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Cell systems and the toxic mechanism(s) of α -synuclein

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Abstract

Mutations in the *SNCA* gene are causal for familial Parkinson disease/Lewy body disease. α -Synuclein is a small acidic protein that binds loosely to the surface of vesicles and may play a role in synaptic dynamics, although its normal function remains somewhat unclear. What is clear is that point mutations or increased expression of wild type α -synuclein causes disease. A great deal of literature supports the overall hypothesis that α -synuclein is damaging to neurons because it is inherently prone to aggregation; mutations or increased concentration of the protein both increase this tendency. An unproven, but popular, contention is that the toxic species are small oligomers that are relatively soluble, which may react with membranes to damage key processes within the cell. The details of this process, especially in determining the order of events and the requirement of particular processes in cell death, are unclear. Derangements in vesicle processing, including synaptic function, protein turnover, mitochondrial function and oxidative stress have all been suggested to occur. Whether there is a sequence of events or whether these are interacting effects is unclear, but the outcome is to trigger cell death, by both apoptotic and non-apoptotic mechanisms depending on the system studied. In this article, we develop a framework for thinking about α -synuclein in terms of initiating events and secondary processes that are required to trigger neuronal dysfunction and cell death.

Without α -synuclein, understanding the relationships between familial and sporadic forms of Parkinson disease (PD) would be extremely difficult. Mutations in the *SNCA* gene produce a range of phenotypes from PD to diffuse Lewy body disease (DLBD), the link being that α -synuclein is deposited in the pathological hallmark of PD/DLBD, the Lewy body. Quantitative mutations, such as triplication of the *SNCA* locus, lead to increased expression of the gene and to a disease with PD/DLBD features (Singleton et al., 2003). All mutations are dominant, suggesting that there is a gain of function and that the point mutations and multiplications have similar mechanisms. This leads to the argument that α -synuclein can be considered a causative agent for PD and related disorders (Cookson, 2005).

These considerations leave us with the simplest possible sketch of the pathogenesis of PD: that α -synuclein is an initiator of damage and the final outputs, after many years, are cell loss and Lewy body formation. Formally, α -synuclein tells us about etiology in familial disease and is likely to be close to the etiology of sporadic disease. We also know the range of disease presentations associated with the etiology. This is often represented diagrammatically with α -synuclein by an arrow pointing at PD, occasionally with a question mark over the arrow to indicate ignorance. The obvious limitation with this level of analysis is that it sidesteps the

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question of *why* α -synuclein causes cell loss. Why synuclein is toxic might be best broken down into two sets of questions. First, what are the immediate biochemical effects of mutations or increased expression of α -synuclein that might be associated with damaging effects? Although not certain, the prevailing thought is that protein aggregation is important in pathogenesis, similar to other 'toxic proteins' associated with a number of neurodegenerative diseases (Taylor et al., 2002). Second, what are the downstream events that mediate the toxic effects of α -synuclein on the neuron? As will be discussed in this review, there are several theories of why α -synuclein might cause neuronal damage and a major challenge to the field is to elucidate those that are critical from those that are secondary effects.

Where cell based models might be useful is in delineating the mechanisms of pathogenesis in simple ways. For example, because it is possible to look at the timing of aggregation relative to downstream events we might be able to order the events related to α -synuclein toxicity. This can be achieved using an inducible system and following toxicity over time (e.g., Tanaka et al., 2001). One might also be able to perform multiple manipulations on cells and establish which are beneficial to α -synuclein toxicity without prejudging which pathways are important, as has been done in yeast (e.g., Willingham et al., 2003). What cell based assays are not are measures of pathogenesis in the intact organism – for these, one will have to use systems where α -synuclein is expressed in the brain, discussed elsewhere in this issue. Here we will discuss only models that use cell based systems, including both mammalian cell cultures and yeast models. The basic supposition of all these models is that α -synuclein can be expressed at sufficient concentration to cause the cell to die or to become dysfunctional. We will discuss the model systems in the context of known or proposed mechanisms involved in cellular toxicity.

Protein aggregation, oligomers and pores

As discussed briefly above, a major theory regarding α -synuclein is that it is prone to form aggregates (Cookson, 2005). The aggregation process initially forms oligomeric species that are relatively soluble; these oligomers might then self-assemble into fibrillar structures that are insoluble. A major, but untested, concept is that the process of fibrillization is important to the formation of Lewy bodies as these organized structures become deposited (Mukaetova-Ladinska and McKeith, 2006). Such inclusion bodies would represent one endpoint of the aggregation pathway but may or may not be the toxic species themselves. A possible culprit for toxicity is the transient oligomeric species formed along the way (Volles and Lansbury, 2003). Most of this work has been developed from systems using recombinant proteins (reviewed in Uversky, 2003).

What have cell models told us about aggregation? Higher order species that might be equivalent to oligomers have been seen in N27 cells expressing A53T α -synuclein (Zhou and Freed, 2005) or in MES neurons exposed to polyunsaturated fatty acids (Sharon et al., 2003). When aggregated species are present, they can be identified using chemical crosslinking methods and are associated with lipid membranes (Cole et al., 2002). However, in most cell models the recovered α -synuclein is predominantly monomeric on SDS-PAGE gels and relatively soluble. This means that the species found in cells are not the types of heavily aggregated, insoluble and deposited forms seen in human brain. In turn, this limits our ability to interpret the cell models in light of aggregation pathways thought to occur in the brain and makes it hard to assess whether, for example, oligomers are the toxic species. A novel approach to this problem is to use fluorescence lifetime imaging-based techniques to probe the organization of α -synuclein within the cell, which has suggested that small oligomers are formed in a head-to-tail orientation (Klucken et al., 2006). It is clear that developing additional novel techniques to address the toxic effects of oligomers is critical to assessing this hypothesis. For example,

there are antibodies against other oligomeric proteins associated with neurodegeneration and similar reagents for α -synuclein might be extremely useful.

Some models do report the presence of α -synuclein-positive inclusion bodies, which presumably represent more aggregated and less soluble forms. In some studies, adding extra stressors increases the frequency of inclusions, such as the mitochondrial inhibitor rotenone (Lee et al., 2002), or co-transfection with other proteins including parkin and synphilin-1 (Engelender et al., 1999). These inclusion bodies might represent aggregated forms of α -synuclein although whether they are equivalent to mature Lewy bodies is not clear.

Perhaps looking for mature Lewy bodies in cells is too much to ask – there is scant evidence for Lewy body formation in some of the animal models (reviewed elsewhere in this volume). What these considerations mean is that we aren't sure of the toxic species in many of the experiments that will be discussed. We know that α -synuclein *can* form oligomers and larger aggregates in cells and in some circumstances these can also form inclusion bodies. However, by definition oligomers are a transient intermediate and are therefore difficult to see so it is still reasonable to question whether α -synuclein aggregation is either sufficient to explain its toxic properties or whether it is a necessary part of its toxicity. An approach that might be useful is to generate artificial mutants with radically different properties and correlate specific properties with toxicity. Such an approach has recently been tried in yeast and has indicated that the rate of synuclein fibrillization did not affect toxicity (Volles and Lansbury, 2007). This, along with other considerations, supports the idea that the fibrillar forms themselves are not the toxic species, but leaves open the idea of whether oligomers may be critical.

Leaving aside evidence about aggregation, what would be the next immediate effect of having oligomers in the cell? Two hints are that (A) some oligomeric species are annular or pore-like and (B) α -synuclein is a lipid-associated protein at least in some structural forms (Volles and Lansbury, 2002; Zhu et al., 2003). Therefore, α -synuclein oligomers might form pores on intracellular membranes such as the plasma membrane. Some indirect evidence for this idea comes from observations that cells expressing α -synuclein have increased but non-specific cation permeability (Furukawa et al., 2006). Although it has not been proven that this is due to partially aggregated synuclein species, these observations would be consistent with the formation of pores.

If pores are forming on the plasma membrane, they might also form on intracellular membranes, providing multiple targets for α -synuclein-toxicity in the cell. Some indirect evidence for this idea comes from studies of vesicles in cultured cells and isolated from the adrenal medulla (Mosharov et al., 2006). All these conditions show evidence of catecholamine leakage from vesicles and would therefore be consistent with the formation of pores or pore-like structures. Given that α -synuclein is concentrated at synaptic vesicles, it seems reasonable to think that in a neuron there would damage to vesicles containing neurotransmitters. This point will be returned to in the section on synapses.

The ER-Golgi connection

A difficulty in deciding how proteins are toxic is the identification of which effects are primary, and therefore critical, and which might be secondary. An important approach is to use relatively unbiased screens of multiple pathways, illustrated in one study using yeast cells to look for modifiers of α -synuclein toxicity (Willingham et al., 2003). The results show that suppressors of α -synuclein toxicity are not randomly distributed in different groups but could be classified by gene ontology category. Significantly over-represented were genes involved in trafficking of vesicles; importantly these were distinct from modifiers of huntingtin toxicity. This result, assuming it holds for mammalian systems, implies that there are some cellular processes that are more important than others in α -synuclein toxicity.

Disruptions of the ER or golgi have also been noted in other models. Prolonged expression of α -synuclein from an inducible promoter in PC12 cells causes upregulation of GRP78 and phosphorylation of eIF-2 α both potential markers of ER stress (Smith et al., 2005). Importantly, the ER stress inhibitor salubrinal or caspase-12 knockdown were able to prevent cell death, suggesting that ER dysfunction plays a causal role in cell death in this model. Fragmentation of the golgi has been seen in COS7 cells transiently overexpressing high amounts of α -synuclein (Gosavi et al., 2002), which may be related to ER stress as the number of misfolded proteins increases.

How early does ER trafficking deficit occur in the mechanism of synuclein toxicity? Returning to yeast, Cooper et al have shown that ER stress, measured by a reporter construct, occurred a few hours after induction of α -synuclein expression and substantially before measurable cell death (Cooper et al., 2006). Importantly, expression of the GTPase Rab1, a mediator of vesicle dynamics, was able to rescue the effects of synuclein in yeast, worms and in mammalian cell cultures. A caveat is that the amount of rescue was not complete, suggesting that there may be multiple mechanisms in more complex systems. Furthermore, these considerations do not yet explain precisely how synuclein affects the ER; for example, is ER damage a consequence of aggregation, either to fibrils or oligomers? Neither do such observations really explain why some groups of neurons are preferentially affected in PD, leaving some open questions for how these processes relate to the disease.

Out to the synapse

When discussing alterations in vesicle dynamics in yeast cells that result from expression of α -synuclein, we have to consider what the equivalent processes are in neurons. Given that the major function of neurons is to produce and release neurotransmitters packaged into vesicles, one possibility for such a process is synaptic transmission. α -Synuclein was originally named, in part, for being a synaptic protein and seems to be largely presynaptic (Maroteaux et al., 1988). Some recent experiments have shown that synuclein is loosely associated with presynaptic vesicles in an activity-dependent manner (Fortin et al., 2005). This is consistent with the data that shows that α -synuclein has a role, however poorly defined, in maintaining synaptic activity (Abeliovich et al., 2000; Cabin et al., 2002; Steidl et al., 2003). Interestingly, some studies suggest that the role of synuclein in modulating neurotransmission in hippocampal slices is revealed under stressed conditions (Martin et al., 2004) and may be dependent on nitric oxide (Liu et al., 2004), although again the mechanisms involved are not fully described.

Several experiments have suggested that toxic expression of α -synuclein has detrimental effects on synaptic function. In a recent model, A30P synuclein decreased exocytosis of catecholamine containing vesicles in primary cells and in chromaffin cells. Although the specifics of the deficit remain to be clarified, a late step in exocytosis was implicated (Larsen et al., 2006), likely to be “priming”, after secretory vesicle trafficking to “docking” sites but before calcium-dependent vesicle membrane fusion. One might imagine that such steps might be approximately equivalent to the blocks of ER-golgi in yeast. Again, though, the details of the mechanism(s) involved and their relationship to toxicity still need to be elucidated. However, given that the molecular machinery of the synapse is known in some detail one imagines that this might be a tractable problem.

Mitochondrial function

A longstanding theory in PD research is that mitochondrial function is an important clue for the preferential cell loss seen for some groups of neurons in the brain. This has been bolstered by the observation that each of three genes that cause recessive parkinsonism are associated, directly or indirectly, with mitochondrial function (Shen and Cookson, 2004; Clark et al.,

2006; Park et al., 2006). Although recessive parkinsonism is a very different phenotype from Lewy body disease, the common facet of prominent nigral cell loss suggests it would be worth examining mitochondrial function in α -synuclein models.

Expression of wild type murine α -synuclein produces morphological changes in mitochondria in GT1-7 cells (Hsu et al., 2000). PC12 cells inducibly expressing α -synuclein show mitochondrial depolarization and induction of mitochondria-dependent cell death (Smith et al., 2005; Tanaka et al., 2001). Similarly, α -synuclein expressing cells (Orth et al., 2003) or worms (Ved et al., 2005) are reported to be more sensitive to the mitochondrial complex I inhibitor rotenone.

Although these studies are consistent with each other and with our concepts about a role of mitochondria in PD, the problem in interpreting them is that there is little evidence that α -synuclein affects mitochondria directly. Thus, the evidence is equally consistent with hypotheses that α -synuclein affects mitochondria, leading to cell death, or affects net sensitivity to cell death, which includes toxins that affect mitochondria. There are reports of α -synuclein binding to cytochrome-C oxidase (COX), a mitochondrial enzyme (Elkon et al., 2002) but how the predominantly cytoplasmic α -synuclein gains access to the inner mitochondrial membrane is not resolved. Therefore, available evidence from cell models does not support a direct effect on mitochondrial function, although one might be found in the future.

Derangements of protein turnover

There are two major systems for protein degradation in mammalian cells, the ubiquitin-proteasome system (UPS) and lysosomal systems including autophagy (reviewed in Rubinsztein, 2006). Several studies in cell culture have implicated an effect of α -synuclein on both of these.

Inhibition of UPS function may be a general mechanism for several proteins associated with disease that are prone to misfolding. Bence *et al.* elegantly demonstrated this a few years ago, showing that net proteasome function is inhibited in cells that have intracellular inclusions of huntingtin (Bence et al., 2001). Such observations were then extended to mutant α -synuclein using over-expression models in cells in culture (e.g., Petrucelli et al., 2002; Stefanis et al., 2001; Tanaka et al., 2001) although it is not clear if this is also true *in vivo* (Chen et al., 2006). The mechanism(s) in play are also unclear. *In vitro* evidence suggests that α -synuclein can block proteasome function directly by binding either to the subunit S6' in the proteasome cap (Snyder et al., 2003) or to the catalytic core of the 20S proteasome (Lindersson et al., 2004). However, there are other plausible mechanisms for decreased proteasome function, such as the depletion of ATP as a result of mitochondrial damage.

In cells, most α -synuclein is not degraded by the proteasome, but rather by lysosomal enzymes (Paxinou et al., 2001; Webb et al., 2003). Some of the earlier studies on proteasome activity in α -synuclein transfected cells also reported alterations in lysosomal function (Stefanis et al., 2001). It has also been shown that chaperone mediated autophagy, a protein degradation pathway that depends on lysosomal function and is tightly regulated by cells, is affected by dominant α -synuclein mutations (Cuervo et al., 2004). However, altered autophagy is a possible consequence of chronic proteasome inhibition (Ding et al., 2003), and thus may be secondary to other changes in the more intact models. This emphasizes the difficulty in assuming that any specific observed cellular change is responsible for neuronal dysfunction resulting from the presence of an aggregating protein.

Overall, there is evidence for alterations in protein degradation that, as we discussed for mitochondrial function, are hard to assign as either primary or secondary effects. Because mitochondrial function impacts protein turnover and vice-versa it is possible that these are

inter-related. Data that show that α -synuclein's toxic effects can be blocked by manipulating function of protein turnover or mitochondrial function are incomplete, although some recent data suggests that induction of autophagy can help clear α -synuclein from the cell (Sarkar et al., 2007).

Oxidative stress and dopamine

Many of the concepts discussed above are common to multiple neurodegenerative diseases. Another commonly discussed mechanism for neuronal damage is oxidative stress, which is often linked specifically to PD because dopamine (DA) is highly prone to undergo oxidation. Additionally, the metabolism of DA produces toxic metabolites (Stokes et al., 1999) and can generate reactive oxygen species (ROS) (Maguire-Zeiss et al., 2005). There is some evidence for a direct connection of DA oxidation to increased α -synuclein aggregation from cellular and cell free systems (Cappai et al., 2005; Xu et al., 2002). In some studies, toxicity of α -synuclein has been shown to be higher in dopaminergic cells than non-dopaminergic cells (Petrucelli et al., 2002) and dopamine synthesis inhibitors can be neuroprotective *in vitro* (Xu et al., 2002). α -Synuclein also modulates expression of DA synthesis enzymes (Baptista et al., 2003) and physically interacts with both the DA transporter (Wersinger et al., 2003; Wersinger and Sidhu, 2003) and the key DA synthesis enzyme, tyrosine hydroxylase (Peng et al., 2005; Perez et al., 2002). While this is an attractive explanation for the selective vulnerability of the substantia nigra, it would not account for the presence of Lewy bodies in non-dopaminergic regions of the brain, such as in DLBD.

Other than dopamine, a variety of compounds induce oxidative stress and can trigger cell death in culture. Some of these have been shown to upregulate α -synuclein such as rotenone (Sherer et al., 2002), which also causes modification to the C-terminus of the protein (Mirzaei et al., 2006), or MPP⁺ (Kalivendi et al., 2004). These complex I inhibitors have the dual effects of inhibiting ATP production and of generating ROS. Because there are two effects, it is difficult to separate whether free radical damage or energetic failure is the primary factor in α -synuclein accumulation and subsequent cell damage. Chronic oxidative stress, presumably without ATP depletion, is reported to drive upregulation of α -synuclein expression in a subset of neurons in primary culture (Quilty et al., 2006). Similar observations of increased expression of α -synuclein have been made for other oxidative toxins (Shavali et al., 2004). Hydrogen peroxide exposure can drive α -synuclein fragmentation and accumulation in the nucleus (Xu et al., 2006) and is thought to be an intermediate driving expression in the MPP⁺ model (Kalivendi et al., 2004), suggesting that this is an oxidative response, not due to ATP depletion. Recombinant synuclein is reported to produce hydrogen peroxide (Turnbull et al., 2001), possibly indicating an important relationship between the protein and subsequent production of oxidative stress.

How different expression levels of synuclein relate to oxidative stress sensitivity (and similar arguments may hold for other stressors) is unclear (discussed in Cookson, 2006). The increased expression from basal to moderate levels due to chronic oxidative stress is associated with decreased sensitivity to apoptosis (Quilty et al., 2006). In other paradigms, synuclein expressing cells become more sensitive to oxidative and nitrative stressors (Jiang et al., 2006; Prasad et al., 2004). The potential reasons for these apparently discrepant results are complicated. In many cases, it is hard to directly compare the amount of synuclein expressed and we know that, in humans, a twofold difference in protein levels is enough to trigger a widespread neurodegenerative disease. Therefore, subtle differences in expression levels may be critically important.

Triggering cell death; is there a role for apoptosis?

Aggregated α -synuclein is unambiguously toxic to neurons, though the means by which this causes cellular death is as unclear as the mechanisms that trigger the initial aggregation. In cellular models increased WT α -synuclein has been shown to correlate with apoptotic markers in neurons (Saha et al., 2000) glia (Stefanova et al., 2001) and lymphoblasts (Kim et al., 2004). Classic markers such as chromatin condensation, nuclear and DNA fragmentation and cytochrome C release may also be observed in yeast expressing WT and mutant forms of α -synuclein (Flower et al., 2005). There is also some rescue of α -synuclein toxicity by non-specific inhibition of the caspases by z-VAD and RNAi knock down of caspase-12 (Cappai et al., 2005). Additionally it is also possible to abrogate α -synuclein toxicity with co-expression of hsp70 in SK-N-SH cells (Yu et al., 2005). Clouding this issue are some instances of α -synuclein acting in a neuroprotective manner (Cappai et al., 2005; Colapinto et al., 2006). Clearly it is important for the normal function of α -synuclein to be better defined for us to understand how mutations presumably cause a loss of normal function and gain of toxic function, and how we may couple this to the apoptotic pathway.

In summary, the overarching theme of all these cellular models is that α -synuclein dysfunction generates ROS, which then activates the apoptotic machinery. While reduction of ROS production has long been seen as a point of intervention where a therapeutic strategy may prove viable, it is doubtful that this would be the case for broad-spectrum anti-apoptotic approaches. Non-apoptotic cell death pathways have received relatively little attention and it is possible that these may also be important, if cellular energy metabolism were compromised.

Summary

Despite there being a great deal of work on how α -synuclein damages neurons, only a few clear principles have been established. In this article, we have followed the following framework for understanding why α -synuclein is toxic. First, it seems likely that there are some very proximal events that initiate toxicity, which relate to the unusual biochemistry of the α -synuclein protein. The prevailing hypothesis is that aggregation of the protein into small soluble oligomeric species is likely to be responsible for its ability to damage cells. This hypothesis is difficult to test at this time, as there is currently no established method to selectively remove partially aggregated synuclein but novel methods are being developed that might allow us to better assess this hypothesis in the future. One might imagine that the aggregation of α -synuclein is related to its normal function, although that also remains unclear. Downstream of the initiation come several intracellular events, some of which may be related to effects on membranes (pore formation, ER stress and synaptic dysfunction). Other events may include alterations in mitochondrial function and/or protein degradation pathways, but which of these is critical for α -synuclein toxicity is unclear. Finally, cell death is triggered, which may include apoptosis but might also invoke non-apoptotic cell death pathways.

Perhaps the major conclusion to be drawn is that we know a surprisingly small amount about why α -synuclein can kill neurons, even in simple systems like cultured cells. Many systems are affected and sorting out primary events from secondary or tertiary ones, and assigning which are required events, has been difficult. However, there have been some notable successes driven by, for example, larger scale approaches in yeast. Clearly, the challenge for the future will be to make finer distinctions as to the critical pathways by which α -synuclein causes neuronal dysfunction and death.

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