The neurotoxicity of amphetamines: Bridging drugs of abuse and neurodegenerative disorders

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Abstract

Amphetamine derivatives are the most commonly abused drugs. These compounds have been known for many years to induce neurotoxicity. However, recent findings have highlighted novel alterations produced by amphetamines in the central nervous system consisting of neuronal inclusions and the involvement of proteins belonging to a multi-enzymatic complex known as the ubiquitin–proteasome system. These ultrastructural and molecular changes are similar to those that occur during degenerative processes that affect the basal ganglia, and in particular Parkinson’s disease, which is characterized by ubiquitin-containing neuronal inclusions in the substantia nigra. This is recently confirmed by the occurrence of ubiquitin immunoreactive structures in the substantia nigra of humans abusing methamphetamine. In this article, we propose that the neurotoxicity of amphetamines and degenerative disorders share a number of steps in their mechanism of action involving the ubiquitin–proteasome system. The fine tuning of this ubiquitous proteolytic pathway is now being elucidated because G-protein-coupled receptors and signaling proteins such as β-arrestin regulate access to this catalytic machinery. The identification of the ubiquitin–proteasome pathway and β-arrestin as molecular targets of neurotoxicity is expected to provide novel therapeutic strategies both for the treatment of drug addiction and the treatment of neurodegenerative disorders.

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Introduction

Abuse of amphetamine derivatives is a growing phenomenon in the Western World. Adding to strong evidence regarding toxicity for monoamine nerve terminals (Seiden, 1985; Gibb et al., 1989; Miller and O’Callaghan, 1994; Battaglia et al., 2002), new data are now showing that amphetamines toxicity involves neuronal cell bodies, where fine ultrastructural alterations progress from whorls, and autophagic granules to neuronal inclusions. These ultrastructural changes extend beyond the dopamine (DA) system and involve also other classes of neurons as shown by light and electron microscopy of medium size GABA cells (Deng et al., 2001; Fornai et al., 2004a,c).

Amphetamine-induced neuronal inclusions recapitulate many immunocytochemical and structural features of those observed in degenerative disorders, and their maturation can be reproduced by continuous exposure to Parkinsonism-inducing neurotoxins such as continuous rotenone (Betarbet et al., 2000; Sherer et al., 2003; Betarbet et al., 2005), continuous MPTP (Fornai et al., 2005b), or chronic combined administration of MPTP and probenecid (Meredith et al., 2002). On the other hand, comparable inclusions are observed following inhibitors of the ubiquitin–proteasome (UP) system (Fornai et al., 2003; McNaught et al., 2004), while their number can be enhanced by concomitant treatment with methamphetamine (MA) and UP inhibitors (Fornai et al., 2004b). Finally, a primary dysfunction of the UP system due to inherited mutations produces degenerative PD (Corti and Brice, 2003), posing the UP system as a molecular bridge conveying common biochemical pathways in MA toxicity and neurodegeneration (Fig. 1). The relevance of these findings translates to humans who are the unique species which spontaneously develop PD characterized by ubiquitin positive inclusions while, in a recent paper, the occurrence of ubiquitinated inclusions was described in the substantia nigra of 37 subject who abused methamphetamine (Quan et al., 2005).

Within this context, it will be considered the roles of the pre-synaptic protein α-synuclein (Fig. 2), G-protein-coupled receptors (GPCRs), and specific signaling proteins, such as β-arrestin (Parruti et al., 1993; Chuang et al., 1996; Iacovelli et al., 2003) which mediates the GPCR signaling to the ubiquitin–proteasome pathway (Shenoy and Lefkowitz, 2003; Shenoy et al., 2001; Wojcikiewicz, 2004). The

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**Fig. 1.** The ubiquitin–proteasome system as a final common pathway for neuronal inclusions in amphetamine toxicity and degenerative disorders. Neuronal inclusions bear several proteins belonging to the ubiquitin–proteasome (UP) pathway, suggesting that the UP system is involved in the pathogenesis of inclusions. This occurs in a number of neurodegenerative disorders as well as in toxicity induced by amphetamine derivatives (MA = methamphetamine; MDMA = Mehtyledioxymethamphetamine). Moreover, while inherited mutations of the UP system lead to familial forms of Parkinson’s disease, a pharmacological blockade of this pathway produces parkinsonism in rodents. The UP system can be saturated by abnormal amount of misfolded proteins, oxidative stress, and altered or inappropriate gene splicing. Thus, under particular conditions, this system may not to be able to cope with increased amounts of substrates. In this way, even in the presence of a normally structured UP system, a non-sufficient enzymatic activity leads to non-digested protein aggregates which further progress as inclusion bodies. Within this context, abnormal or excessive amount of α-synuclein or excessive by-products of DA metabolism (as occurs following treatment with amphetamine derivatives) may sort comparable effects via recruitment of the same final common pathway. In fact, the same effects (membraneous whorls and neuronal inclusions) may be observed when the UP system is pharmacologically inhibited (as in the case of lactacystin or epoxomycin). Since the UP catalytic activity of the enzymes is tightly related to the amount of ATP, when a slight and long-lasting inhibition of the mitochondrial respiratory chain occurs (either continuous rotenone or MPTP administration), comparable effects are described. Altogether, these altered physiological conditions of the cell are prototypical example of wide convergent pathways operating both in the genesis of neuronal degeneration and amphetamines toxicity. This may represent a key to disclose biochemical pathway responsible for disease onset to be targeted in future therapeutic development.
involvement of the UP system and its recruitment by specific GPCR and β-arrestin represents an attractive target for novel therapeutic strategies.

New vistas on Parkinson’s disease (PD)

Despite existing trivial differences concerning the clinical aspect, the neuropathology, and the etiology between neurodegenerative disorders, there are many common immunocytochemical, biochemical, and structural features which recently emerged. Most of these disorders are characterized by eosinophilic inclusions, which, at ultrastructural level possess an electron dense structure, which is not limited by external membranes, and stains for a variety of UP antigens (see later). Progress in neurogenetics allowed to dissect multiple causes for what once was simply known as PD, which is now split into several different disorders (Corti and Brice, 2003). Despite multiple divergent causes, the pathogenesis of these diseases converges to common effectors mechanisms (Greenamyre and Hastings, 2004).

Common elements consist of alterations in protein handling which is preceded by formation of polyubiquitin chains and the occurrence of protein deposits containing α-synuclein (Golbe and Mouradian, 2004). Thus, in PD and some degenerative disorders, neuronal inclusions contain both α-synuclein and various components of the UP. The frequent involvement of α-synuclein led to collect this class of disorders as “synucleinopathies” (Golbe and Mouradian, 2004).

**New vistas on amphetamines toxicity: neuronal inclusions as key pathological correlates**

Both MA and its derivative, 3,4-methylenedioxymethamphetamine (MDMA, ‘ecstasy’), are known to be toxic for dopamine nerve terminals, thus replicating striatal DA loss occurring in Parkinsonism (Seiden, 1985; Kalant, 2001). Recent findings shed new vistas on amphetamines toxicity indicating that MA and MDMA induce neuronal inclusions in the substantia nigra and corpus striatum of mice (Fornai et al., 2004a,c). These inclusions appear at first as membranous whorls, and autophagic granules as described in vitro in cultured midbrain neurons (Cubells et al., 1994; Larsen et al., 2002) and PC12 cells (Fornai et al., 2004a,c). A time course analysis demonstrates the evolution from whorls to non-membrane limited inclusions (Fornai et al., 2004c). Recent data suggest a comparable evolution for neuronal inclusions occurring in PD, which now are hypothesized to start as immature bodies named “aggreosomes” (Olanow et al., 2004). Again, experimental PD induced by continuous MPTP exposure leads to a comparable time-progression of the same inclusions (Fornai et al., 2005b). Moreover, immunocytochemistry of “mature” MA-induced inclusions reveals a striking analogy with those occurring in PD. MA-induced bodies found in the substantia nigra are immunopositive for α-synuclein, ubiquitin, ubiquitin conjugating enzyme (E1), a type of ubiquitin-ligase (parkin), and a variety of proteins belonging or related to the UP pathway (Fornai et al., 2004c). Interestingly, as Lewy bodies (LB) in PD, MA-induced inclusions in DA containing neurons are
exclusively cytoplasmic, while their occurrence in striatal GABA cells extends to the nucleus consistent with what happening in degenerative HD.

It is noteworthy that MA-induced inclusions accompany but are not synonymous of neurotoxicity and they might also reflect a defensive mechanism aimed at clearing harmful proteins. This point is crucial, since most reports indicate that nigral neurons do not die following administration of MA and we failed to observe nigral cell loss in vivo following a dosage of MA sufficient to produce inclusions. For instance, the dose of MA which produces the highest number of inclusions does not produce cell death (Fornai et al., 2004c). Again, inclusions found in striatal neurons occur in the absence of cell loss and nigral LB are found in surviving cells in PD. In this way, inclusions might be viewed as a beneficial defensive mechanism, which is recruited when high levels of oxidized, misfolded proteins occur in the cell, which maintains its viability. In line with this, LB are not “inorganic stones”, but preserve a fair degree of enzymatic activity.

Dopamine has been implicated in the pathophysiology of LB (Conway et al., 2001; Sulzer, 2001), thus providing a further link with MA-induced inclusions. Since MA is known to produce high amounts of cytosolic DA which is converted to DA-quinone (Sulzer, 2001), in keeping with this, MA toxicity against nerve terminals (Gibb et al., 1989) and MA-induced inclusions require endogenous DA. The mechanism by which endogenous DA participates in the formation of neuronal inclusions is also supposed to involve α-synuclein.

The role of DA

The role of DA and DA metabolites can be demonstrated by the protective effects obtained by neuroprotection induced by transient depletion of nigrostriatal DA or enhanced neurotoxicity induced by DA-increasing drugs. Methamphetamine-induced neuronal inclusions can be suppressed following administration of the TH inhibitor, αMPt. In fact, blocking DA synthesis with αMPt partially prevents inclusions formation unless DA synthesis is restored by the adding L-DOPA. This can be achieved also by blocking the conversion of DA to NE (by using the DA β-hydroxylase inhibitor fusaric acid) (Fornai et al., 2004c). The role of DA might be related to the spontaneous oxidation of DA into DA-quinones (Sulzer, 2001); in fact, the blockade of oxidative deamination using the non-selective monoamine-oxidase inhibitor pargyline worsens neurotoxicity as does L-DOPA administration (Fornai et al., 2004c). This lends substance to a recent hypothesis on the occurrence of toxic adducts between DA-quinones and α-synuclein as a critical biochemical step in PD (Conway et al., 2001; Sulzer, 2001). In keeping with this, DA neurons are selectively vulnerable to various neurotoxins which increase cytosolic DA content. This is the case of methamphetamine (Schmidt et al., 1985), malonate (Moy et al., 2000), or the absence of vesicular DA transporter especially when this occurs with a normal expression of membrane DA transporter (Gainetdinov et al., 1998; Jones et al., 1998; Fumagalli et al., 1999; Miller et al., 1999). There is a growing body of evidence which focuses on free cytosolic DA as a critical agent for DA containing neurons. In summary, in vivo and in vitro data joined together indicate that DA containing cells represent per se a system in a delicate metabolic equilibrium. This instability is an intrinsic property of these cells, which is due to the presence of high levels of DA in their cytosol (Conway et al., 2001; Sulzer, 2001; Miller et al., 1999). DA might also be implicated in the development of inclusions observed in medium-sized striatal spiny neurons, which do not synthesize catecholamines. However, one should take into account that striatal neurons develop MA-induced neuronal inclusions but do not synthesize DA and are exposed to extracellular DA peaks; this is quite different when compared with nigral and PC12 cells which constantly synthesize DA. Thus, in considering striatal neuronal inclusions as a consequence of increased DA activity, one should hypothesize that DA receptors placed on striatal neurons might be crucial in transducing the signaling which triggers formation of inclusions within striatal cells. Alternatively, oxidative species formed within DA terminals might diffuse to striatal GABA neurons.

The role of α-synuclein (Fig. 2)

Punctiform mutations in the α-synuclein gene (A53T and A30P and E46K) cause early-onset inherited PD (Polymerepoulos et al., 1997; Kruger et al., 1998; Zarranz et al., 2004), which is autosomal dominant suggesting a toxic gain of function. In line with this, inherited PD is also produced by increased amount of otherwise normal α-synuclein (Farrer et al., 2004). This suggests a role of α-synuclein in the pathogenesis of PD as witnessed, by the fact that α-synuclein is a major component of LB in sporadic PD (Spillantini et al., 1997). In keeping with this, expression of α-synuclein in Drosophila results in neuronal loss and LB-like inclusions (Feany and Bender, 2000). Again, injection of either human wild-type or mutant α-synuclein-expressing viral vectors into the nigrostriatal pathways or in cell cultures causes neurodegeneration and α-synuclein containing inclusions (Kirik et al., 2003; Stefanis et al., 2001).

It has been suggested that the UP system tags α-synuclein for catalytic degradation which might be saturated due to high amounts of damaged/overexpressed α-synuclein thus producing protein aggregates (Chung et al., 2001). Accordingly, expression of mutant forms or overexpressing normal forms of α-synuclein produce a severe impairment of the UP system (Kirik et al., 2003).

A report by Conway et al. (2001) suggests that α-synuclein and DA might interact to stabilize the protofibrils inhibiting their conversion to fibrils (Rochet et al., 2004). Protofibrils accumulation and fibrils inhibition seem to be a consequence of covalent modification by the DA-derived quinone (DAQ). This could apply very well to the elevated cytoplasmic DA concentration produced by MA. In line with this, MA-induced inclusions depend on the modulation of DA metabolism.
Mirroring again what occurs in PD, we recently found that administration of MA and MDMA produces in mice an increased expression of α-synuclein within DA containing neurons (Fornai et al., 2005b) (Fig. 2). In this way, amphetamines operate either increasing the amount of normal α-synuclein and/or altering α-synuclein protofibrils (Fig. 2), thus encompassing the same mechanisms which are responsible for different inherited forms of PD.

**Focus on common mechanisms**

*The role of the ubiquitin–proteasome system in inclusions formation*

As represented in Fig. 1, different mechanisms converge to alter the UP system and a primary dysfunction of this enzymatic pathway leads to accumulation of misfolded proteins which trigger the formation of aggregates and hence neuronal inclusions. The presence of ubiquitinated proteins and various components of the UP pathway within neuronal inclusions is one of the hallmarks of neurodegeneration. The UP system becomes abnormally activated during oxidative stress, abnormal proteolytic cleavage, and altered or inappropriate gene splicing. In these conditions, the UP system may not be able to degrade damaged proteins, thus producing their accumulation in the cell and ultimately neuronal dysfunction up to cell death (Chung et al., 2001). In line with this, a primary reduction in the efficacy of the UP system increases the storage of aggregated proteins and could explain the accumulation of ubiquitin and UP substrates expressed in diseased neurons (Bence et al., 2001). Interestingly, genetic studies described a few mutations of the UP leading to familial PD like, UchL-1, and parkin (Kitada et al., 1998; Leroy et al., 1998). These mutations decrease the enzymatic activity of the UP, while an impairment of the proteasome is described also in sporadic forms of PD (McNaught et al., 2003).

These studies suggest a tight association between impairment of the UP and occurrence of a selective damage to the nigrostriatal pathway featured by subcellular alterations similar to PD. If this is correct, then a blockade of the UP system achieved by exogenous pharmacological inhibitors should sort similar selective and deleterious effects.

In a recent study, we found that intrastriatal administration of proteasome inhibitors, at doses determining a significant inhibition of the proteasome activity causes neuronal damage, which selectively affects striatal DA axons and spares both striatal serotonin levels and GABA neurons. This is associated with marked DA cell loss within the ipsilateral substantia nigra pars compacta (Fornai et al., 2003). In spared nigral neurons, we found the presence of α-synuclein, parkin, E1, and ubiquitin-immunostained inclusions. Altogether, these findings demonstrate that striatal UP inhibition leads to a selective involvement of the DA component and produce a retrograde toxicity for nigral DA cells. Confirming these findings, in a recent paper (McNaught et al., 2004), it has been described that systemic administration of proteasome inhibitors determines a selective loss of the nigrostriatal DA innervation.

Altogether, these experimental approaches allow to dissect part of the molecular mechanisms regulating the dynamics of inclusion formation within the context of the disease progression and provide the evidence for selectivity, shape, and fine structure of the nigrostriatal damage. In fact, on this basis, it has been hypothesized that rough proteasome-derived intracellular structures and centrosome-related bodies represent the first aggregate which further develops as LB (Olanow et al., 2004).

In order to deeply analyze the fine structure of this progressive phenomenon, we reproduced in vitro the time course of inclusions in which ubiquitin and α-synuclein co-localize and we described the ultrastructure of these formations (Fig. 3). These findings were extended in vivo by demonstrating the occurrence of immature inclusions (whorls) within nigral cells which stain for parkin, ubiquitin, E1, and α-synuclein. These intracellular inclusions when observed at electron microscopy resemble analogous inclusions we observed in vivo in mice following MA (Fornai et al., 2004c) or MDMA (Fornai et al., 2004a). Using a combined approach, we demonstrated how these “early stage aggregates” mature into classic inclusion. In fact, the so-called vacuoles, and autophagic granules first described at light microscopy in vitro after MA administration (Cubells et al., 1994; Larsen et al., 2002) correspond to what we found as membrane whorls at transmission electron microscopy which further develop as authentic inclusions (not membrane limited).

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Fig. 3. Confocal images show co-localization of ubiquitin and α-synuclein in fluorescent spots correspond to membraneous whorls at electron microscopy. Confocal microscopy of PC12 cells after administration of MA (1 μM). (a) Ubiquitin immunofluorescence in green and (b) α-synuclein immunofluorescence in red show intense and localized spots. A co-localization of ubiquitin and α-synuclein is shown when merging the single staining as yellow spots (c). These correspond to membraneous whorls when the cells are examined at ultrastructural level (d). (a, b, c) scale bars = 2 μm; (d) scale bars = 0.200 μm.
The role of GPCRs signaling in inclusions formation

The presence of a final common pathway for amphetamine’s neurotoxicity and neurodegenerative disorders leads to unravel innovative neuroprotective strategies aimed at modulating selective substrates for the UP system. While at first α-synuclein stems as a major target, one should also consider that physiological recruitment of the UP system involves the biochemical manipulation of membrane proteins such as neurotransmitter receptors. In line with this, Shenoy et al. (2001) recently demonstrated that internalized catecholamine receptors are polyubiquitinated, thus becoming a target of the UP system once bound to β-arrestin. This is an adaptor protein directly involved in the signaling and regulation of the majority of GPCRs, which plays a crucial role in the process of receptor internalization (Parruti et al., 1993; Iacovelli et al., 2003). β-Arrestin is involved in the fate of internalized receptors towards their degradation in the UP system (Shenoy and Lefkowitz, 2003). We recently showed that β-arrestin is ubiquitinated following treatment of PC12 cells with MA. Additionally, using electron microscopy and confocal microscopy, we found the presence of β-arrestin within MA-induced inclusions (De Blasi et al., 2003). These findings indicate that β-arrestin is involved in the formation of DA-dependent ubiquitinated inclusions via overstimulated DA receptors. This mechanism might be crucial to explain the occurrence of MA-induced inclusions in striatal GABA neurons (Fornai et al., 2004c). In fact, these cells do not possess endogenous DA but bear a high density of DA receptors which are overstimulated by amphetamine-induced DA release. In striatal neurons, a preferential interaction takes place between β-arrestin and DA receptors (Macey et al., 2004). This new concept recalls the opinion shed a few years ago by Jakel and Maragos (2000) on the detrimental effects of extracellular DA for striatal GABA neurons either as an extracellular oxidant or via the stimulation of DA receptors. Similarly, exposure to DA is known to antagonize an overstimulation of GPCRs; or (iii) modulating the molecular processes involved in receptor trafficking to the UP system.

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