



Modulation of α -Synuclein Expression in Transgenic Animals for Modelling Synucleinopathies - is the Juice Worth the Squeeze?

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Studies of various animal models have made a substantial contribution to the recent progress in understanding the molecular and cellular basis of neurodegenerative disorders. Modelling of neurodegeneration by genetic alteration of laboratory animals became one of the most powerful tools of modern experimental neurology. The crucial event in pathogenesis of neurodegenerative diseases known as synucleinopathies is modification of α -synuclein metabolism caused by missense mutations, increased expression of the gene, or impaired degradation or intracellular compartmentalisation of the protein. Therefore, manipulations with expression of α -synuclein in laboratory animals were widely used for creating models of these diseases. In the present review we discuss strong and weak sides of such models, what has been already learned from studies of these animals and what types of models might be useful to further our knowledge about pathogenesis of different synucleinopathies.

Keywords: Synuclein; Synucleinopathy; Inclusions; Transgenic animals; Animal models of diseases; Aggregation; Cell death; Multiple system atrophy; Motoneurons; Motor dysfunction; Parkinson's disease; Dopaminergic neurons

INTRODUCTION

In the last decade substantial progress has been achieved in understanding molecular and cellular events associated with neurodegeneration. The pivotal role of particular proteins in the development of specific pathologies has been revealed, for example, amyloid β -protein precursor (APP) and tau for Alzheimer's disease, superoxide dismutase (SOD) for ALS and huntingtin for Huntington's disease. The crucial moment for studies of aetiology and pathogenesis of Parkinson's disease (PD) was the revealing of α -synuclein as a causative factor for both familial and idiopathic forms of this disease (Polymeropoulos *et al.*, 1997; Spillantini *et al.*, 1997). Further studies demonstrated that mishandling of this protein by various types of neurons or glial cells is associated with several neurological disorders, including dementia with Lewy bodies (DLB), multiple system atrophy (MSA) and certain others. These diseases were coalesced into a new group named synucleinopathies (Goedert and Spillantini, 1998; Spillantini and Goedert, 2000; Galvin *et al.*, 2001; Marti *et al.*, 2003). The major histopathological feature of synucleinopathies is accumulation of fibrillated α -synuclein that forms characteristic inclusions in the perikaria, neurites and, sometimes, nuclei of affected cells.

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Accumulation and aggregation of α -synuclein can be caused by many factors, including increased expression due to duplication or triplication of the gene, reduced degradation of the protein or its disturbed transport to synaptic terminals, where α -synuclein normally resides and functions. Rare missense mutations (A53T, A30P and E46K have been identified so far) affect intrinsic properties of α -synuclein; this could lead to pathology associated with familial forms of PD. However, for idiopathic forms of PD and other synucleinopathies that comprise the majority of cases, some external factors should trigger changes of the metabolism of normal α -synuclein protein.

Although inclusions are the most prominent feature of synucleinopathies, their role in dysfunction and death of neurons is not clear. A growing body of evidence suggests that intermediates of α -synuclein aggregation, oligomers and protofibrils, but not final products of this process, fibrils, are toxic for neurons (reviewed in Caughey and Lansbury, 2003; Dev *et al.*, 2003; Fink, 2006; Uversky, 2007). Another key and still unanswered question is why particular cell populations are more sensitive than others to α -synuclein pathology. Most importantly, poor understanding of the mechanisms of α -synuclein-induced neurodegeneration precludes development of therapies that might target dysfunction of this protein.

Neurodegeneration is an extremely complicated process and clear understanding molecular and cellular events leading to pathology requires combination of clinical and experimental studies. Although *in vitro* and tissue culture studies were quite useful for revealing certain intracellular mechanisms of neurodegeneration, only modelling of particular types of neurodegenerative processes in laboratory animals could provide a tool for experimental studies in the context of the whole organism. Various animal models were used for studying synucleinopathies but the most obvious way of creating such models - by genetic manipulation of α -synuclein expression - appeared to be not the most straightforward and fruitful. In this review we describe what has been achieved in these studies and what questions are waiting for better models that should help in finding comprehensive answers.

Pan-Neuronal Expression of α -Synuclein in Transgenic Mice

The first attempt to model synucleinopathy in transgenic mice was made by Masliah and colleagues at the turn of the century (Masliah *et al.*, 2000). A relatively strong, although non-specific PDGF β gene promoter was used to control expression of the wild type human α -synuclein. This allowed achieving relatively high levels of human protein expression in the nervous system of five independent mouse lines. Progressive accumulation of cytoplasmic and nuclear inclusions in neurons of deep cortical layers, CA3 area of the hippocampus, ophthalmic bulb and, rarely, substantia nigra, was observed in all these mice from the age of two months. These inclusions were positive for ubiquitin and human α -synuclein but not endogenous mouse α -synuclein. In contrast to Lewy bodies and other inclusions characteristic for human synucleinopathies, inclusions in transgenic mouse neurons consisted of amorphous aggregates and not α -synuclein fibrils (Masliah *et al.*, 2000; 2001). Only animals of line D with the level of human α -synuclein expression approximately five times higher than in other lines developed pathology - at the age of 12 months these animals displayed a marked decrease of the level and activity of tyrosine hydroxylase in the striatum, and motor dysfunction that could be revealed in a rotarod test. However, no loss of dopaminergic neurons was found in the substantia nigra of these mice (Masliah *et al.*, 2000). Further studies of line D in several laboratories revealed other but usually subtle changes of the phenotype (Hashimoto *et al.*, 2001; 2004; Masliah *et al.*, 2001; 2005; Klucken *et al.*, 2004; Winner *et al.*, 2004; 2008; Bar-On *et al.*, 2006), including negative effect of human α -synuclein expression on survival but not proliferation of neuronal precursor cells (Winner *et al.*, 2004; 2008). Interestingly, co-expression of α -synuclein and β -amyloid peptides had substantial effect on pathological changes in the nervous system of double transgenic mice. Fibrillated human α -synuclein was found in neuronal inclusions along with amorphous protein, and progressive degeneration of synapses and neurons of cholinergic system developed in the brain of ageing animals (Masliah *et al.*, 2001). Therefore, neuronal pathology in double transgenic mice resembled pathology characteristic to dementia with

Lewy bodies and Lewy body variant of Alzheimer's disease. In another laboratory PDGF β promoter was used to regulate expression of either wild type or A53T mutant human α -synuclein (Sharon *et al.*, 2001; 2003). Both studies focused on interaction of α -synuclein with polyunsaturated fatty acids, and although progressive accumulation of soluble oligomers in the brain of transgenic mice was observed (Sharon *et al.*, 2003), changes of the phenotype have not been reported for any of several independent lines of these transgenic animals.

High levels of α -synuclein expression in the majority of neurons throughout the nervous system of transgenic mice was also achieved in a number of laboratories by using a prion protein (PrP) gene promoter of either hamster (Gomez-Isla *et al.*, 2003; Fortin *et al.*, 2004) or mouse origin (Giasson *et al.*, 2002; Lee *et al.*, 2002; Gispert *et al.*, 2003). Several animal lines expressing various levels of wild type or mutant (A53T or A30P) α -synuclein were generated in each laboratory. Pathological phenotypes were often observed in mice expressing mutant variants of the protein while animals expressing in their neurons even high levels of wild type protein were completely spared of pathology (Giasson *et al.*, 2002; Lee *et al.*, 2002; Gispert *et al.*, 2003; Gomez-Isla *et al.*, 2003). However, even with mutant proteins results were not very consistent. Homozygous transgenic mice from two independent lines expressing A53T human α -synuclein developed motor dysfunction with paralysis that led to eventual death at the age of approximately 16 months. However, hemizygous animals from one of these lines never developed any sign of pathology, while the onset of pathology for hemizygous mice from another line was around 22-28 months (Giasson *et al.*, 2002).

Similar results were reported by Lee *et al.* (2002) who also observed pathological phenotype in transgenic mice expressing A53T human α -synuclein. In studied mouse lines an inverse correlation was found between the level of this transgene expression and the onset of the development of lethal neuronal pathology. Histological changes that included α -synuclein-positive and ubiquitin-positive inclusions in neuronal cell bodies and processes, damaged neuronal mitochondria, and apoptotic death of neurons were observed in many areas of the nervous system (Lee *et al.*, 2002; Martin *et*

al., 2006). However, as in the previously described study (Giasson *et al.*, 2002), these changes were most common for motoneurons of the spinal cord. The clinical manifestation of the pathology was mainly caused by dysfunction and death of these neurons. The dopaminergic nigrostriatal system of these mice was generally unaffected, although changes of dopamine neurotransmission were reported in the later study (Unger *et al.*, 2006). No pathology was found in mice with low level of A53T or high level of either wild type or A30P human α -synuclein expression (Lee *et al.*, 2002).

Different results were obtained in another laboratory that used PrP promoter-driven expression of human α -synuclein transgenes (Gomez-Isla *et al.*, 2003). Five lines expressing different forms of this protein were produced but in four of them expression of wild type or A53T or A30P protein did not trigger pathology in transgenic mice even at the age of 20 months although expression levels of human proteins were substantially higher than the level of endogenous mouse α -synuclein. Only mice from the line Tg5093 with the highest level of A30P α -synuclein expression displayed pathological phenotype - homozygous animals developed progressive motor dysfunction from the age of 4-7 months, whereas much later onset (8-16 months) was typical for hemizygous animals. However, no pathological inclusions, deposits or other evidence of human α -synuclein aggregation were found even in homozygous Tg5093 mice that accumulated large amounts of this protein in their neurons and developed obvious clinical signs of pathology. A widespread gliosis was the only histopathological manifestation of degenerative changes in the nervous system of these mice. Although minimal if any changes were observed in the nigrostriatal system, dopaminergic neurons of substantia nigra of Tg5093 animals were more sensitive to the toxic effect of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) but not rotenone (Gomez-Isla *et al.*, 2003; Nieto *et al.*, 2006). Similar observations were made by another group - expression of A53T human α -synuclein induced progressive motor dysfunction without any signs of human α -synuclein aggregation in the nervous system of transgenic mice (Gispert *et al.*, 2003). This phenotype was not noticed in mice of a line that expressed lower levels of the same transgene, however ablation of the

expression of endogenous mouse α -synuclein by cross breeding of these mice with α -synuclein null mutant mice, resulted in spinal motoneuron dysfunction and paralysis at around 16 months of age (Cabin *et al.*, 2005). This result might suggest that at least for some neuronal populations expression of α -synuclein during development makes them protected from certain neurotoxic insults in post-natal period.

A promoter of the mouse Thy-1 gene was also widely used for generation of mice with pan-neuronal expression of α -synuclein. In contrast to unsuccessful attempts with PrP promoter-driven expression, Thy-1 promoter was associated with reproduction of certain pathological changes in mice expressing not only mutant but also wild type human α -synuclein (van der Putten *et al.*, 2000; Rockenstein *et al.*, 2002; Fleming *et al.*, 2004; Chandra *et al.*, 2005). From the age of 3 months mice expressing either wild type or A53T human α -synuclein developed progressive motor dysfunction accompanied by pathological changes in synapses, axons and cell bodies of neurons, predominantly motoneurons of the spinal cord and the brainstem (van der Putten *et al.*, 2000). Relatively early changes in the sensory and motor systems were also revealed by various behavioural tests in another transgenic mouse line but no pathological inclusions were found in neuronal cell bodies or processes of these mice (Rockenstein *et al.*, 2002; Fleming *et al.*, 2004). No substantial dysfunction of dopaminergic neurons of the substantia nigra was observed, which is not surprising because it is well known that expression levels of Thy-1 promoter-driven transgenes in these neurons is usually quite low. Nevertheless, further studies of these mice revealed increased sensitivity to subtoxic doses of MPTP (Song *et al.*, 2004), paradoxical effect of 3,4-L-dihydroxyphenylalanine (L-DOPA), abnormal responses to apomorphine and amphetamine (Fleming *et al.*, 2006), and stimulation of α -synuclein aggregation by paraquat (Fernagut *et al.*, 2007), which all might reflect sub-pathological changes in their nigrostriatal system.

In contrast to transgenic mice described above, in heterozygous animals from several lines produced in another laboratory, accumulation of either wild type or A30P human α -synuclein in neuronal cell bodies and neurites was accompanied by its aggre-

gation, as revealed by biochemical analysis (Kahle *et al.*, 2000; 2001). However, only in one of these lines aggregation and hyperphosphorylation of α -synuclein lead to the development of intracellular inclusions, dysfunction of motor and cognitive functions, and astrocytosis predominantly in the spinal cord and the brainstem. The onset of these changes was substantially earlier for homo- than for hemizygous animals (Neumann *et al.*, 2002; Freichel *et al.*, 2007). Changes of the activity of certain mitochondrial enzymes (Poon *et al.*, 2005) and increased tau phosphorylation (Frasier *et al.*, 2005) were also found in the nervous system of these mice but sensitivity of their dopaminergic neurons to MPTP toxicity was not altered (Rathke-Hartlieb *et al.*, 2001).

Several mouse lines with roughly similar levels of expression (5-10 times higher than the level of endogenous α -synuclein expression) of wild type, A30P or A53T human α -synuclein or wild type mouse α -synuclein under control of Thy-1 promoter were used to compare the ability of these proteins to trigger pathological changes (Chandra *et al.*, 2005). Age-related progressive neurodegeneration with α -synuclein aggregation, astrocytosis, spinal motoneuron loss and paralysis of limbs were observed in mice expressing either form of human protein. However, the penetrance of phenotype development at 16 months of age was over 70% in lines expressing mutant forms and only 16% in lines expressing wild type human α -synuclein (Chandra *et al.*, 2005; Gallardo *et al.*, 2008). Pathology did not develop in mice expressing even high levels of wild type mouse α -synuclein (Chandra *et al.*, 2005).

Recently, consequences of Thy-1 promoter-driven expression of a new type of modified human α -synuclein have been checked in transgenic mice. A mutation that results in the substitution of tyrosine to cysteine at position 39 of α -synuclein molecule has not been found in association with any of hereditary synucleinopathies but *in vitro* studies have demonstrated that the ability of Y39C α -synuclein to form dimers via disulfide bonds substantially increases its propensity to aggregate and the ability to cause cytotoxicity. Consequently, even a relatively low level of transgene expression (approximately 150% of the level of endogenous α -synuclein expression) triggered accumulation of

α -synuclein oligomers, formation of intraneuronal inclusions and apoptotic death of neurons (Zhou *et al.*, 2008). These molecular and cellular changes were followed by progressive decline of motor and cognitive functions in ageing mice. However, as for the majority of mouse lines discussed above, substantia nigra neurons were not affected in these mice and pathological changes were restricted to the cerebral cortex and spinal cord.

Studies of transgenic mouse lines with pan-neuronal expression of human α -synuclein isoforms made substantial contribution to our understanding molecular mechanisms of the development of synucleinopathies. For instance, it has been clearly demonstrated that expression of wild type human α -synuclein could trigger neuronal pathology but only when the level of transgene expression in neurons is substantially higher than the level of endogenous mouse α -synuclein expression in these cells. Even lines with high levels of human wild type protein expression show late onset and low penetrance of pathology suggesting that for the development of pathological changes in the nervous system of these mice accumulation of wild type human α -synuclein should be complemented by some additional unidentified internal and/or environmental factors. These results are in agreement with analysis of familial forms of synucleinopathies caused by duplications or triplications of α -synuclein gene that also revealed incomplete penetrance of the disease for carriers of these mutations (Singleton *et al.*, 2003; Farrer *et al.*, 2004). The absence of pathology in mice expressing high levels of mouse α -synuclein is consistent with *in vitro* data that demonstrated lower propensity to aggregate for mouse protein compared to human protein (reviewed in Uversky and Fink, 2002; Fink, 2006; Uversky, 2007). This also suggests that the development of pathology could not be triggered simply by enhancing a normal function of α -synuclein. Another clear conclusion that could be drawn from the analysis of the above described mouse lines is that the expression level threshold required for triggering pathology is substantially lower for proteins carrying Parkinson's disease-associated amino acid substitutions than for wild type human protein. This could be considered as a direct experimental confirmation of a causative role for corresponding gene mutations in aetiology of familial forms of Parkinson's disease.

Aggregation of human α -synuclein is common for transgenic animals that developed clinical signs of neurological pathology although in several lines such pathology was not accompanied by aggregation of this protein and, conversely, mice from other lines with multiple α -synuclein-positive inclusions in their nervous system did not develop any pathology. Therefore, studies of these mouse models failed to clarify the role of α -synuclein aggregation in the development of clinical signs of pathology. Even those of transgenic mouse lines that displayed obvious progressive neurological phenotype with histopathological changes similar to changes typical for synucleinopathies had one serious disadvantage as models for these diseases - localisation of pathology. In the majority of cases, the spinal cord was the most affected region of the nervous system of these mice, and the observed phenotypical changes mainly reflected damage or loss of motoneurons and their axons, while spinal pathology is not a common feature of human synucleinopathies. Further studies are required to reveal if this was the result of high levels of expression of all utilized transgenes in spinal motoneurons or an increased sensitivity of these cells to toxic effects of human α -synuclein.

Cell Type Specific Expression of α -Synuclein in Transgenic Mice

A number of attempts were made to create transgenic mouse models of a particular synucleinopathy. For creation of an animal model of PD several laboratories expressed various forms of α -synuclein specifically in dopaminergic neurons. Similarly, specific expression of this protein in oligodendrocytes was applied for modelling of MSA.

Expression in Dopaminergic Neurons

A common approach for producing mice with dysfunction of the dopaminergic system was specific expression of human α -synuclein in dopaminergic neurons using tyrosine hydroxylase (TH) promoter. Studies of the first sets of such transgenic mouse lines generated independently in two laboratories produced rather disappointing results. Animals expressing either wild type or mutant (A53T or A30P) human α -synuclein did not develop motor or other behavioural dysfunction, and no histopathological changes were observed in their nervous

systems despite accumulation of the human protein in cell bodies and processes of dopaminergic neurons (Matsuoka *et al.*, 2001; Rathke-Hartlieb *et al.*, 2001). Also, no changes were found in striatal dopamine metabolism, number of dopaminergic neurons in the substantia nigra, or their sensitivity to the toxic effect of MPTP (Matsuoka *et al.*, 2001; Rathke-Hartlieb *et al.*, 2001; Manning-Bog *et al.*, 2003). Although paraquat treatment induced aggregation of the wild type or mutant form of human α -synuclein in dopaminergic neurons, they were resistant to the toxic effect of this herbicide (Manning-Bog *et al.*, 2003). No pathological changes were revealed in studies of several other more recently generated mouse lines with TH promoter-driven expression of human α -synuclein (Wakamatsu *et al.*, 2007; 2008).

Changes in the dopaminergic system were also minimal in a line of mice expressing wild type human α -synuclein under control of TH promoter that was produced in another laboratory (Richfield *et al.*, 2002). However, mice expressing under control of this promoter a double mutant human α -synuclein (with both A53T and A30P amino acid substitutions) developed a motor deficit accompanied by progressive axonal and synaptic pathology, changes of dopamine metabolism, dysfunction of ubiquitin-proteasome system, and loss of neurons in their nigrostriatal system (Richfield *et al.*, 2002; Thiruchelvam *et al.*, 2004; Chen *et al.*, 2006). Dopaminergic neurons of the substantia nigra were more sensitive to combined toxicity of herbicides paraquat and maneb but did not have pathological inclusions or aggregates of α -synuclein (Thiruchelvam *et al.*, 2004). Different results were obtained in another laboratory that produced transgenic mice expressing the same double mutant human α -synuclein under control of three different promoters, including TH promoter. These mice did not develop any pathological phenotype, and only progressive structural and functional changes of mitochondria were found in their neurons. Neither pathological inclusions, nor loss of neurons or their synapses, nor astrogliosis was observed (Maskri *et al.*, 2004; Stichel *et al.*, 2007). It is not clear why results of these two laboratories are so contradictory.

Truncated α -synuclein species lacking C-terminal sequences have increased propensity to aggregate

in vitro and are abundant in pathological inclusions found in brains of patients suffering from synucleinopathies. Nevertheless, aggregated α -synuclein was not found in dopaminergic neurons of mice expressing a C-terminally truncated, α 1-130, variant of human protein under control of TH promoter (Wakamatsu *et al.*, 2008). As in the case of mice described above (Richfield *et al.*, 2002), in the absence of aggregates these mice still developed selective loss of dopaminergic neurons in the substantia nigra, reduced striatal dopamine level and, consequently, motor dysfunction. However, these changes were not progressive and neuronal loss took place soon after activation of transgene expression at the early embryonic stage (Wakamatsu *et al.*, 2008). In contrast, mice with TH promoter-driven expression of another C-terminally truncated, α 1-120, variant of human α -synuclein developed progressive decrease of locomotor activity, which correlated with decreasing level of striatal dopamine and formation of pathological inclusions in dopaminergic neurons in the substantia nigra (Tofaris *et al.*, 2006). No loss of these neurons was revealed, although the recent study suggested that their sensitivity to stress could be increased (Tofaris *et al.*, 2006; Michell *et al.*, 2007).

In summary, expression of α -synuclein in transgenic mice under control of TH promoter turned to be inefficient for creating mouse models of PD. Triggering of some pathological changes in dopaminergic neurons was achieved only when artificially engineered mutant forms of human protein were used but the whole spectrum of PD pathology has not been reproduced in any of these models. The most probable explanation of these failures is the low level of TH promoter-regulated expression of transgenes. Because of this low expression, accumulation of exogenous α -synuclein in mouse dopaminergic neurons does not reach the level required for triggering pathological changes. It is well documented that rodent dopaminergic neurons are more resistant than human neurons to toxic drugs causing PD, like MPTP. It is feasible that a pathological threshold level of α -synuclein is also substantially higher for mouse neurons than for human neurons and, therefore, successful modeling of pathology in mice requires stronger dopaminergic neuron-specific promoters for expression of α -synuclein.

Expression in Oligodendrocytes

Attempts to create mouse models of MSA were much more successful. The approach for modelling MSA was based on ectopic expression of wild type human α -synuclein in mouse oligodendrocytes because α -synuclein-positive inclusions in these cells are the major histopathological hallmarks of this idiopathic disease. Aggregation and hyperphosphorylation of α -synuclein, and formation in the perinuclear area of oligodendrocytes of inclusions that resembled inclusions in oligodendrocytes of MSA patients were achieved in several mouse lines with transgene expression regulated by oligodendrocyte-specific proteolipid protein (PLP) gene promoter (Kahle *et al.*, 2002). However, these animals did not develop any motor dysfunction, and degeneration of neurons or axons was not noticed in their nervous system, probably due to low levels of transgene expression. Nevertheless, they appeared to be more sensitive to oxidative stress because a mitochondrial inhibitor, 3-nitropropionic acid (3-NP), triggered development of substantially more severe neurological pathology in these transgenic mice than in control wild type mice (Stefanova *et al.*, 2005). Importantly, regional localisation of neurodegeneration and atrophy within the brain of transgenic mice closely resembled localisation of pathology typical for MSA. These changes along with widespread astrogliosis and microglia activation led to gradual decline of animal motor performance in various behavioural tests (Stefanova *et al.*, 2005; 2007a,b,c).

Another, 2',3'-cyclic nucleotide-3'-phosphodiesterase (CNP), gene promoter also produced relatively low total brain level of human α -synuclein expression in transgenic mice, but because this expression was restricted to oligodendrocytes, the level of ectopic protein in these cells was substantially higher than the level of endogenous mouse α -synuclein (Yazawa *et al.*, 2005). Aggregation of human α -synuclein and progressive accumulation of its fibrils observed in oligodendrocytes led to their degeneration and death as well as autophagocytosis of axonal myelin sheets. Interestingly, endogenous α -synuclein was also involved in the development of pathological changes in neurons of these mice - abnormal accumulation of mouse protein was found in axons and presynaptic terminals. The outcome of above changes was the loss of both

glial and neuronal cells in many regions of the brain of transgenic mice and the impairment of their motor behaviour (Yazawa *et al.*, 2005).

Pathological phenotype was even more pronounced in a line of transgenic mice expressing very high level of human α -synuclein under control of the myelin basic protein gene promoter (Shults *et al.*, 2005). In contrast to mice from other lines with lower levels of transgene expression that lived longer than 18 months, all mice from this line developed severe neurological alterations at 2-4 months of age, resulting in death at the age of 6 months. Histological pathology in the brain of affected animals included astrogliosis, and structural changes of axons and their myelin sheets followed by degeneration of these structures. It has been suggested that the above changes were triggered by dysfunction of oligodendrocytes, which in turn was caused by aggregation of human α -synuclein in these cells and formation of inclusions similar to inclusions seen in oligodendrocytes of MSA patients (Shults *et al.*, 2005).

Why did overexpression of α -synuclein in relevant cell populations produce respectable models of MSA but not PD pathology? It is feasible that neurons expressing α -synuclein during the embryonic period become "tolerant" to accumulation of this protein at the later stages of development. In contrast, oligodendrocytes that normally express very little, if any, α -synuclein are much more sensitive to forced accumulation of this protein, which resembles the situation with ectopic expression of α -synuclein in invertebrates neurons (see below). Nevertheless it should be noted, that low levels of exogenous α -synuclein expression are still not sufficient for triggering pathology in oligodendrocytes, *i.e.*, a threshold level of expression and accumulation should be reached. In this aspect, it would be interesting to reveal if mutant forms of α -synuclein that induced pathology in neurons much more effectively than wild type α -synuclein would be also more effective in triggering pathology in oligodendrocytes where transport of this protein from cell body to presynaptic terminals is not an issue.

Invertebrate Models of Synucleinopathies and the Role of Endogenous α -Synuclein

Substantial pathological changes were observed in several studies of mice expressing human

α -synuclein but lacking endogenous mouse α -synuclein (Cabin *et al.*, 2005; Chandra *et al.*, 2005; Tofaris *et al.*, 2006; Gallardo *et al.*, 2008). Although only one study directly demonstrated that the severity of pathology increases at α -synuclein null background (Cabin *et al.*, 2005), it has been suggested that the endogenous protein has neuroprotective function(s). A possible scenario is that expression of endogenous α -synuclein in developing embryonic neurons makes them tolerant to this protein, *e.g.*, certain intracellular pathways become permanently active (or permanently inactive), which helps these cells to combat, to some extent, increased expression of this protein at later stages of life. In contrast, neurons that never express α -synuclein during development and therefore never get prompted to develop such defense mechanisms become vulnerable to this protein. Importantly, the vulnerability to α -synuclein toxicity does not equate with increased sensitivity to other toxic insults; conversely, increased resistance to α -synuclein toxicity does not necessarily make cells generally more robust. Moreover, a particular combination of active/inactive signalling pathways might increase sensitivity to one and resistance to another type of toxic factor (see below). However, other mechanisms of neuroprotection by endogenous α -synuclein could also be suggested and our current knowledge is far from sufficient in choosing between many possible explanations.

However, the absence of endogenous α -synuclein could explain why invertebrates that lack any synuclein-like proteins readily develop pathological features typical for human synucleinopathies upon ectopic expression of α -synuclein in their neurons. Expression of wild type, A53T or A30P human α -synuclein in transgenic *Drosophila melanogaster* flies resulted in aggregation of α -synuclein, formation of intracellular inclusions, progressive loss of dopaminergic neurons and changes of locomotor behaviour (Feany and Bender, 2000). Similarly, loss of dopaminergic neurons and their axons, reduction of dopamine level and motor dysfunction were characteristic for transgenic *Caenorhabditis elegans* worms, expressing wild type or mutant human α -synuclein (Lakso *et al.*, 2003; Kuwahara *et al.*, 2006).

Neither neuronal loss nor α -synuclein oligomers, fibrils or inclusions were found in the nervous sys-

tem of transgenic flies expressing a deletion mutant of human α -synuclein (lacking an internal fragment between amino acids 71 and 82) that shows decreased propensity to aggregate *in vitro*. In contrast, expression of a C-terminally truncated protein led to its aggregation into large intraneuronal inclusions and neuronal death (Periquet *et al.*, 2007). These results suggested that aggregation and inclusion formation are required for neurotoxic effects of ectopically expressed α -synuclein. However, studies of another line of transgenic flies demonstrated that co-expression of a chaperon, Hsp70, could rescue dopaminergic neurons from α -synuclein-induced death but has no effect on α -synuclein aggregation or inclusion formation in surviving neurons (Auluck *et al.*, 2002). Therefore, studies of transgenic flies, as described in the above studies of transgenic mice, failed to resolve ambiguities about the role of aggregation in α -synuclein-induced neurotoxicity.

Protective Function of Endogenous α -Synuclein and its Role in the Development of Neurodegeneration

In the healthy nervous system α -synuclein is localised nearly exclusively in presynaptic terminal, suggesting that it has important functions in this neuronal compartment (Maroteaux *et al.*, 1988; Maroteaux and Scheller, 1991; Jakes *et al.*, 1994). It has been suggested that α -synuclein plays an important role in synaptoprotection (Chandra *et al.*, 2005) and, therefore, α -synuclein dysfunction could lead to degeneration of synapses followed by retrograde death of neurons. Increased expression of α -synuclein or expression of mutant forms trigger aggregation and compromise axonal trafficking of this protein (Saha *et al.*, 2004). Consequently, α -synuclein stacks in the neuronal cell body, and its synaptic pool gets gradually depleted, which makes synapses progressively vulnerable to various stress and other environmental factors. According to this hypothesis the loss of protective function of endogenous α -synuclein is a substantial if not crucial event in the development of neurodegeneration in synucleinopathies. Another obvious conclusion is that animals lacking α -synuclein should develop synaptic pathology followed by axonal and, finally, neuronal degeneration. However, this might be a simplistic conclusion,

particularly if one takes into consideration that synapses and neurons of invertebrates survive perfectly well and function without α -synuclein or any other synuclein-like proteins. Indeed, mice with targeted inactivation of α -synuclein gene independently produced in several laboratories did not display any signs of neurodegeneration, although slight abnormalities of synaptic and mitochondrial functions and reduced numbers of neurons in certain populations have been reported (Abeliovich *et al.*, 2000; Cabin *et al.*, 2002; Dauer *et al.*, 2002; Chandra *et al.*, 2004; Robertson *et al.*, 2004; Ellis *et al.*, 2005). Moreover, animals supplied by Harlan UK to many laboratories as C57Bl6 wild type mice were found to carry a homozygous deletion of a locus that includes the whole α -synuclein gene (Specht and Schoepfer, 2001).

A possible explanation for the absence of noticeable alterations in the nervous system of α -synuclein null mutant mice is a functional compensation by two other closely related members of the synuclein family, β -synuclein and γ -synuclein. However, this idea got a substantial blow when no obvious phenotypical changes were observed in double (α/γ (Robertson *et al.*, 2004) or α/β (Chandra *et al.*, 2004)) or even in triple (our unpublished observations) synuclein null mutant mice. How is it possible that mouse neurons are so indifferent to the presence of such an evolutionary conserved and highly expressed protein? It could be suggested that developing embryonic neurons of null mutant mice switch on certain intrinsic mechanisms that compensate for the absence of synucleins. Activation of these mechanisms allows neurons to survive during critical developmental periods and function in adult nervous system. Although there is no experimental data that could give a clue about the nature of these intracellular mechanisms and their relationship with mechanisms that confer neurons tolerant to α -synuclein (see above), one may speculate that activation of compensatory pro-survival processes/ pathways could make neurons less sensitive to certain toxic insults. Indeed, dopaminergic neurons of substantia nigra pars compacta of α -synuclein and γ -synuclein null mutant mice were found to be resistant to toxic effects of MPTP, paraquat, 3-NP and malonate (Dauer *et al.*, 2002; Drolet *et al.*, 2004; Robertson *et al.*, 2004; Fornai *et al.*, 2005; Klivenyi *et al.*, 2006; and our

unpublished data). However, the same neurons might be more sensitive to other factors or changes of intracellular metabolism, for instance to overexpression of α -synuclein later in the development (see above). Another example is accelerated development in mice lacking two members of the synuclein family, α -synuclein and β -synuclein, of neurodegeneration triggered by the absence of synaptic co-chaperone CSP α (Chandra *et al.*, 2005). Moreover, increased expression of functional α -synuclein in CSP α null mutant mice rescued degeneration of synapses. These results directly demonstrated that α -synuclein could protect synapses and neurons from particular pathological processes.

Interestingly, substantial impairment of synaptic function was observed in the nigrostriatal system of ageing α -synuclein null mutant animals (our unpublished data). This suggests that although dopaminergic neurons are able to compensate for the absence of α -synuclein, this requires mobilisation of other protective systems, whose efficiency declines in an ageing organism. As the result, neuronal compartments that are normally rich in α -synuclein, first of all presynaptic terminals, become unprotected, which leads to their dysfunction and degeneration.

A growing body of evidence suggests that depletion of the synaptic pool of α -synuclein contributes to the development of neurodegeneration in patients and in animal models of synucleinopathies but it is not clear how substantial this contribution could be. Currently available tools are not adequate for approaching the problem - new animal models are desperately needed that will allow tightly regulated conditional inactivation of α -synuclein expression *in vivo*. This will eliminate the impact of developmental compensation and should clarify the role of endogenous α -synuclein in the adult, ageing and degenerating nervous system.

CONCLUSIONS

Studies of animals with modified expression of α -synuclein produced a wealth of data that substantially improved our understanding of aetiology and pathogenesis of synucleinopathies. However, many important questions still remain unanswered due to the complexity of the process and relative

simplicity of animal models created so far. New more sophisticated models with tightly regulated and conditional expression of α -synuclein as well as other members of the family are urgently required. To get better juice one should squeeze better fruits.

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