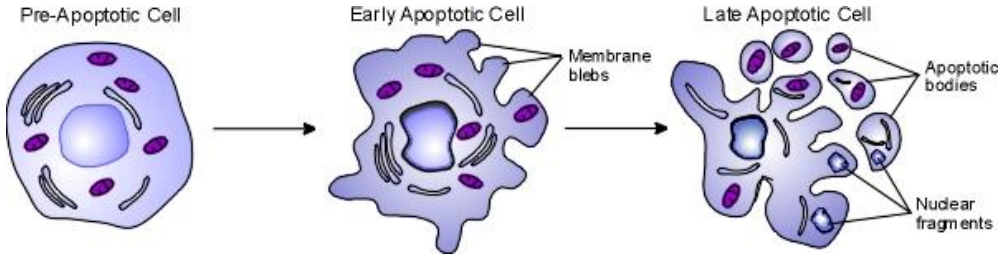


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Figure 3.4 Diagrammatic representation of the event of apoptosis.

3.4.2 Mechanism of apoptosis

The mechanism of apoptosis is involved in an energy dependent cascade of molecular events. There are two main mechanisms that can initiated apoptosis, the extrinsic pathway and the intrinsic pathway (Figure 3.5). Initiation of either pathway results in the same regulatory and effector mechanisms. Initiation leads to the expression of regulatory apoptotic genes that give rise to the initiator and effector caspases. Once an initiator caspase is cleaved, the caspase cascade is activated. This cascade causes the cleavage of effector caspases, typically caspase 3, which responsible for the cleavage of a number of so-called death substrates that lead to the well-known characteristic hallmarks of an apoptotic cell including DNA fragmentation, chromatin condensation, membrane blebbing and other morphological and biochemical changes (Pond ; Portt, Norman et al. 2011)

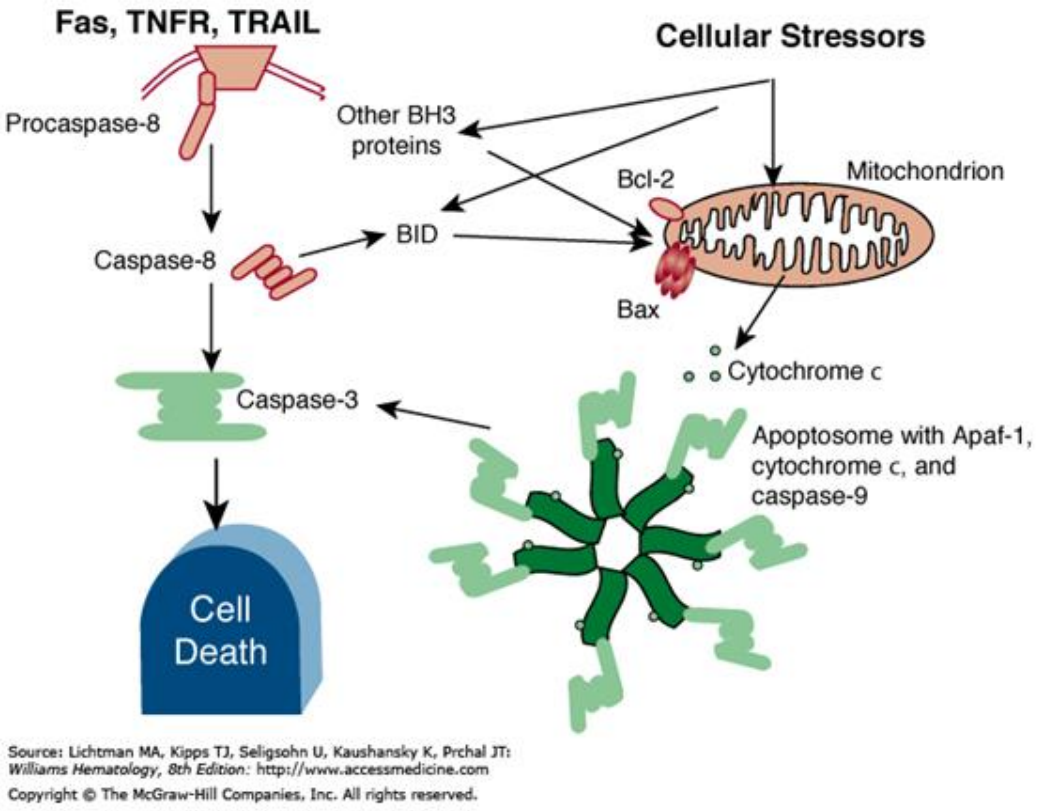
The extrinsic pathway or the death receptor pathway is initiated apoptosis by transmembrane receptor-mediated interactions. The death receptor that induced apoptosis in this pathway belong to the tumor necrosis factor (TNF) receptor gene superfamily such as TNF receptor and Fas receptor, which play important roles in controlling cell death in the immune system. These receptors directly activate a distinct initiator caspase, caspase-8 results in the activation of effector caspases and nucleases (e.g., caspase-3, -6, and -7, caspase-activated DNase) (Elmore 2007; Galluzzi, Brenner et al. 2008).

The intrinsic pathway or mitochondria mediated pathway is controlled by mitochondria, which collect and integrate pro- and anti-apoptotic signals incoming from other organelles as well as from the extracellular microenvironment. Cellular stresses such as decreased oxygen, increased reactive oxygen species, endoplasmic reticulum (ER) stress, lysosomal stress, DNA damage and calcium (Ca^{2+}) overload or the absence of certain growth factors, hormones which lead to failure of suppression of death programs are able to activate this pathway of apoptosis by favoring mitochondrial membrane permeabilization (MMP). The release of pro-apoptotic molecules, such as cytochrome-c from the intermembrane space to cytoplasm activates downstream effector caspases, ultimately leading to cell death. The control and regulation of these apoptotic mitochondrial events occurs through members of the Bcl-2 family of proteins (Cory and Adams 2002) that are either pro-apoptotic or anti-apoptotic (Pond ; Elmore 2007; Galluzzi, Brenner et al. 2008).

3.4.3 Significant of apoptosis

Apoptosis is important process in survival of organism. It is a process in which individual cells die for the good of the whole. Apoptosis is part of developmental processes, cell turn over, regulation and function of the immune system. There are a variety of stimuli and conditions that can trigger apoptosis such as oxidative stress, radiation, DNA damaged, or chemical-induced cell death. Apoptosis eliminate damaged cell or infected cell that may interfere normal function of organism to maintain multicellular biological systems. Inappropriate apoptosis either too little or too much is lead to pathological conditions. Defects in apoptosis can result in

cancer, autoimmune diseases and spreading of viral infections while excessive apoptosis can cause neurodegenerative disorders, AIDS and ischaemic diseases (Elmore 2007; Cooper and Hausman 2009).



Figrue 3.5 Two main signaling cascades lead to apoptosis. Extracellular signaling from the death receptors tumor necrosis factor receptor (TNFR), Fas, and TNF-related apoptosis-inducing ligand (TRAIL) lead to activation of caspase-8, which leads to activation of caspase-3 and other end effectors of apoptosis. Cell stressors and related signals lead to mitochondrial alterations resulting in caspase-3 activation through the interaction of Apaf-1, cytochrome *c*, and caspase-9. Bcl-2 opposes the pathway that involves mitochondria (Kaushansky and Williams 2010).

3.5 Relation of METH to Apoptosis and Autophagy

METH induced extensive dopaminergic cell death is thought to depend on that it can trigger an excessive release of dopamine, increasing oxidative stress, and thus, inducing mitochondrial disruption and apoptotic cell death. Many studies have documented the fact that METH can cause neuronal apoptosis through cross-talks between the mitochondria, endoplasmic reticulum, and receptor mediated death pathways (Jayanthi, Deng et al. 2001; Cadet, Jayanthi et al. 2003; Cadet, Jayanthi et al. 2005; Jayanthi, Deng et al. 2005). It has been shown that METH treatment cause increases in pro-apoptotic proteins, Bax, Bad and Bid, but decreases in antiapoptotic proteins Bcl-2 and Bcl-XL (Jayanthi, Deng et al. 2001; Deng, Cai et al. 2002). These proteins are known to interact in the mitochondrial membrane and are involved in either activating or inhibiting the mitochondria-dependent cell death pathway (Breckenridge and Xue 2004). The alterations of pro or anti apoptotic proteins form METH administration cause the formation of channels lead to the reduction of mitochondrial membrane potential and the release of mitochondrial cytochrome c into the cytosol with subsequent activation of the caspase cascade (Deng, Cai et al. 2002).

It is also consistent with the study from Wu et. Al. (2007) that METH induced a decrease in mitochondrial membrane potential, an increase in intracellular oxidative stress, and mitochondrial mass increasing in the neuroblastoma cells (Wu, Ping et al. 2007). The increase in mitochondrial mass may accelerate the activation of METH-induced apoptosis as it may provide more membrane in which the pro-death proteins are able to form channels that allow cytochrome c release (Breckenridge and Xue 2004).

In addition to disruption of mitochondria, METH causes cell death through interaction of the mitochondria- and ER-dependent death pathways (Jayanthi, Deng et al. 2004). METH induced oxidative stress causing endoplasmic reticulum (ER) stress and dysfunctions. Sustained release of Ca^{2+} from the ER stores initiate calcium-dependent apoptosis via the permeabilization of the outer mitochondrial membrane cause release of pro apoptotic factors disrupts cellular metabolism (Kroemer, Galluzzi et al. 2007).

METH-induced neuronal apoptosis has also been linked to stimulation of the death receptors pathway. METH was shown to increase the expression of FasL, which is a member of the tumor necrosis factor (TNF) superfamily of cytokines that is involved in mechanisms of neuronal apoptosis, in striatal neurons (Jayanthi, Deng et al. 2005). Thorburn et. Al. (2004) found that there are cleavages of caspases 8 and 3, which are mediators of FasL/Fas apoptosis pathway after METH administration (Thorburn 2004).

Although several lines of evidence suggested that the mechanism underlying METH induced neuronal damage mostly depends on ROS production and apoptosis. Many studies reported the association of autophagy in this process. The effects of METH on the autophagic pathway are thought to depend on the excess of oxidative species generated within the cell. Particularly within the DA neuron, these effects become severe as METH is specific to dopamine targets and the tendency of dopamine to self-oxidize and generate free radicals (Pasquali, Lazzeri et al. 2008) (Figure 3.6).

Several studies have demonstrated that neurotoxic doses of METH can cause autophagy in vitro and in vivo (Larsen, Fon et al. 2002; Fornai, Lenzi et al. 2004; Kanthasamy, Anantharam et al. 2006; Lazzeri, Lenzi et al. 2006; Castino, Lazzeri et al. 2008). For example, METH caused vacuolation of endocytic compartments and formation of autophagic granules within cytoplasm of midbrain neuronal cells (Cubells, Rayport et al. 1994; Larsen, Fon et al. 2002). Kanthasamy et al. (2006) have reported drug-related autophagic changes in mesencephalic DA cell cultures (Kanthasamy, Anantharam et al. 2006). Kongsuphol et al. (2009) found that METH treatment in SK-N-SH dopaminergic cell line inhibits the phosphorylation of mTOR, the negative regulator of autophagy and increase the expression of LC3-II, a protein associated with the autophagosome membrane, in a dose-dependent manner (Kongsuphol, Mukda et al. 2009). Immortalized mesencephalic cells treated with METH also show characteristic vacuoles in their cytoplasm (Cadet, Ordonez et al. 1997). METH-induced accumulation of cytoplasmic inclusions resembling autophagic vacuoles has also been reported in PC12 cells, in nigrostriatal dopaminergic neurons and in striatal GABAergic neurons (Fornai, Lenzi et al. 2004; Lazzeri, Lenzi et al. 2006). On that basis, autophagy was considered essential in

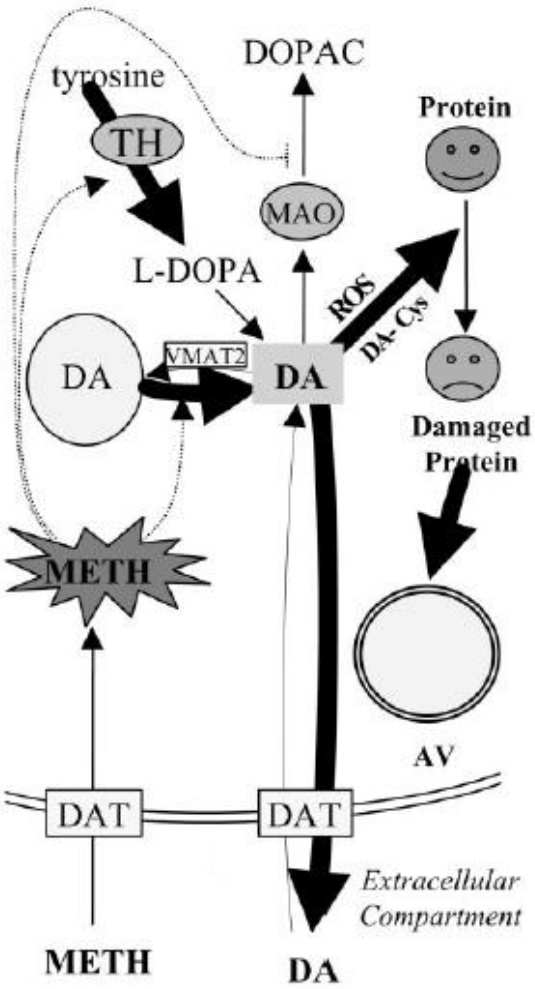


Figure 3.6 Proposed model for METH-induced neurotoxicity (Larsen, Fon et al. 2002). Proposed model for METH-induced neurotoxicity in ventral midbrain dopminergic neurons. METH is taken up by the plasma membrane DAT. Once inside the neuron, METH promotes dopamine release from synaptic vesicles and stimulates TH activity to synthesize even more dopamine. Cytosolic dopamine is metabolized by monoamine oxidase (MAO) to DOPAC and/or released into the extracellular milieu. METH is known to inhibit monoamine oxidase, resulting in additional increase in cytosolic dopamine. Excess cytosolic dopamine is oxidized to reactive dopamine metabolites, such as cysteinyl-DA, resulting in damaged or dysfunctional proteins and lipids. These damaged constituents are sequestered within autophagic vacuoles (AV) for degradation.

METH-induced neurotoxicity, but its neurotoxic or protective role has not been clearly addressed.

Because autophagy plays a critical role in the degradation of oxidatively damaged proteins, it is possible that METH-induced dopamine dependent production of ROS promotes protein misfolding and aggregation, resulting in the upregulation of autophagic degradation in dopaminergic neurons as a part of the protective mechanism against drug toxicity. This idea is supported by findings showing that inhibition of autophagy either by pharmacologic (3-methyladenine) or genetic manipulation of the class III phosphatidylinositol-3 kinase-mediated signaling prevented the removal of α-synuclein aggregates and eventually lead to caspase-dependent cell death following bax-mediated permeabilization of mitochondria in METH-treated dopaminergic neuronal-derived cell line (the rat pheochromocitoma PC12 cells and the human neuroblastoma SH-SY5Y) (Castino, Lazzeri et al. 2008).

3.6 Relation of caffeine to Apoptosis and Autophagy

Caffeine was known to induce apoptosis in many cell lines and this mechanism is responsible for the cytotoxic effect of caffeine. Caffeine was shown to substantially increase apoptosis and mitochondrial damage in human cancer cell lines HL-60 and U937 (Dai, Yu et al. 2001). Lu et. al. (2008) reported that caffeine induced ROS trigger cell injury through activation of mitochondria-dependent apoptosis cell death signaling and inactivation of the survival signal apoptosis in human and mouse osteoblast cell line(Lu, Lai et al. 2008). Caffeine treatment was reported to increase caspase-3 enzyme activity in SK-N-MC human neuroblastoma (Jang, Shin et al. 2002). He et. al. (2003) illustrated that the mechanism of induction of apoptosis in JB6 Cl41 and human non-small cell lung cancer H358 cell line cells by caffeine involves activation of p53, Bax, and caspase 3 (He, Ma et al. 2003).

Over than involvement in apoptosis pathway, caffeine has also been reported to inhibit some kinase activities, including various forms of phosphoinositol-3 kinase and mammalian target of rapamycin (mTOR) (Foukas, Daniele et al. 2002; Kudchodkar, Yu et al. 2006). Sinha et.al. (2013) showed that caffeine is a potent

stimulator of hepatic autophagic flux involving downregulation of mTOR signaling and alteration in hepatic amino acids and sphingolipid levels (Sinha, Farah et al. 2013). Saiki et.al. (2011) reported that caffeine enhance autophagic flux in a dose-dependent manner in SH-SY5Y, HeLa and PC12D cell lines. It has been suggested that the induction of autophagy is mediated mainly on PI3K/Akt/mTOR/p70S6 signaling (Saiki, Sasazawa et al. 2011). However, the exact mechanism by which caffeine induces autophagy is still not clearly understood.

In summary, METH is known to cause apoptosis concurrent with the incretion of LC3 protein level. This is similar to the effect of caffeine that can induce both apoptosis and autophagy. Sinchai et al. (2011) demonstrated that caffeine potentiates methamphetamine-induced toxicity possibly mediated via oxidative stress and apoptosis pathway. However the involvement of autophagy in this process have not been addressed, more studies regarding the mechanism of caffeine potentiating effect of METH is needed to be done.