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Amphetamine toxicities

Classical and emerging mechanisms

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The drugs of abuse, methamphetamine and MDMA, produce long-term decreases in markers of biogenic amine neurotransmission. These decreases have been traditionally linked to nerve terminals and are evident in a variety of species, including rodents, nonhuman primates, and humans. Recent studies indicate that the damage produced by these drugs may be more widespread than originally believed. Changes indicative of damage to cell bodies of biogenic and nonbiogenic amine–containing neurons in several brain areas and endothelial cells that make up the blood–brain barrier have been reported. The processes that mediate this damage involve not only oxidative stress but also include excitotoxic mechanisms, neuroinflammation, the ubiquitin proteasome system, as well as mitochondrial and neurotrophic factor dysfunction. These mechanisms also underlie the toxicity associated with chronic stress and human immunodeficiency virus (HIV) infection, both of which have been shown to augment the toxicity to methamphetamine. Overall, multiple mechanisms are involved and interact to promote neurotoxicity to methamphetamine and MDMA. Moreover, the high coincidence of substituted amphetamine abuse by humans with HIV and/or chronic stress exposure suggests a potential enhanced vulnerability of these individuals to the neurotoxic actions of the amphetamines.

Keywords: amphetamine; methamphetamine; MDMA; neurotoxicity; apoptosis; excitotoxicity; neuroinflammation; proteasome; ubiquitination; neurodegeneration; drug abuse

Introduction

Methamphetamine (METH) and its derivative, 3,4-methylenedioxymethamphetamine (MDMA), are widely abused psychostimulant drugs. The acute effects of these drugs include euphoria, alertness, decreased appetite, increased locomotor activity, and hyperthermia. Long-term abuse of METH and MDMA may result in psychosis, aggressiveness, and neurotoxicity. METH in particular has a very high abuse potential owing primarily to its strong euphoric properties. According to the recent National Institute on Drug Abuse (NIDA) reports[1–3] the abuse of METH and MDMA is an extremely serious and growing problem in the U.S. and worldwide. METH and MDMA use among significantly diverse populations has been documented. For instance, young adults who attend "raves" or private clubs are increasingly using amphetamines. METH use is also high among persons infected with human immunodeficiency virus (HIV).[4] Although the acute effects of these drugs are relatively well known, the long-term consequences and possible neurotoxicities associated with the administration of these drugs are unclear.

Amphetamines are substrates for transporters associated with the uptake of the biogenic amines dopamine (DA), norepinephrine (NE), and serotonin (5-HT). They either diffuse into or are taken up by nerve terminals via these transporters and subsequently cause a reverse transport of monoamines from the cytoplasm into the synaptic cleft. Amphetamines also promote DA and 5-HT release from storage vesicles and prevent the uptake into vesicles, thus increasing the cytoplasmic concentrations of the neurotransmitter and

making them more readily available for reverse transport. In addition, the amphetamines also increase synaptic levels of monoamines by inhibiting their reuptake.[5–8] The net result of the acute action of the amphetamines is an increased neurotransmission of DA, 5-HT, and NE. METH and MDMA differ in their affinities for monoamine transporters. MDMA has a greater affinity for the 5-HT transporter (SERT) versus the DA transporter (DAT) than amphetamine or METH.[9] Consequently, MDMA causes a greater release of 5-HT than DA. In addition, the substituted amphetamines also increase the release of glutamate (GLU),[10–12] which probably contributes to the neurotoxicity profiles of these drugs.

In rodents and nonhuman primates, administration of either a large single dose or repeated high doses of METH or MDMA produces longlasting deficits in markers of DA and 5-HT nerve terminals (i.e., the levels of a neurotransmitter, its metabolites, biosynthetic enzymes, receptors, and transporters)[13–20] while sparing NE terminals.[17,21] Amphetamines also produce astrogliosis,[22–24] and METH[18] but not MDMA[21] displays morphological signs of axonal degeneration. Early studies have shown that METH most severely affects DA terminals in the striatum,[13,16,18,25,26] whereas DA terminals in the nucleus accumbens, olfactory bulb, frontal cortex, and hypothalamus are minimally affected or unaffected.[15,16] The reasons for this difference are unclear but could be related to the varied densities of DAT in these regions. In contrast to DA terminals, 5-HT terminals in various brain regions including hippocampus, prefrontal cortex, amygdala, and striatum are equally sensitive to the toxic effects of METH.[15,16,20,27] MDMA differs from METH in that it is selectively neurotoxic to 5-HT terminals in multiple brain areas in rodents and nonhuman primates[19,28–32]; however, it can produce DA deficits in mice.[24]

A persistent reduction in most DA markers[33–37] and SERT[38,39] also has been observed in human chronic METH users. Similarly, decreases in SERT have been observed in multiple brain regions in chronic MDMA users.[40] Because of many animal studies and more recent studies on humans suggesting that the amphetamines have long-term consequences, efforts have been directed toward the understanding of the mechanisms that contribute to the neurotoxicity of the amphetamines. This review

will examine the new characteristics and emerging mechanisms purported to contribute to the neurotoxic profiles of the substituted amphetamines, METH and MDMA.

Classical aspects of METH and MDMA toxicity

Studies on the toxicity of METH and MDMA to monoaminergic terminals indicate that amphetamine toxicity involves the occurrence of at least three acute events: an increase in extracellular and intracellular DA, an increase in extracellular GLU, and hyperthermia. The major classical molecular mechanisms by which these events subsequently produce long-term effects include oxidative stress, excitotoxicity, and mitochondrial dysfunction. These mechanisms interact and result in the augmentation of their consequences. Those studies have been reviewed previously[41–48] and therefore will not be discussed in detail. This review, however, will focus on new and emerging aspects that in combination with the more classic mechanisms summarized in the following, broaden the scope of the pharmacological action of the amphetamines and contribute to their long-term toxicity.

Oxidative stress

Several studies using animal models have supported the involvement of oxidative stress in METH and MDMA neurotoxicity (reviewed in Refs. 44 and 48). For instance, METH and MDMA produce reactive oxygen species (ROS) and reactive nitrogen species (RNS) and lipid peroxidation products. Neurotoxic effects of amphetamines can be attenuated by free radical scavengers and antioxidants or overexpression of antioxidant enzymes. Both METH and MDMA decrease the levels of antioxidants in DAergic and/or 5-HTergic terminals. The presence of oxidative stress has also been documented in human METH users.[49,50] Research on mechanisms leading to amphetamine-mediated oxidative stress indicate that an early event in METH toxicity is an increase in intracellular DA levels resulting from amphetamine-mediated disruption of vesicular proton gradient and vesicular monoamine transporter function.[46] This is followed by an overproduction of toxic metabolites of DA oxidation, including free radicals and quinones.[51,52] For MDMA, which is neurotoxic only to 5-HT

terminals, it is believed that DA-derived ROS are generated in 5-HT terminals either after SERT-mediated uptake of released DA[53] or by the synthesis of DA from tyrosine.[54] Alternatively, toxic metabolites of 5-HT oxidation or MDMA itself can also mediate MDMA toxicity.[55–57]

Excitotoxicity

Excitotoxicity includes a succession of several events: excessive GLU release, activation of GLU receptors, increase in intracellular calcium levels, activation of a variety of calcium-dependent enzymes, generation of free radicals and nitric oxide (NO), and activation of apoptotic pathways, culminating in failure of cellular organelles, such as mitochondria and endoplasmic reticulum (ER), breakdown of cytoskeletal proteins, and DNA damage.[58–60] Several studies support a role for excitotoxicity in mediating METH neurotoxicity to striatal terminals. For example, high-dose METH causes a release of GLU in rat striatum[10,61] via activation of the striatonigral pathway.[62] Inhibition of this release protects against METH toxicity to terminals.[62] Agonists of metabotropic GLU receptor 5 (mGluR5)[63] and inhibitors of NO synthase (NOS)[64] attenuate METH toxicity to striatal DA terminals independently from hyperthermia. Increases in striatal levels of nitrate[65] and 3-nitrotyrosine[66] suggest that high-dose METH increases the levels of NO. METH increases breakdown of microtubule-associated protein, tau,[67] and another cytoskeletal protein, spectrin,[68] in rat striatum *in vivo* and cortical neurons *in vitro*.[69] A role for excitotoxicity in mediating MDMA toxicity is less clear.[48] Nevertheless, the mechanism by which excitotoxicity mediates the toxicity of the amphetamines appears to be NO-mediated nitration of proteins associated with DA and 5-HT terminals.[48]

Mitochondrial function

Administration of both METH and MDMA impairs mitochondrial function. More specifically, toxic doses of METH inhibit mitochondrial electron transport chain enzyme complexes, complex I,[70] complex II–III,[71] and complex IV,[72] in the striatum and other DA-containing brain areas. High-dose MDMA has been shown to decrease mitochondrial complex I–II in rat striatum[44] and complex IV in rat striatum, nucleus accumbens, and substantia nigra.[72] In addition, MDMA causes

oxidative stress in mitochondria and deletions in mitochondrial DNA coding for complex I and IV in several brain areas.[73] A correlation between impairment of mitochondria and amphetamine toxicity to monoaminergic terminals has been provided by several studies. For example, coadministration of METH[74] or MDMA[75] with an inhibitor of energy metabolism synergistically depleted striatal DA or 5-HT, respectively. Conversely, coadministration of amphetamines with energy substrates attenuated the neurotoxicity to DA and 5-HT nerve endings.[75,76] The underlying mechanism of the impairment of mitochondrial function appears to involve increases in ROS and RNS[64] and/or increases intracellular calcium,[43,44,48] which may be mediated by GLU.

Hyperthermia

Hyperthermia occurs after the administration of high doses of both METH and MDMA,[77–79] and its occurrence is important for development of amphetamine neurotoxicity to DA and 5-HT terminals. For example, multiple injections of high-dose METH at room temperature produced a significant depletion of DA in the striatum; however, equivalent doses of METH administered in a cold environment blocked striatal DA and 5-HT depletions in mice.[78] Similarly, MDMA toxicity to 5-HT terminals during hyperthermic and hypothermic conditions also can be enhanced and attenuated, respectively.[79] Hyperthermia by itself does not decrease striatal DA levels in rodents.[80] Instead, it is envisioned to enhance the enzymatic and/or nonenzymatic reactions initiated by high-dose METH or MDMA treatment. Hyperthermia might interact with other known mediators of METH neurotoxicity, such as increased GLU neurotransmission and oxidative stress. In fact, GLU receptor antagonists, such as MK-801, have been shown to reduce body temperature and provide neuroprotection.[81–83] Similarly, inhibition of METH-induced hyperthermia decreases the formation of ROS in the striatum that, in turn, attenuates the damage to DA terminals.[84]

New and emerging aspects of the toxicity of amphetamines

As noted in the preceding, a classic mechanism underlying the toxicity of the amphetamines involves oxidative stress to DA and 5-HT terminals.

However, a more current and emerging focus has been on the toxic effects of the amphetamines to nonmonoaminergic cell bodies, as originally suggested and demonstrated by several groups in the 1980s and 1990s.[19,21,85–90]

Emerging mechanisms that may be related to both terminal and cell body damage produced by the amphetamines are processes linked to excitotoxicity, inflammation, proteolytic/proteasomal dysfunction, apoptosis, alterations in trophic support, HIV infection, and the influence of environmental stress. The review that follows will cover this current literature while incorporating these mechanisms into our understanding of the classic processes involved in damage to DA and 5-HT terminals.

Most studies of the mechanisms of METH and MDMA neurotoxicity have, until recently, investigated the toxic effects on DA and 5-HT terminals. Despite significant damage to these terminals, METH and MDMA appear to spare the monoamine-containing cell bodies from which these terminals arise.[18,91] Some studies, however, have reported that amphetamines could produce neurodegeneration of nonmonoaminergic cell bodies in several brain areas. For instance, high binge doses of METH[87] and MDMA[19] produce a loss of DA cells in the substantia nigra of mice and a loss of 5-HT cells in dorsal raphe nucleus in nonhuman primates, respectively. In addition, METH, MDMA and D-amphetamine damage a population of nonmonoaminergic neurons and their processes in rat parietal cortex (somatosensory cortex).[21,85,88,90,92] In mice, high-dose METH leads to cell death in a variety of brain areas including the striatum, cortex (frontal, parietal, and piriform), indusium griseum, medial habenular nucleus, hippocampus, tenia tecta, and fasciola cinerea.[93,94] More recently, a low dose of METH has been shown to damage cell bodies in rat prefrontal cortex of behaviorally sensitized rats,[95] whereas an escalating binge dose of METH damages pyramidal neurons in the frontal cortex, CA3 and dentate gyrus regions of the hippocampus, and calbindin interneurons of the striatum.[96] Finally, there are several more recent reports of amphetamine toxicity to DA-containing neurons and their terminals in mouse olfactory bulb[97,98] and rat retina.[99]

The mechanisms underlying the damage to cell bodies have yet to be elucidated. Nevertheless, in-flammatory cytokines, the ubiquitin proteasome system (UPS), environmental stress, HIV, neurotrophic factors, and apoptotic proteins have recently emerged as mediators of the toxicity of amphetamines that may explain both the terminal and somatic degeneration observed after exposure to these drugs.

Excitotoxicity to nonmonoaminergic cell bodies

Studies of mechanisms underlying METH toxicity to neuronal cell bodies are relatively recent and indicate that an early event in METH toxicity to nonmonoaminergic striatal and somatosensory cortical neurons might be a release of GLU that initiates a chain of events culminating in apoptosis.

Striatal GABA neurons and interneurons

Approximately 90% of the neurons in the striatum are GABAergic medium spiny projection neurons, which contain either substance P and dynorphin or enkephalin. The remaining 10% are interneurons, of which the GABA-parvalbumin, somatostatin (SST)/NOS, and cholinergic interneurons are the most prevalent.[100] It is the GABA neurons that express enkephalin and parvalbumin in the rat and mouse striatum that are damaged by METH.[101–103]

Excitotoxicity mediated by GLU was suggested by several studies as a mechanism for cell death produced by METH. Along these lines, striatal neurons express GLU receptors,[104,105] and METH causes a release of GLU in rat striatum[10,61] via activation of the striatonigral pathway.[62] Indirect evidence suggests that METH produces an increase in NO in the striatum[65,66] and induces toxicity to GABAergic neurons via mitochondrial dysfunction and ER stress,[106] both of which are mediated by GLUergic and calcium-dependent mechanisms. Specifically, ER stress involves the rapid activation of calcium-dependent calpain and its substrate caspase-12, as well as an increase in the expression of other proteins indicative of ER dysfunction, namely, GRP78, BiP, and CHOP.[106] In parallel, METH causes a release of cytochrome *c*, smac/DIABLO, and apoptosis-inducing factor (AIF)[107] from mitochondria to the cytosol, presumably the result of damage to mitochondria. In fact, mitochondrial dysfunction has been shown to mediate METH-induced apoptosis in an immortalized rat striatal cell line.[108] METH

also activates the calcium-dependent protease, cal-pain, to cause spectrin proteolysis.[68] These events are in conjunction with the activation of several effector caspases and prodeath transcription factors, including the NFAT-family transcription factors[103] that lead to apoptosis. Along similar lines, GLU excitotoxicity produces caspase-dependent and caspase-independent (AIF mediated) apoptosis in neuronal cells *in vitro*.[109,110] Thus, the convergence of GLU and calcium-dependent and -independent mechanisms that also involve the mitochondria can mediate the observed death to striatal cells after METH exposure.

METH-induced apoptosis of striatal GABA neurons also depends on DA. Administration of DA receptor antagonists prevents DA terminal damage and apoptosis in mouse striatum,[111] whereas administration of a D1 receptor antagonist decreases the number of terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling–positive cells and inhibits upregulation of NFAT transcription factors in rat striatum.[103] DA may contribute indirectly to excitotoxicity in GABA neurons via regulation of (i) extracellular GLU,[61,112] (ii) *N*-methyl-ᴅ-aspartate (NMDA) receptors,[113,114] and/or (iii) substance P signaling[115] as well as via ROS formation in the extracellular space.[116,117] The activation of NMDA receptors by GLU to induce NO in SST/NOS interneurons could further increase the release of GLU and DA in the striatum,[118] resulting in a feed-forward mechanism that promotes METH toxicity.

Substance P also may contribute to METH-induced apoptosis of striatal GABA neurons. Pharmacological blockade of the substance P receptor, neurokinin 1 (NK-1R), attenuates METH-induced damage to DA terminals[119] and neuronal apoptosis in the striatum.[94] Deletion of the NK-1R–expressing interneurons (SST/NOS and cholinergic) from the striatum prevents METH-induced apoptosis but does not prevent DA terminal damage.[120] Most NK-1R–expressing terminals form asymmetric synapses with dendrites and dendritic spines,[121] suggesting that substance P modulates excitatory GLUergic neurotransmission. Therefore, substance P may mediate neuronal apoptosis via regulation of GLU release from its afferents and/or via activation of NOS. Collectively, the available data suggest that damage to striatal GABA neurons is mediated by excitotoxicity.

Somatosensory cortex

Administration of amphetamines can cause degeneration of a population of nonmonoaminergic cortical neurons and their processes in layers II/III and IV of rat primary somatosensory cortex.[21,22,85,88,90] The damaged neurons have been identified as pyramidal or stellate cells[88,90,92] confined to the cytochrome oxidase–rich areas.[92] The morphology, localization, absence of monoaminergic markers,[85] and substantial decrease in GLU immunoreactivity in affected areas[22] suggest that these neurons are GLUergic. The cortical damage produced by METH occurs via an excitotoxic mechanism, as evidenced by the findings that METH induces a rapid increase in NMDA receptor binding[122] and that NMDA receptor antagonism[89] or removal of excitatory sensory input from rat whiskers to somatosensory cortex[123] decreases the rapid Fluoro-Jade staining in this cortical area.

Overall, the toxicity induced by amphetamines appears to be more widespread than originally believed and includes damage to cell bodies as well as terminals. An increase in extracellular GLU may mediate the damage to both targets, but the terminals may be more susceptible because of the occurrence of DA-mediated intracellular oxidative stress and other factors, such as proinflammatory mediators that converge upon the terminals.

Inflammation

METH-induced GLU release may also serve to activate inflammatory mediators of METH toxicity to monoaminergic as well as nonmonoaminergic neurons. For instance, GLU receptor antagonism decreases,[124,125] whereas GLU receptor stimulation increases, microglial activation. Thus, activation of GLU receptors increases the production of proinflammatory cytokines interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), and IL-6.[126–129] In turn, cytokines can increase extracellular GLU levels by either inhibition of GLU uptake[130] or an increase in GLU release from activated microglia.[131] Thus, the interactions between the cytokines and GLU may form a feed-forward cycle to promote neurotoxicity.

METH and MDMA trigger inflammation in brain areas that exhibit DA and 5-HT terminal degeneration. METH elicits microglial activation in rat and mouse striatum[132–136] rat cortex (including somatosensory and frontal cortices)[96,136–138] and

hippocampus[137,139] but not in areas where DA levels are unaffected by METH, such as substantia nigra.[132,134] METH-induced microglial activation occurs in the rat somatosensory cortex[138] but not mouse somatosensory cortex.[140] Microglial activation has also been detected in the brains of human METH users[141] and nonhuman primates administered METH.[142]

Microglia might be involved in the toxic effects of METH to DA terminals and GABA neurons via a release of proinflammatory and prooxidative stress molecules into the extracellular space. In mouse striatum, a single dose of METH increased mRNA levels of IL-6, TNF-α, and IL-1α.[143,144] Interestingly, METH-induced microglial activation appears to depend on newly synthesized and released DA. Thus, a decrease in DA synthesis or an increase in cytosolic DA can decrease and increase, respectively, microglial activation in mouse striatum.[140] These results are in conflict with the finding that DA itself inhibits the activation of microglia *in vitro*.[145] In contrast, DA quinones are powerful activators of microglia.[146] Therefore, it can be envisioned that nonenzymatic degradation of DA that is released after METH results in production of DA quinones[147] and, in turn, activates striatal microglia to provide a proinflammatory stimulus for neurodegeneration of both DA terminals and striatal cell bodies.

The ability of MDMA to induce microglial activation is more equivocal. For example, MDMA-induced microgliosis was detected in male[133] but not female[136] mouse striatum and was absent in rat striatum or cortex.[137] In contrast, MDMA increased production of IL-1β in rat frontal cortex,[148,149] whereas intracerebroventricular administration of IL-1β potentiated MDMA-induced 5-HT toxicity in the cortex.[146] An explanation for the varied results and the limited potential of MDMA to induce microglial activation might stem from the fact that MDMA also has an immunosuppressive action that involves suppression of proinflammatory cytokines via an increase in IL-10 production.[150] However, central injections of proinflammatory factors interferon γ[151] and lipopolysaccharide[152] before or immediately after METH administration can attenuate METH toxicity to striatal DA terminals through a decrease in extracellular GLU concentrations,[153,154] or a decrease in extracellular and intracellular DA levels.[155] These data suggest that the initial and acute upregulation of inflammatory cytokines might be

protective by upregulating the buffering capacity of either neurons or glia to counter the excessive and prolonged increases in GLU or DA. In contrast, the neurotoxic effects of the cytokines may be related to the magnitude of their increase after the induction of the GLU excitotoxicity cascade.

Astrocytes can also play a role in substituted amphetamine toxicity through modulation of GLU-mediated excitotoxicity and inflammation. Astrocytes regulate extracellular concentrations of GLU, mainly by uptake of the neurotransmitter. They can also release GLU upon activation through an increase in intracellular calcium.[156] For METH, the activation of cortical astrocytes appears to be caused by GLU release and protein kinase C activation and is inhibited by GLU receptor antagonists.[157] Under normal physiologic conditions, however, astrocytes suppress microglial activation through the release of anti-inflammatory cytokines and neurotrophic factors.[124] For example, astrocytes suppress microglial activation by releasing the anti-inflammatory cytokines transforming growth factor β (TGF-β) or IL-10.[158,159] On the other hand, IL-1 and TNF-α are known to be involved in the development of central nervous system inflammation through, among other factors, the induction of chemokines from astrocytes.[160] Therefore, astrocytes can mediate either an increase or decrease in inflammation depending on the cytokine that is released. More information is needed to identify the specific conditions under which astrocytes may be pro- or anti-inflammatory.

Oxidative stress plays a key role in substituted amphetamine toxicity, as noted in the preceding. Moreover, oxidative stress and inflammation are intimately linked,[48,161] but the exact relationship between the two in mediating amphetamine toxicity is unclear. However, edaravone, a free radical scavenger, blocked METH toxicity to DAergic terminals, the increase in protein oxidation as evidenced by 3-nitrotyrosine immunoreactivity, and the activation of astrocytes, but it did not affect the activation of microglia,[162] suggesting that METH-induced activation of microglia and inflammation is independent of oxidative stress. In fact, a variety of intracellular signaling molecules that have been identified to be involved in METH toxicity, such as GLU, DA-quinones, matrix metalloproteinases (MMPs), substance P, and α-synuclein, can induce microglial activation[124,146,161] independent of the formation of free radicals. However, oxidative stress can

activate microglia to release MMP-3 and α-synuclein,[161] thus providing another means by which microglia are activated. The self-perpetuating cycle of oxidative stress and inflammation is further promoted by the diminished capacity of microglia under prooxidant conditions to store iron,[163] thereby potentially exacerbating Fenton reaction and iron-dependent oxidative stress that mediates METH toxicity.[164] Taken together, activated microglia can initiate, exacerbate, and perpetuate METH neurotoxicity.

The time courses of microglial activation and increases in inflammatory markers vary relative to indices of neurotoxicity. For example, microglial activation in the striatum occurs 1–3 days after METH[132–136] and precedes degeneration of DAergic terminals.[135,138] On the other hand, rat striatal GABA-enkephalin neurons exhibit an upregulation of FasL, a member of the TNF superfamily of cytokines, that appears as soon as 2–4 h after one high dose of METH.[103] Interestingly, Bowyer *et al.*[135] reported the relatively early appearance of phagocytic microglia with Fluoro-Jade C–labeled striatal neurons in mice 12–24 h after one high dose of METH. These findings suggest that damage to striatal cell bodies appears before the neurodegeneration of DA terminals, but it is unknown whether damage to GABA neurons plays a causal role in DA terminal degeneration or is simply an independent event. Regardless of the temporal relationship between the activation of microglia and the appearance of neurodegeneration, microglia are emerging as new players in the toxicity of the amphetamines that, at the minimum, perpetuate excitotoxic events that eventually lead to neurodegeneration. Although factors that promote and perpetuate toxicity have historically been the focus of studies on the neurotoxic amphetamines, more recent efforts have been directed toward endogenous protective systems, such as the UPS, and neurotrophic factors that are emerging as targets whose functions may be compromised by these drugs.

Ubiquitin proteasomal system

Recent studies have shown that the substituted amphetamines promote the dysregulation of the UPS, which may further contribute to neurotoxic and apoptotic events. A decrease in the activity of the UPS can lead to the accumulation of unwanted proteins and has been implicated in the etiology of various neurodegenerative disorders.[165] Furthermore, identified mediators of amphetamine neurotoxicity described in the foregoing, such as GLU-induced NOS activity, mitochondrial dysfunction, and oxidative stress, are known to affect or be affected by the UPS. Inhibition of the proteasome can block inducible NOS degradation[166] and potentially increase NO production, NO-mediated nitrosative stress, damage to the ubiquitin ligase, parkin,[167] and protein misfolding,[168] all of which can potentiate the inhibition of the proteasome.[60,169,170] Conversely, proteasomal inhibition can produce an impairment of the mitochondria and a release of proapoptotic proteins.[171] Therefore, on the basis of the overlap between mediators of amphetamine toxicity and events associated with the UPS, these studies suggest the view that amphetamines can lead to unwanted accumulation of protein through a dysregulation of the UPS.

Administration of high METH or MDMA doses causes formation of intracellular inclusions in the nucleus of medium-sized GABA neurons and cytoplasm of neurons of the substantia nigra pars compacta of mice.[117,172–176] The inclusions in GABA neurons stain for ubiquitin and enzymatic components of the UPS (including E3 ligase parkin) but usually not for α-synuclein, whereas inclusions found in substantia nigra neurons stain for α-synuclein, a hallmark of Lewy bodies frequently observed in Parkinson's disease and other degenerative disorders. Occurrence of ubiquitinated inclusions was also reported in the substantia nigra of 37 subjects who abused METH.[177] The specific cause of the inclusions is unknown, but neuronal inclusions can occur when the UPS is inhibited pharmacologically.[178,179] Moreover, oxidative stress commonly leads to inclusion formation, and the inclusions produced by METH, MDMA, and MPTP[180] are ultrastructurally similar to those produced by DA-mediated oxidative stress.[117,172,173] In addition, inclusion formation is decreased upon administration of antioxidant/iron-chelating agent, S-apomorphine.[175]

It is hypothesized that striatal neuronal inclusions are a consequence of amphetamine-mediated increases in DA release followed by overstimulation of DA D1 receptors.[117,181] The underlying mechanism is thought to involve β-arrestin that is present together with ubiquitin in inclusions after exposure

of PC12 cells to METH.[182] Because β-arrestin is involved in the internalization of DA and mGlu5 receptors,[183–185] it suggests the possibility that activation of these receptors contributes to the formation of inclusions in striatal GABA neurons. In addition, DA and non–DA-derived ROS might diffuse to GABA neurons and inhibit the function of proteasome.[117]

α-Synuclein, a presynaptic protein involved in various degenerative disorders including Parkinson's disease, might also contribute to DA-dependent inclusion formation in nigral cells after toxic amphetamine administration. Increases in α-synuclein levels are known to be toxic to DA neurons *in vitro*[186] and *in vivo*.[187] Administration of METH and MDMA increases expression of α-synuclein in DA neurons in the substantia nigra of mice.[176] It is possible that covalent modification of α-synuclein by DA-derived quinone[188,189] after amphetamine administration promotes the formation of toxic α-synuclein aggregates.[190]

Misfolded protein aggregates or damaged organelles that accumulate cannot be degraded by the UPS. This function is reserved for the lysosomal system and the process of microautophagy. Autophagic vacuole formation by the lysosomal system will remove oxidized and damaged organelles (such as mitochondria) and misfolded protein aggregates produced by METH. Conversely, inhibition of autophagy is deleterious to cells because of a diminished ability to clear α-synuclein aggregates after METH exposure, eventually resulting in caspase-dependent cell death.[191]

Now it is unclear whether a dysfunction of the UPS system is a consequence or a cause of the toxicity to the amphetamines. It remains to be determined if the excitotoxic, oxidative, and inflammatory mediators discussed earlier directly target the UPS and thus disrupt the normal, ongoing removal of unwanted proteins to ultimately produce the demise of cell bodies and terminals. A likely scenario, however, is that the damage produced by the amphetamines is ultimately dependent upon the balance of factors that promote toxicity (e.g., excitotoxic glutamatergic events, prooxidant processes, inflammation) and endogenous protective systems (such as the UPS), antioxidants, and growth-promoting molecules (such as neurotrophic factors) that can be targeted by toxic insults.

Neurotrophic factors

Several neurotrophic factors can act as survival-promoting proteins. These factors include neurotrophins, glial cell line–derived neurotrophic factor (GDNF) family, and TGF-α.[192] Neurotrophins comprise a family consisting of four members: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4.[193] The GDNF family includes GDNF, neurturin, artemin, and persephin.[194] Of these, GDNF was the first neurotrophic factor demonstrated to protect DA terminals against METH neurotoxicity in animal models.[195–197] Recently, neurturin and artemin are two other GDNF family members that have been shown to protect against METH toxicity in the rat.[198] The protective action of GDNF might involve regulation of DA release[196] and/or attenuation of METH-mediated oxidative stress: GDNF has been shown to upregulate striatal antioxidant enzymes *in vivo*[199] and reduces levels of free radicals in cultured mesencephalic neurons.[200] Conversely, a study by Boger *et al.*[201] demonstrated that a partial GDNF gene deletion increased the susceptibility of mice to METH neurotoxicity during young adulthood and increased age-related deterioration of motor behavior and DA function.

In contrast to the protective effects of several of the growth factors, ciliary neurotrophic factor provides no protection against METH toxicity to DA neurons.[198] In non-DAergic primary rat cortical neurons, METH-triggered apoptosis was attenuated by BDNF through the PI3K–Akt but not MAPK–Erk pathway.[202] Overall, these results indicate that GDNF may play a greater role in protecting DA terminals against METH toxicity, whereas BDNF may be more potent in the protection of non-DA neurons.

Blood–brain barrier dysfunction

Recent studies have begun to demonstrate another emerging consequence of exposure to high doses of the amphetamines. Administration of MDMA or METH has been shown to increase blood–brain barrier (BBB) permeability in rodents. MDMA-induced damage to the BBB was observed in the striatum and hippocampus.[43] Moderate to high doses of METH disrupt the BBB in several brain regions, including the cortex, hippocampus, thalamus, hypothalamus, cerebellum, amygdala, and

striatum[135,203–206] that, in turn, are augmented by hyperthermia and seizures.[135,205,206] Although it is unclear whether there is a relationship between the damage to the BBB and the damage to neurotransmitter systems, the damage to the BBB appears to contribute to striatal neuron degeneration rather than DA terminal damage.[135]

The mechanisms underlying the damage to the BBB produced by the amphetamines have not been elucidated. However, the amphetamines can cause hyperthermia[77–79] and produce ROS,[164] both of which trigger BBB breakdown.[207] Consistent with these findings, administration of antioxidants attenuates the effects of amphetamines on the BBB[205] and further implicates oxidative stress in the effects of amphetamine at the BBB.

Another possible mediator of the damage to the BBB could be the MMPs, whose functions are to degrade tight junction proteins[208] present in the extracellular matrix that supports the endothelial cells of the BBB.[209] METH has been shown to increase the release of MMP-1 and the MMP activator, urokinase plasminogen activator, in neuron–astrocyte cocultures.[210] METH also alters the expression of several tight junction proteins and increases the permeability of brain-derived primary microvascular endothelial cells.[211] High doses of METH also increase the levels of MMP-9 in the hippocampus.[212] The activation of the MMPs is thought to occur through several mechanisms, including oxidative stress[213] and cytokine production.[214,215] Collectively, these findings suggest that amphetamine-mediated oxidative stress followed by activation of MMPs and breakdown of tight junctions mediate BBB disruption. Because both activation of MMPs[216] and oxidative stress[161] can induce inflammation, these events in conjunction with the MMPs could be accompanied by an increase in cytokine production within microglia[217] to perpetuate damage and increase the permeability of the BBB. The consequences of the breakdown in the BBB are widespread and may enhance the vulnerability of the brain to toxins and infection, such as those produced by HIV. This and the fact that the BBB breakdown can be mediated by other toxic mechanisms, such as oxidative stress, neuroinflammation, and hyperthermia, suggests it as a new and important contributing factor to the toxicity of amphetamines.

Interactions of amphetamines and HIV

The comorbidity of drug abuse and HIV infection is well known. Early findings of decreases in post-mortem levels of DA and homovanillic acid in the caudate nucleus and substantia nigra neuron degeneration in HIV patients suggested that HIV infection might damage nigrostriatal DA neurons.[218,219] It was subsequently found that HIV injured not only these two regions but also other brain areas, such as prefrontal cortex, parietal cortex, nucleus accumbens, and hippocampus, thus increasing the vulnerability of these areas to METH toxicity in HIV-infected METH users.[220] Along these lines, intrastriatal injections of the HIV protein, Tat, damage both efferent and afferent projections of the rat striatum and/or substantia nigra neurons,[221–224] common targets of the toxic effects of METH.

Similar mechanisms mediate the toxicity to the amphetamines and HIV. Oxidative stress, mitochondrial dysfunction, inflammation, and caspase-dependent neuronal apoptosis[220,225] all contribute. Similar to METH, Tat potentiates GLU toxicity via interaction with the NMDA receptor,[226] causes neuronal cell death via activation of the D1 receptor,[227] and decreases DAT function.[228]

The combined effects of HIV and chronic METH exposure converge to produce neuronal damage and inflammation. *N*-Acetylaspartate, myoinositol, and brain metabolites are increased more in HIV-positive METH abusers than in HIV patients with no METH abuse.[229] Langford *et al.*[230] found decreased blood flow; an increased microglial response; and more pronounced losses of synaptic vesicle–associated protein, synaptophysin, and the interneuron-associated protein, calbindin, in HIV-infected METH abusers relative to HIV-infected non-METH abusers. Similarly, Chana *et al.*[231] reported that HIV-positive METH users have greater losses of frontal cortex calbindin and parvalbumin interneurons than do HIV non-METH abusers and that these effects are associated with cognitive impairment. In addition, METH has been shown to enhance HIV infection of macrophages, the primary target of the virus, and decrease IFN-α in these cells *in vitro*.[232]

The mediators of the damage produced by the combination of HIV and METH are being actively investigated. Tat and METH synergistically impair mitochondria in a variety of cellular targets,

including DAergic neurons[233]; a non-DAergic, calbindin-positive hippocampal cell line[234]; and human fetal neurons.[235] This effect on mitochondria is accompanied by oxidative stress and can be blocked by antioxidants.[234,235] In HIV-positive rodent striatum, METH produces a synergistic increase in oxidative stress markers, expression of several inflammatory cytokines (e.g., IL-1α, IL-1β, and TNF-α), augmented activity of redox-responsive transcription factors,[236,237] and toxicity to striatal DA terminals.[224,235,238] These findings indicate that HIV infection increases susceptibility of DAergic and non-DAergic neurons to METH neurotoxicity. Moreover, oxidative stress, inflammation, and possibly excitotoxicity might interact to exacerbate toxicity in HIV-infected METH users.

Both METH and HIV increase permeability of the BBB via damage to tight junction proteins.[211] In HIV-positive METH abusers, METH-induced increases in BBB permeability might facilitate an increased transport of HIV-infected leukocytes across the BBB. In fact, both METH and HIV protein gp120, alone and in combination, significantly increase transendothelial migration of immunocompetent cells across the BBB.[211] Conversely, HIV-induced increases in BBB permeability might facilitate an increased transport of METH. Finally, METH may contribute to HIV-induced BBB breakdown by stimulating release and/or activation of MMPs. Levels of MMP-2, -7, and -9 are higher in cerebrospinal fluid of HIV-infected individuals,[239,240] and both METH and Tat increase the release of MMPs *in vitro*.[210] Overall, the common mechanisms underlying the toxic effects of METH and HIV appear to accurately predict an additive if not a synergistic damage to neurons and endothelial cells of the BBB. Therefore, the dangerous consequences of the comorbidity of amphetamine abuse with HIV infection can be extended to include potentiated and exacerbated damage to multiple cells in the central nervous system.

Interactions of amphetamines and environmental stress

The stress response involves a release of glucocorticoid hormones via activation of the hypothalamic–pituitary–adrenal axis as well as a release proinflammatory cytokines via activation of the immune system.[241,242] In experimental animals, chronic stress potentiates the toxicity of neurotoxins[243–247] and can cause neurodegeneration by itself.[248] Chronic stress also exerts neurotoxic effects in humans.[249,250] Several neurochemical effects are common to the amphetamines and stress and include oxidative stress, excitotoxicity, mitochondrial dysfunction, depletion of energy stores, increase in glucose utilization, inflammation, and hyperthermia.[48,241,242] In fact, stress can potentiate METH-induced excitotoxicity[247,251] and hyperthermia.[252]

Stress may also contribute to the toxic effects of the amphetamines through the mechanisms summarized in previous sections, such as trophic factor expression, UPS function, and HIV infections. For example, exposure to a variety of stressors decreases the levels of NGF in rat hippocampus.[253] In an astroglial cell line, corticosterone reduces basal levels of NGF secretion and stimulated NGF secretion triggered by IL-1β and TGF-β1.[254] In addition, corticosterone-induced cell death can be prevented by administration of BDNF[255] or insulin-like growth factor[256] in cultured rat hippocampal neurons. For UPS activity, the proteasome regulates glucocorticoid receptor activity via regulation of the trafficking of the receptor. Inhibition of the proteasome blocks glucocorticoid receptor translocation to the nucleus,[257] which would increase expression and signaling of the receptor at the plasma membrane. Conway-Cambell *et al.*[258] have demonstrated that the proteasome also regulates glucocorticoid receptor activity via the rapid degradation of the activated glucocorticoid receptor. In regard to its interaction with HIV, HIV-positive patients have increased hypothalamic–pituitary–adrenal axis activity[259] that, in turn, can potentially increase the toxic effects of stress and the amphetamines as well as their combined exposures. Overall, there are multiple overlapping mechanisms between stress and the amphetamines that predict an augmentation of neurotoxicity produced by their combined exposure, such as that observed in individuals with posttraumatic stress disorder that have a high comorbidity with substance abuse.[260]

Concluding remarks

There is mounting evidence that the characteristics of amphetamine-induced toxicity extend beyond the selective damage to DA and 5-HT terminals to include neuronal and endothelial cell bodies.

The underlying mechanisms have yet to be elucidated, and the consequences of this extended damage remain to be determined. However, the causes of the newly identified consequences to cell bodies most likely involve a convergence of excitotoxic, proteolytic, inflammatory, and bioenergetic processes that interact with and contribute to the previously established role of oxidative stress. Although basic experimental studies have provided clear, interpretable roles for each of these causative processes, we now know that each process does not occur in isolation. Moreover, the frequent comorbidities of the abuse of the amphetamines with other exposures, such as environmental stress, hyperthermia, and HIV infection, add to the complexity and severity of the toxicity. More studies are needed that take into account and model the more realistic scenario involving their concurrent exposures, comorbidities, and how they interact before effective therapeutic interventions can developed.

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Conflicts of interest

The authors declare no conflicts of interest.

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