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CHAPTER 5

Viral vector-mediated overexpression of α-synuclein as a progressive model of Parkinson's disease

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Abstract: The discovery of the role of α-synuclein in the pathogenesis of Parkinson's disease (PD) has opened new possibilities for the development of more authentic models of Parkinson's disease. Recombinant adeno-associated virus (AAV) and lentivirus (LV) vectors are efficient tools for expression of genes locally in subsets of neurons in the brain and can be used to express human wild-type or mutated α-synuclein selectively in midbrain dopamine neurons. Using this approach, it is possible to trigger extensive PD-like cellular and axonal pathologies in the nigrostriatal projection, involving abnormal protein aggregation, neuronal dysfunction, and cell death that develop progressively over time. Targeted overexpression of human α-synuclein in midbrain dopamine neurons, using AAV vectors, reproduces many of the characteristic features of the human disease and provides, for the first time, a model of progressive PD that can be applied to both rodents and primates.

Keywords: Synuclein; Dopamine; Nigrostriatal system; Motor impairment; Adeno-associated virus; Lentivirus; Viral vectors; Animal models

Introduction

The commonly used animal models of Parkinson's disease (PD) are based on systemic or local administration of neurotoxins, such as 6-hydroxydopamine (6-OHDA), 1-methyl-1,2,3,4-tetrahydropyridine (MPTP), or rotenone. Although highly useful, these models have a limitation in that they address only one element of the underlying neuropathological changes, i.e., free radical damage and the associated mitochondrial dysfunction. Moreover, the neurotoxin models are essentially non-progressive and do not replicate all aspects of the disease.

There is, therefore, a strong need to develop new animal models that more faithfully reproduce the progressive neuropathological changes seen in

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idiopathic PD. The discovery of α-synuclein (α-syn), in 1997, as a key player in the pathogenesis of familial PD (Polymeropoulos et al., 1997), and as a major component of the characteristic protein inclusions—Lewy bodies and dystrophic neurites—that develop over time in the brains of PD patients (Spillantini et al., 1997), has opened new interesting possibilities to replicate PD-like changes in animals. The new models that have been developed over the last years are based on overexpression of human wild-type or mutated α-syn using either transgenic techniques (transgenic flies and mice; reviewed in the chapter by Magen and Chesselet in this volume), or local intracerebral injections of viral vectors carrying the α-syn gene.

A large number of transgenic mouse lines overexpressing α-syn have been generated during the last decade. Although very interesting as models of more generalized synucleinopathy, none of the transgenic mouse lines have so far reproduced the prominent, progressive neurodegenerative changes that are the hallmarks of human PD. In this regard, overexpression of α-syn by viral vector delivery offers a valuable alternative approach. Two vector systems have been explored for this purpose: recombinant adeno-associated virus (AAV) and recombinant lentivirus (LV). These vectors transduce neurons in the adult brain with high efficiency, and the expression of the inserted genes is long-lasting, over many years. These tools are also attractive in that they can be used for gene delivery in a broad range of animal species, including mice, rats, pigs, and monkeys.

The viral vector approach was initially explored in rats using injections of AAV or LV vectors encoding either wild-type or mutant human α-syn unilaterally into the substantia nigra (SN) (Kirik et al., 2002; Klein et al., 2002; Lo Bianco et al., 2002). In these studies α-syn was efficiently encoding in the nigral dopamine (DA) neurons, accompanied by cellular and axonal pathologies and DA neuron cell loss that developed progressively over time. Subsequently, the AAV vectors, in particular, have been used with similar success also in mice (Theodore et al., 2008; St Martin et al., 2007) and marmosets (Eslamboli et al., 2007; Kirik et al., 2003). These studies show that targeted overexpression of human α-syn in midbrain DA neurons, using AAV vectors, reproduces many of the characteristic features of the human disease and provides for the first time a model of progressive PD that can be applied to both rodents and primates. In this chapter we provide an overview of the results obtained so far using this disease modeling approach, and the mechanisms most likely to be involved in the development of neuronal dysfunction and neurodegeneration in the AAV-α-syn model; in addition, we discuss some of the key technical aspects and pitfalls in the use of AAV vectors for this purpose.

Induction of PD-like neuropathological changes by AAV-mediated overexpression of α-syn

AAV vectors are particularly useful for targeted gene delivery in the rodent brain. They have a high tropism for midbrain DA neurons, and since they can be produced in high titers, efficient transduction can also be obtained with small injection volumes. In rats, a single 2–3 μl injection of vectors of the AAV2 serotype has been shown to transduce the nigral tyrosine hydroxylase (TH)-positive neurons with 80–90% efficiency within the transduced area (Kirik et al., 2002; Yamada et al., 2004). Other AAV serotypes, notably AAV2/5 and AAV2/6 (i.e., generated with a transfer plasmid carrying AAV2-inverted terminal repeats packaged in an AAV5 or 6 capsid), have been used with similar success (Azeredo da Silveira et al., 2009; Gorbatyuk et al., 2008; Sanchez-Guajardo et al., 2010; own unpublished observations). The transgene expression in midbrain DA neurons achieved with LV vectors is clearly lower, usually no more than 50%. The extent of DA neuron cell loss obtained LV-mediated overexpression of wild-type or mutated α-syn is also less pronounced, typically between 25 and 35% (Lauwers et al., 2003, 2007; Lo Bianco

et al., 2002), as compared an average of to 50–60% seen in rats treated with AAV vectors (see below).

Cell death

In the rat AAV-α-syn model the average loss of TH-positive neurons, as reported in several studies from independent laboratories using various serotypes of AAV, is relatively consistent and ranges between 50 and 60% reduction in the TH-positive cell numbers in the SN (Chung et al., 2009; Gorbatyuk et al., 2008; Kirik et al., 2002, 2003; Maingay et al., 2006; Yamada et al., 2004, 2005), accompanied by a similar level of TH-positive innervation in the striatal target areas (Fig. 1). The time course of cell loss, however, has been variable: from 8 weeks in the Kirik et al. (2002) and Gorbatyuk et al. (2008) studies, to 3–4 months in the Yamada et al. (2004) and Chung et al. (2009) studies. This difference may, at least in part, be due to the level of α-syn expression obtained with the different vector constructs (the strong expression from the synthetic chicken β-actin (CBA) promoter in the first two studies, as compared with the cytomegalovirus (CMV) and synapsin promoters in the two latter studies). Gorbatyuk et al. (2008) have reported that the level of expression of human wild-type or mutated α-syn in the SN was about fourfold higher than the endogenous α-syn in rats injected with the CBA-driven vector construct. Similar data are not available for the alternative vector constructs, but the expression levels obtained is likely to be lower with the CMV and synapsin promoters.

In rats, the cellular pathological and neurodegenerative changes induced by intranigral AAV-α-syn are associated with the development of motor impairments (Eslamboli et al., 2007; Kirik et al., 2002; Maingay et al., 2006; Yamada et al., 2005). As predicted from the highly variable cell loss (30–80% at 2 months and beyond), overt behavioral impairments were seen only in a subset of the AAV-α-syn-treated animals (~25% in the experiments in rats carried out in our center) (Fig. 2). Interestingly, the impact of α-syn overexpression seems to be different between the two major DA neuron subtypes, leading to prominent cell loss in the A9 cells of the SN, while the A10 cells in the adjacent ventral tegmental area (VTA) survive, despite similar levels of α-syn expression (Maingay et al., 2006). Signs of α-syn toxicity, including the formation of α-syn-positive inclusions and dystrophic neurites, also developed in the transduced VTA neurons, but this did not seem to affect the survival of the cells in the same way as in the nigral cells.

In mice, the nigral cell loss obtained with AAV-α-syn vectors is clearly less pronounced. In the studies performed to date, the loss of TH-positive neurons in the SN, as observed at 2–3 months after injection, is in the order of 20–25% (St Martin et al., 2007). This is in line with our own unpublished data. Consistent with this level of cell loss, the treated mice have in our hands shown no or only subtle behavioral impairments. Also in mice, the VTA neurons seem to be less affected.

Axonal pathology

AAV-mediated overexpression of human α-syn provides an interesting model for progressive PD-like cellular pathology, including the formation of α-syn-positive cytoplasmic inclusions and prominent axonal pathology (Fig.1e). The axonal changes develop early after vector injection, precede DA neuron cell loss and persist in the striatum over many months (Chung et al., 2009; Eslamboli et al., 2007; Kirik et al., 2002, 2003; Klein et al., 2002; Maingay et al., 2006; St Martin et al., 2007; Yamada et al., 2004, 2005). In surviving nigral neurons that express the human α-syn transgene, the denritic projections in the SN pars reticulata are truncated with distorted morphology, and in the projection areas in the striatum the pre-terminal axons display swollen and distorted profiles (Fig. 1g), filled with α-syn-positive aggregates (Fig. 1e). In addition there is a notable

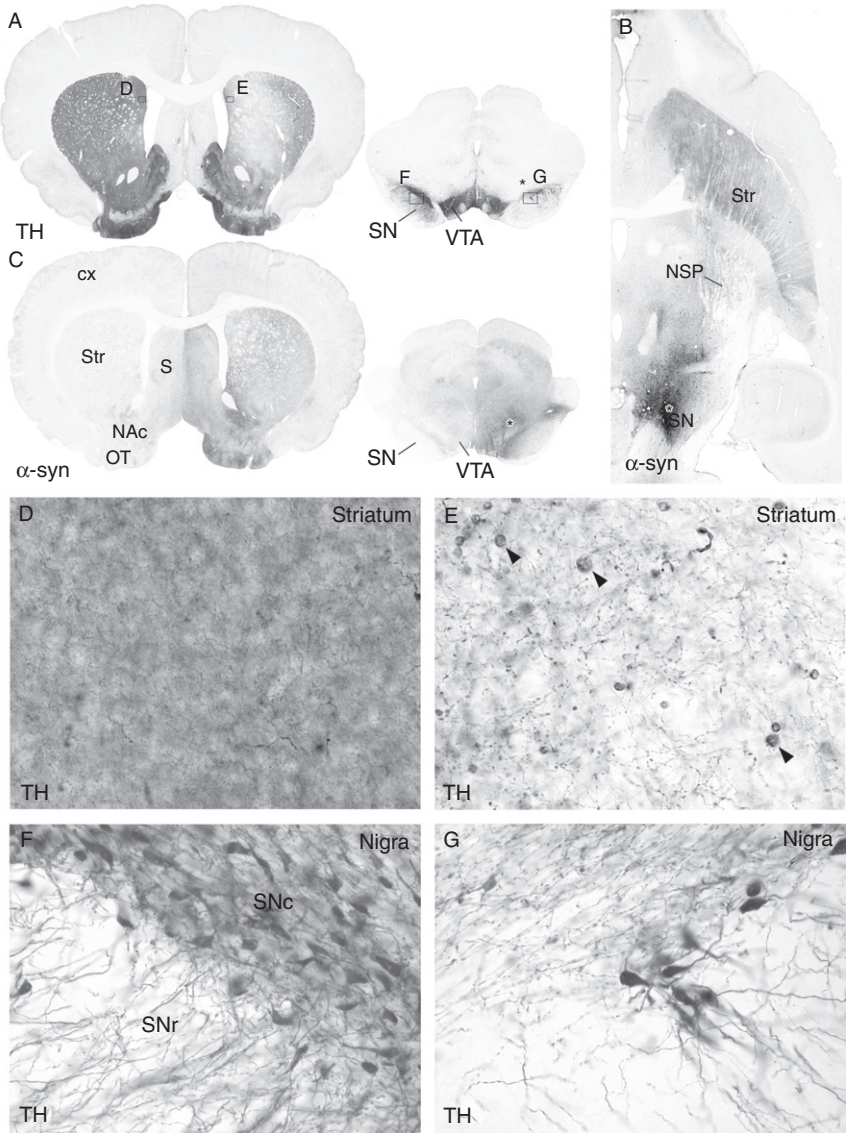

Fig. 1. Overexpression of human wild-type α-syn in the rat midbrain leads to PD-like neurodegeneration in the nigrostriatal pathway (NSP), 8 weeks after injection of a single deposit of an AAV6-α-syn vector into the right substantia nigra (SN). Immunohistochemical staining against TH shows reduced DAergic innervation in the striatum in the injected side (a, e) compared to the intact side (a, d). Intra-cytoplasmic inclusions immunoreactive for TH are present in DAeric dystrophic terminals (see arrow heads in panel e), and a substantial loss of TH-positive neurons in the SN on the injected side (a, g) compared to the contralateral side (d), while the A10 neurons in the ventral tegmental area (VTA) are largely spared. Immunohistochemical staining against α-syn (using an antibody specific for human α-syn) shows overexpression of α-syn in the nigrostriatal system; in the midbrain DA neurons, along the NSP and in the axonal terminals in the striatum (Str) (b, c). Asterisk in panel b marks the injection site. Cx, cortex; NAc, nucleus accumbens; OT, olfactory tubercle; SNc, SN pars compacta; SNr, SN pars reticulata.

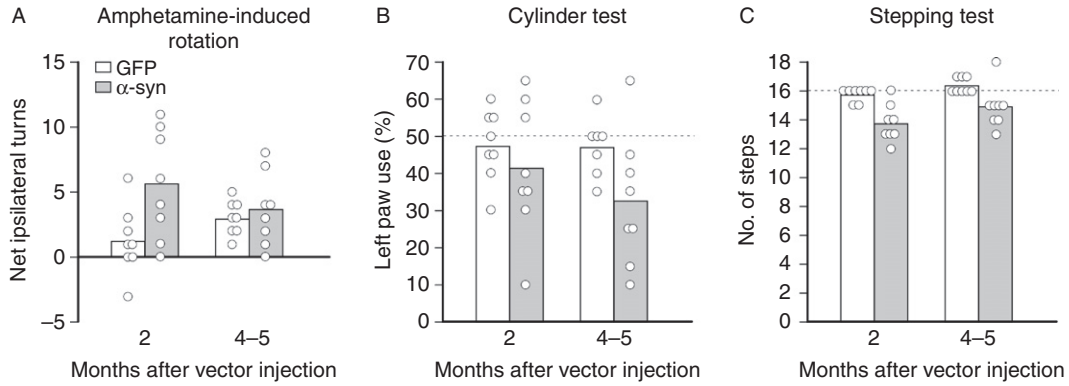

Fig. 2. Changes in motor behavior in rats given unilateral intranigral injections of either AAV6-α-syn or AAV6-GFP ($n = 8$ in each group). Although the mean performance of the AAV6-α-syn-injected animals did not differ from the AAV6-GFP-injected ones in either the amphetamine-induced rotation test (a), the cylinder test (b), and the stepping test (c), clear impairments were observed in a subset of α-syn-overexpressing animals. This effect was most pronounced in forelimb use in the cylinder test, where half of the animals showed impairment in the 4–5-month test. Dotted lines in b and c indicate level of performance in normal controls.

loss of fine-caliber TH-positive terminals in the target regions of the affected nigra neurons (Fig. 1a–c).

The morphology of these α-syn-containing dystrophic axons, which is remarkably similar to those observed in brains from PD patients (Braak and Braak, 2000; Galvin et al., 1999), seems compatible with intra-axonal buildup due to impaired axonal transport (Saha et al., 2004). Indeed, in a recent study, Chung et al. (2009) have shown that the appearance of dystrophic axonal swellings along the nigrostriatal projection is accompanied by altered levels of proteins involved in axonal transport and vesicle exocytosis, pointing to an early and persistent impairment of both axonal transport and synaptic function.

Immune and inflammatory changes

AAV-mediated overexpression of either wild-type or A53T mutant α-syn in midbrain DA neurons induces an early and persistent immune/inflammatory reaction similar to what has been reported in PD patients (Chung et al., 2009; Sanchez-Guajardo et al., 2010; Theodore et al., 2008). These changes are characterized by an early activation of microglia, expression of neuroinflammatory markers and infiltration of lymphocytes at a time when α-syn is fully expressed (4 weeks), but before any significant cell loss has occurred. Sanchez-Guajardo et al. (2010) have described two distict patterns of inflammatory/immune changes depending on the extent of cell death: (1) an early, transient change in the number of activated microglia and long-lasting major histocompatibility complex (MHC) II expression associated with persistent α-syn-induced neuropathology, but no cell death; and (2) a second, more protracted microglia response correlated with long-lasting CD68 expression and infiltration of CD4+ and CD8+ lymphocytes, which is seen in cases where α-syn has induced both pathological changes and cell death. At the level of the striatum, the development of prominent α-syn-induced axonal pathology is associated with the appearance of activated microglia, increased MHC II expression, and elevated levels of pro-inflammatory cytokines (interleukin 1β, interferone γ and tumor necrosis factor α) (Chung et al., 2009; Sanchez-Guajardo et al., 2010) (Fig. 3). These data show that overexpression of human wild-type or mutant α-syn, in

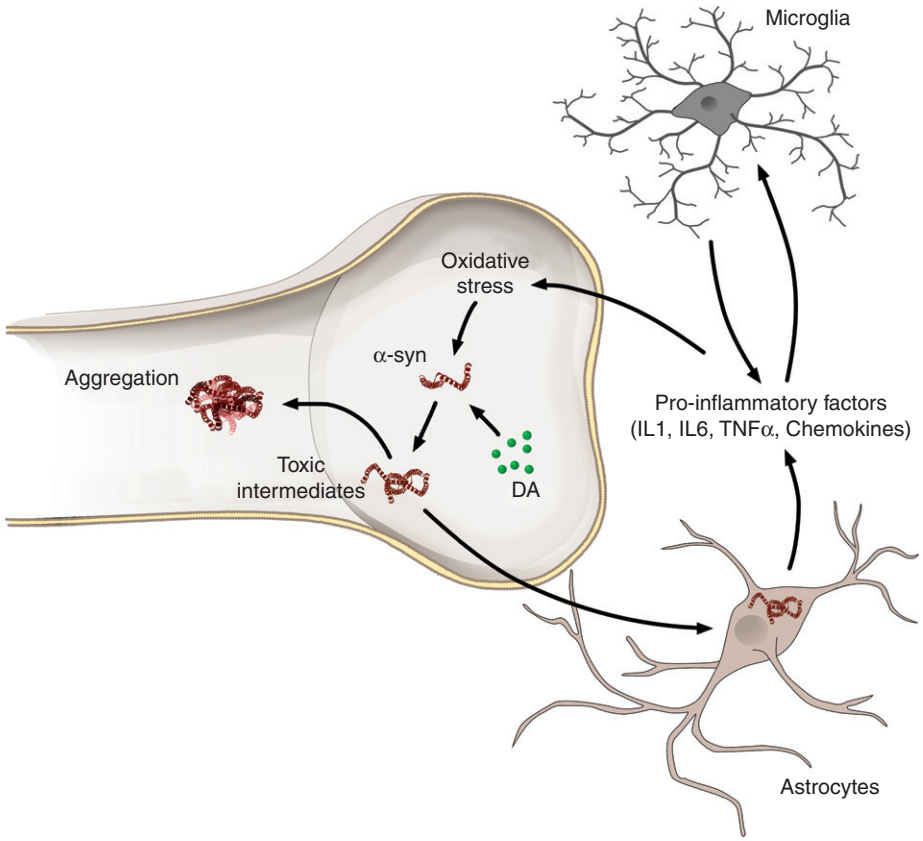

Fig. 3. A growing body of evidence suggests that the presence of ongoing inflammation may contribute and hasten the progression of PD. This is supported by evidence of activated microglia, accumulation of cytokines, oxidative damage in post-mortem PD brains as well as the increased expression of genes encoding pro-inflammatory cytokines in the SN. In parallel, post-mortem analysis has shown the presence of α-syn inclusions in astrocytes in the striatum of PD patients. Mice overexpressing human α-syn display increased microglial burden and higher levels of inflammatory cytokines prior to cell loss. This inflammatory environment represents a source of oxidative stress to which DA neurons are particularly susceptible. In addition, recent findings suggest a direct transfer of α-syn from DA neurons to astrocytes, leading to the production of pro-inflammatory mediators. In this model, astrocytes and microglia act as key players in the induction of oxidative stress and the production of toxic α-syn intermediates in the α-syn-overexpressing DAergic neurons, probably acting on both the cell body and the axon terminal level.

the absence of overt neuronal cell death, is sufficient to trigger a sustained neuroinflammatory response, including both microglial activation and adaptive immunity, similar to that seen in progressive PD in patients. In humans the presence of ongoing inflammation may contribute to neurodegeneration and drive the progression of PD. This is supported by evidence of activated microglia and accumulation of cytokines, in post-mortem PD brains (McGeer and McGeer, 2008), and by experiments in transgenic mice showing that the induction of a neuroinflammatory response (by local injection of lipopolysaccharide (LPS)) is sufficient to induce α-syn aggegation, cytoplasmic inclusions, and cell death in nigral DA neurons (Gao et al., 2008). In addition, a microarray study has revealed increased expression of genes encoding pro-inflammatory cytokines in the SN (Duke et al., 2007) and

positron emission tomography (PET) imaging studies have demonstrated increased microglial activation in various brain regions, including the striatum, of patients with idiopathic PD (Gerhard et al., 2006).

Enhancement of α-syn toxicity by post-translational modifications

As illustrated schematically in Fig. 4, the damage caused by α-syn overexpression is likely to depend on the formation of toxic intermediates (oligomeres or protofibrils) that may overwhelm or bypass the ubiquitin-proteasome system/lysosomal degradation system. The formation of these toxic α-syn intermediates is promoted by oxidative damage and disease causing genetic mutations. In addition, post-translational modifications of α-syn have been implicated in this process. AAV-mediated overexpression of post-translationally modified forms of α-syn has been used to generate improved models of PD and to further investigate

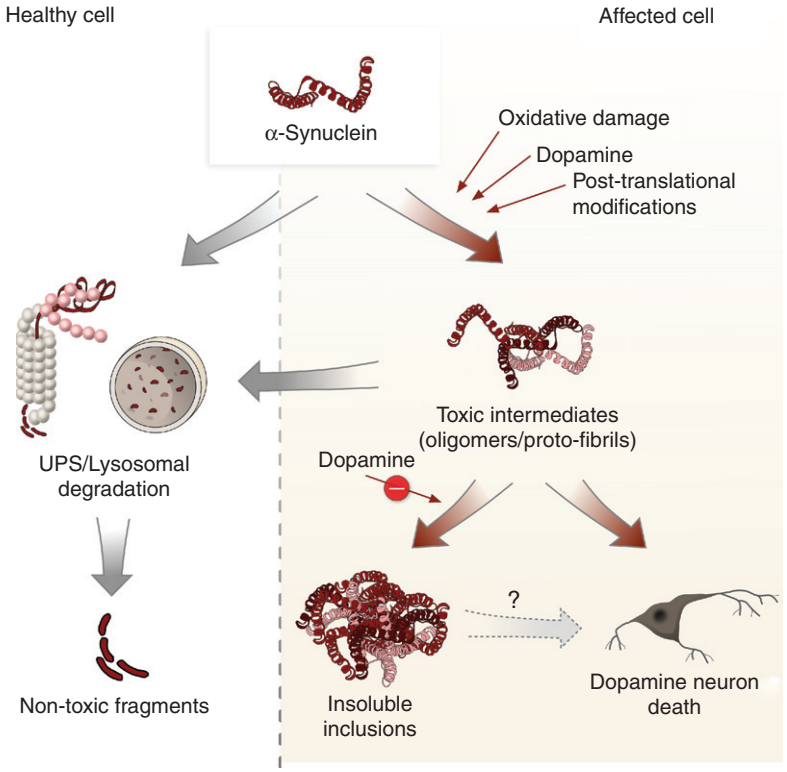

Fig. 4. Cellular mechanisms of α-syn-mediated DAergic cell death. In a healthy cell where the levels are within physiological range, α-syn can be degraded via the intracellular degradation pathways (i.e., chaperone-mediated autophagy, macroautophagy or the ubiquitin-proteasome system (UPS)). α-Syn protein is prone to aggregate and form intermediate species under stress conditions (i.e., oxidative and nitrosative stress), or by post-translational modifications. Moreover, α-syn is known to interact with DA or its toxic metabolites leading to the formation of oligomeres or protofibrils. These intermediates are suggested to be the toxic to the cell, unless they are degraded by the endogenous pathways or neutralized by forming insoluble fibrillar inclusions. A widely accepted view postulates that the formation of insoluble aggregates is part of the survival mechanism rather than the cause of neuronal death. In the AAV-α-syn model, where the levels of α-syn in nigral DA neurons are elevated to levels severalfold above normal, increased formation of toxic intermediates is proposed to be a key player in the induction of the inflammatory response (as illustrated in Fig. 3), the formation of inclusions and aggregates (as shown in Fig. 1), and cell death.

the mechanisms underlying α-syn-mediated neurodegeneration. Most of the α-syn located in Lewy bodies in PD brains and related synucleinopathies, and in the aggregates formed in transgenic models of PD, is phosphorylated at serine residue at position 129 (S129) (Anderson et al., 2006; Fujiwara et al., 2002; Hasegawa et al., 2002; Kahle et al., 2000; Neumann et al., 2002; Takahashi et al., 2003), and this is the case also in the α-syn-positive aggregates formed in the AAV-α-syn model (Yamada et al., 2004). Although the presence of phosphorylated α-syn in pathological accumulations suggests that S129-phosphorylated α-syn is closely linked to PD pathology, the exact mechanism by which phosphorylation at S129 modulates α-syn aggregation and toxicity *in vivo* is not clear. Studies in *Drosophila* have shown that blocking the phosphorylation at S129 residue of the α-syn protein, using S129A mutant α-syn that cannot be phosphorylated, suppresses DAergic neurodegeneration. Whereas when the phosphorylation mimic S129D mutation was introduced, the neurotoxicity was enhanced (Chen and Feany, 2005).

In order to gain a better understanding of the role of phosphorylation in α-syn toxicity and aggregation in rodents, several groups have used AAV vectors to overexpress S129D mutant forms of α-syn in the rat midbrain DAergic neurons. Interestingly, however, AAV-mediated expression of S129D phosphomimic α-syn in rats led to similar or lower levels of toxicity in the SN as compared to the AAV-mediated S129A α-syn expression (Azeredo da Silveira et al., 2009; Gorbatyuk et al., 2008; McFarland et al., 2009). At this point, as these site-specific mutations do not replicate the biological consequences of the actual phosphorylation event on the natural serine residue, the role of S129 phosphorylation in the induction of α-syn aggregation and neurotoxicity remains controversial. Recently, α-syn phosphorylation at S87 has been documented in post-mortem analysis of synucleinopathy patient brains, as well as in brains of α-syn overexpressing transgenic mice (Mbefo et al., 2010). Interestingly, when micelle-bound α-syn is phosphorylated at S87, it underwent a conformational change and represented a lower affinity to lipid vesicles, suggesting that phosphorylation of this site might have a role in interactions with other molecules rather than the aggregation process. Further studies are warranted to clarify the effects of phosphorylation of α-syn in the mechanism of neurodegeneration *in vivo*.

Besides phosphorylation, many other post-translational modifications, such as oxidation, C-terminal truncation, ubiquitination, and nitration, have been implicated in the mediation of α-syn aggregation and toxicity. Using transgenic mouse models several groups have shown that truncated α-syn can lead to increased DAergic dysfunction and pathology (Daher et al., 2009; Tofaris et al., 2006; Wakamatsu et al., 2008), and evidence from *in vitro* studies suggests that the C-terminal-truncated α-syn is more prone to aggregate and that it can promote aggregation of full-length α-syn (Crowther et al., 1998; Liu et al., 2005). In line with these observations, we have shown that AAV-mediated co-expression of wild-type human full-length α-syn and C-terminal-truncated α-syn in the rat SN promotes the accumulation of pathological full-length α-syn protein and leads to increased DAergic cell death, suggesting that changes in truncation of α-syn can be responsible from the pathogenesis and progression of PD (Ulusoy et al., 2010).

Mechanisms of α-syn toxicity in DA neurons

The results obtained in the AAV-α-syn model show that overexpression of human α-syn can induce α-syn-positive cytoplasmic and axonal inclusions and progressive neurodegenerative pathological changes in midbrain DAergic neurons. Our own observations suggest that these degenerative changes are most prominently expressed in the nigral DA neurons and were not seen in any of the non-DAergic neuron systems that were efficiently transduced by the same AAV-α-syn vector (Kirik et al., 2002). The impact of α-syn overexpression in midbrain DA neurons

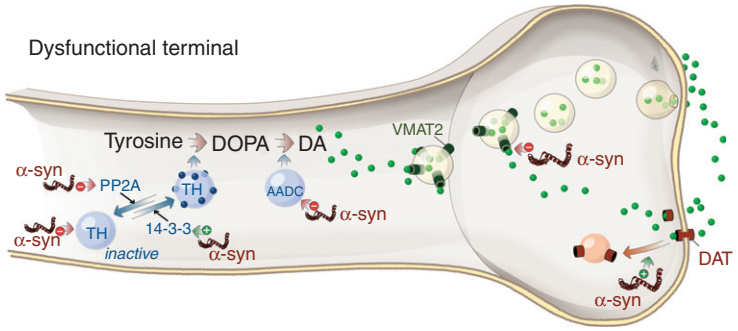

Fig. 5. α-Syn interacts with enzymes involved in DA synthesis, including TH, 14-3-3, PP2A, and AADC, thus taking part in the regulation of the production of the neurotransmitter. DA storage, release and uptake are also influenced by α-syn. Based on this model, overexpression of α-syn in midbrain DA neurons, as seen at early time points in the AAV-α-syn model, will lead to a state of impaired DA neurotransmission due to reduced synthesis and storage of the transmitter and impaired synaptic DA release. The subsequent formation of α-syn aggregates, on the other hand, may lead to an impairment of normal α-syn function in the cell, which in turn may result in the synthesis of larger amount of DA and a reduction in the storage capacity and a leakage of DA from the synaptic vesicles. In addition, reduced bioavailability of α-syn at the synapse could promote the presence of the DAT at the membrane and increase its efficacy for DA reuptake. Taken together, this may induce a dysfunctional state, leading to an increased concentration of free cytosolic DA and a source of detrimental oxidative stress.

is twofold: a suppression of DA synthesis and storage (as reflected in reduced TH enzymcl activity and striatal DA levels) at a stage when α-syn has a diffuse cytoplasmic distribution, followed by the appearance of cytoplasmic inclusions, dystrophic neuritis, and cell death. These data point to interesting interactions between α-syn and DA, in the maintenance of DA neurotransmission, in the regulation of DA homeostasis at the synapse level, and in the formation of toxic α-syn derivatives and cell death. In the AAV-α-syn model these three mechanisms, in combination with the α-syn-induced inflammatory response (see above), may interact to induce the progressive pathological changes that develop over time in the transduced DAergic neurons.

Effects on DA synthesis

In DAergic neurons, the regulation of DA synthesis, storage, release, and re-uptake has been shown to depend, at least in part, on the function of α-syn (Fig. 5). There is considerable evidence that the activity of the TH enzyme is modulated by α-syn, in particular by regulation of its phosphorylation state. In this line, a reciprocal interplay between α-syn and 14-3-3 proteins has been suggested for the regulation of TH activity (Xu et al., 2002). The chaperone 14-3-3 protein binds to the active phosphorylated form of TH and is required for maximal phosphorylation of the enzyme (Ichimura et al., 1987, 1988). Interaction between 14-3-3 protein and TH maintains the latter in its active form by protecting it from dephosphorylation and increases its half-life in DA neurons (Toska et al., 2002). Conversely, it has been demonstrated that α-syn co-localizes with and binds directly to TH with the functional consequence of a reduced enzymatic activity and a decrease in DA synthesis. Indeed, α-syn has the property to bind to TH in its dephosphorylated form and thereby maintains the enzyme in its inactive form (Alerte et al., 2008; Perez et al., 2002). In addition, it was shown that α-syn might also regulate TH activity indirectly by acting on protein phosphatase 2A (PP2A). Overexpression of α-syn in DA cells leads to unaltered expression of PP2A, while the activity of the

phosphatase is increased in parallel with α-syn expression (Peng et al., 2005; Perez et al., 2002). This suggests that the inactivation of TH seen at early time points in AAV-α-syn-transduced DA neurons (Kirik et al., 2002) may be due to dephosphorylation of the TH enzyme induced by PP2A.

Effects on DA storage and release

Different studies support that α-syn is involved in synaptic vesicle recycling, DA storage and release in the presynaptic terminal by maintaining a reserve pool of synaptic vesicles. This function is suggested by the observation that the synaptic response to paired stimulus depression of DA release, which is capable of depleting the release pool of vesicles, was impaired in α-syn knockout mice, and the time for replenishing docked vesicles from reserve pool was slower in these transgenic animals (Abeliovich et al., 2000). Further evidence in support of a role for α-syn in vesicular recycling and transmitter release comes from *in vitro* studies of hippocampal neurons (Cabin et al., 2002) and PC12 and chromaffin cells (Larsen et al., 2006), possibly mediated via an interaction with phospholipase D2, which is an important regulator of synaptic vesicle recycling (Payton et al., 2004). In support, Nemani and colleagues (2010) have reported that even modest non-toxic overexpression of α-syn, similar to what was found in PD patients with a triplication of the α-syn gene locus (Miller et al., 2004), has an inhibitory effect on neurotransmitter release.

α-Syn has been implicated also in the regulation of DA storage into synaptic vesicles. This process is critical as the low pH environment within the vesicle stabilizes DA and therefore prevents its oxidation and the formation of toxic reactive intermediates in the cytoplasm. Volles and colleagues have shown that α-syn aggregates can permeabilize synaptic vesicles and postulated that this caused cytosolic leakage of DA (Volles and Lansbury, 2002; Volles et al., 2001). In addition, an *in vitro* study has provided evidence that the A53T mutant, but not wild-type α-syn, can modulate the vesicular monoamine transporter-2 (VMAT2) (Lotharius and Brundin, 2002; Lotharius et al., 2002). LV-induced overexpression of A53T mutant α-syn in an immortalized human mesencephalic cell line (MESC2.10) resulted in down-regulation of VMAT2, decreased potassium-induced and increased amphetamine-induced DA release, enhanced cytoplasmic DA and increased intracellular levels of superoxide. This finding is in line with a reduction of VMAT2 that was observed by PET imaging in the brain of PD patients (Frey et al., 1996).

Effects on DA re-uptake

The DA transporter (DAT) plays a major role in the regulation of the synaptic content of DA and therefore is a key component of DA neurotransmission. Thus, enhanced activity or expression of DAT on the plasma membrane will result in an increase in intracellular levels of DA and an increased risk for oxidative damage to DA neurons.

In vitro studies have shown that DA uptake is modulated by α-syn expression. Under basal conditions, α-syn appears to be a negative regulator of the DAT function due to a decrease in DA uptake velocity, while no changes in the affinity for the neurotransmitter or the level of expression were observed (Adamczyk et al., 2006; Wersinger et al., 2003). Co-immunoprecipitation studies revealed that this effect results from a direct interaction between the non-amyloid component domain of α-syn and the last 22 amino acids of the C-terminal tail of the DAT (Lee et al., 2001; Wersinger et al., 2003).

Due to its ability to bind to membrane lipids α-syn can also affect the trafficking of DAT by sequestrating the transporter away from the plasma membrane, limiting the re-uptake of released DA (Lee et al., 2001; Wersinger and Sidhu, 2003). Wersinger and Sidhu (2005) have observed that disruption of the interaction

between α-syn and microtubules, using microtubule-destabilizing agents, leads to an enhanced cell surface recruitment of the DAT, suggesting that the regulation of DAT activity by α-syn is due, at least in part, to its ability to tether the transporter to the microtubular network.

Together, these studies suggest a model of α-syn-mediated pathogenesis linked to aberrant regulation of DAT function, leading to increased cytoplasmic levels of DA. In support of this idea Wersinger et al. (2003) have observed that the attenuation of DAT function by α-syn has a protective effect on DA-mediated oxidative stress and cell death.

Dopamine-dependent toxicity

Aggregation of the α-syn protein is a characteristic feature of PD, but the consequence of this process on cell survival and function remains unclear. *In vitro* studies have shown that, at high concentration, monomers of α-syn are prone to self-aggregate into insoluble fibrils via an oligomeric state (Volles and Lansbury, 2002), and that the disease-related mutant variants (A53T and A30P) of α-syn are prone to aggregate faster than the wild-type form (Conway et al., 2000). Although monomers of α-syn can self-aggregate, various intracellular factors, among them the interaction with DA, oxidative stress, and post-translational modifications, have been shown to speed up this process (Fig. 4). In support, Xu and colleagues showed that accumulation of α-syn in cultured human DA neurons led to cell death that required DA synthesis and was driven by reactive oxygen species (Xu et al., 2001). Overexpression of α-syn in non-DAergic human cortical neurons, by contrast, had no toxic effect, but rather exhibited neuroprotective property.

Oxidative stress has been shown to play an important role in the facilitation of α-syn aggregation. DA neurons may be particularly susceptible since the free radical production is high in these types of cells, due to their content of both DA and iron. Interestingly, α-syn protein is itself a target for oxidative damage. Nitration of the protein by reactive oxygen and nitrogen species has been shown to produce toxic intermediates that are prone to aggregate (Giasson et al., 2000). Indeed, in the presence of compounds generating free radicals, cells overexpressing α-syn produce intra-cytoplasmic inclusions of ubiquitinated α-syn (Hashimoto and Masliah, 1999; Ostrerova-Golts et al., 2000), and administration of the mitochondrial inhibitor rotenone (which causes the generation of reactive oxygen species) has been reported to lead to the formation of α-syn aggregates and the loss of DA neurons in rats (Sherer et al., 2003).

In the AAV-α-syn overexpression model it is likely that cytoplasmic DA plays a role in the induction of toxic damage. Free cytosolic DA can interact with α-syn in the generation of toxic species and thus contribute to the susceptibility of α-syn to aggregate *in vivo*. As described above, this may be further aggravated by the neuroinflammatory response induced by toxic α-syn monomers (see Fig. 3), leading to microglial activation and increased production of reactive oxygen species, such as microglia-derived nitric oxide and superoxide (Gao et al., 2008). It has been shown that DA binds to two different parts of the α-syn protein, including the non-amyloid component region (Herrera et al., 2008). Depending on the state of aggregation, this results either in the inhibition of α-syn fibrillation or in the disaggregation of fibrils into oligomers that are likely to be the most toxic species (Cappai et al., 2005; Conway et al., 2001; Herrera et al., 2008; Li et al., 2004; Mazzulli et al., 2006; Norris et al., 2005).

Studies on neuroprotection in the AAV-α-syn model

Mutations in the parkin gene has been linked to one of the most common causes of familial Parkinson's disease, and it has been suggested that

this protein, which is a ubiquitin-proteasome E3 ligase enzyme, may play a role in the defense of the cell against α-syn toxicity. Based on this idea several studies have been performed to explore the possible neuroprotective properties of parkin in the α-syn overexpression model (Lo Bianco et al., 2004a; Yamada et al., 2005; Yasuda et al., 2007). According to Lo Bianco and colleagues LV-mediated overexpression of parkin reduces nigral TH neuron loss, induced by α-syn overexpression, from 31 to 9% when cotransfected in the midbrain DAergic neurons in rats (Lo Bianco et al., 2004b). In a similar study, performed with AAV vectors to co-express α-syn and parkin, Yamada et al. (2005) demonstrated protection from α-syn-induced DAergic cell loss, from about 50 to 20%, and prevention of the development of motor impairments (Yamada et al., 2005). When tested in the 6-OHDA model of PD, on the other hand, AAV-mediated overexpression of parkin in rat SN led to improvement of motor functions in the 6-OHDA-lesioned rats, but without detectable neuroprotection, as observed at 12 weeks after lesion (Manfredsson et al., 2007). In another study, Verkammen et al. (2006) reported that LV-mediated gene transfer of parkin in the rat 6-OHDA lesion model resulted in a significant (~80%) neuroprotection at 3 weeks after the lesion, although the protective effect of parkin diminished to about 5–10% at later time points.

Glial cell-line-derived neurotrophic factor, GDNF, and its functional analogue *neurturin*, have been shown to provide functional recovery and nigral DA neuron protection in rodent and primate toxin models of PD (Eslamboli et al., 2005; Georgievska et al., 2002; Hoffer et al., 1994; Horger et al., 1998; Kearns et al., 1997; Kirik et al., 2001; Kordower et al., 2000, 2006; Rosenblad et al., 1999, 2003; Tomac et al., 1995). Several clinical studies, performed either by direct delivery of recombinant GDNF protein into the brain or by AAV-mediated overexpression of neurturin into the putamen, have so far failed to show convincing evidence of neuroprotection in PD patients (Ceregene, 2009; Gill et al., 2003; Manning-Bog et al., 2006; Nutt et al., 2003). Although there is solid evidence that GDNF can act as an efficient neuroprotective agent in the 6-OHDA or MPTP lesion models, the two studies that have been performed in the rat AAV-α-syn overexpression model have so far given negative results (Lo Bianco et al., 2004a; own unpublished observations). This discrepancy of results obtained in the toxin and AAV-α-syn models is a concern and raises critical questions regarding the predictive value of the toxin models currently used for testing of novel neuroprotective strategies.

Use of AAV vectors for overexpression of α-syn in non-dopaminergic systems

A particular advantage of AAV vector delivery is that it makes it possible to target α-syn overexpression to selected brain structures, also outside the SN. The non-motor symptoms of PD, such as cognitive decline, mood disturbances, olfactory deficits, and balance and sleep disturbances, are likely to reflect, at least in part, the involvement of brain structures outside the midbrain DAergic system. Neuro-pathological studies of the distribution of α-syn-positive Lewy bodies and Lewy neurites in PD patients point to a progressive involvement of structures outside the nigrostriatal system, including serotonin neurons of the raphe nuclei, noradrenergic neurons of the locus coeruleus, cholinergic neurons of the basal forebrain, and ultimately also multiple forebrain, olfactory and cortical areas (Braak et al., 2003). In the attempts to model PD made so far, the AAV-α-syn vector injections have been targeted to the midbrain DA neurons alone. In future studies, however, a combination of nigral AAV-α-syn injections and overexpression of α-syn in selected non-DAeric structures, such as the serotonergic, noradrenergic and cholinergic systems, may be used to model a wider range of PD-related pathological changes. In particular, it will be interesting to explore the impact of α-syn pathology in cortical and/or basal forebrain cholinergic neurons on

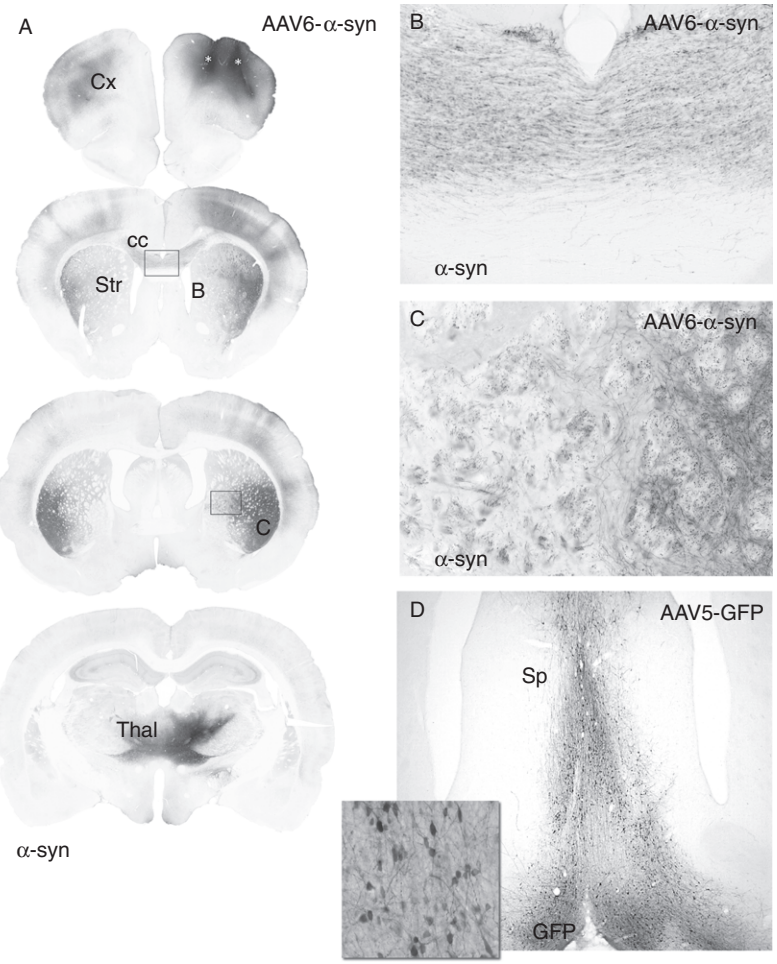

Fig. 6. Overexpression of human wild-type α-syn in neocortex (a–c) and septum (d). Unilateral injection of AAV6-α-syn in sensorimotor cortex results in a good spread of the vector, as illustrated by the strong expression of the protein in the cortical region around the injection site, and transport of α-syn along the corticostriatal and corticothalamic pathways (a). α-Syn-immunopositive fibers (visualized with an antibody specific for human α-syn) are found in the contralateral cortex (a), the corpus callosum (b), the ipsi- and contralateral striatum (a, c), and the ipsi- and contralateral thalamus (a). Panel d shows the efficient transduction of neurons in the septum-diagonal band area obtained with an AAV-GFP vector of serotype 5. Efficient transport of the GFP protein was observed along the septo-hippocampal pathway and its terminals in the hippocampus (not shown). Inset in panel d shows transduced medial septal neurons in higher magnification. CC, corpus callosum; Cx, cortex; Sp, Septum; Thal, thalamus.

cognitive functions and the effect of α-syn overexpression in locus coeruleus and raphe nuclei on non-motor aspects of behavior, such as mood and sleep.

In preliminary experiments (in part unpublished) we have seen that α-syn can be very effectively expressed in cortical projection neurons by intracortical injection of an AAV6-α-syn (Fig. 6a–c). Similarly, we have found that neurons located in the medial septum-diagonal band region or striatum can be efficiently transduced with AAV5 vectors (Fig. 6d–f), illustrating the

versatility of AAV vector delivery for modeling multiple aspects of neurodegenerative diseases *in vivo*. It should be kept in mind, however, that different serotypes of AAV vectors have different propensity to transduce neurons in different brain regions. Our own observations indicate that the AAV5 serotype is considerably more efficient in transducing rat cortical and hippocampal pyramidal neurons than the AAV2 serotype (Kirik and Bjorklund, 2003), and comparisons made after vector injection in the rat and monkey striatum indicate that the AAV2 vector is less efficient in transducing striatal neurons than either the AAV1, AAV5, or AAV8 serotypes (Davidson et al., 2000; Dodiya et al., 2010; Reimsnider et al., 2007). The volume of tissue transduced by single deposits of the AAV2 vector is also much less than that for the other serotypes tested due to more limited diffusion of AAV2-type vectors. The experience gained from studies in adult rats indicate that AAV2 vectors are quite efficient for use in SN, but for gene transfer to neurons in other brain regions other serotypes, such as 1, 5, 6 or 8, are preferred.

Technical aspects of AAV-mediated α-syn delivery

Successful application of AAV vectors for disease modeling depends on a number of factors related to the quality, purity, and titers of the vector preparations, as well as on the correct handling and use of the vectors in the stereotaxic surgery. The technical details of producing high-quality AAV vectors have been thoroughly reviewed earlier (Grimm et al., 1998; Ulusoy et al., 2008; Zolotukhin et al., 1999, 2002). In this section we will focus on a number of practical issues related to the application of AAV vectors in the brain.

Induction of PD-like pathology and DA neuron cell death by AAV-α-syn vector delivery depends on achieving a sufficient level of α-syn expression, in the absence of any non-specific toxicity. The expression level is primarily determined by the titer of the vector. In rats and mice, it is possible to obtain transduction of a major part of the SN-VTA region with a single 2–3 μl deposit, provided that the vector is of high titer. With low-titer vector preparations it is difficult to obtain transduction of more than a part of the SN, leading to partial effects. On the other hand, it is critical that the purification procedures in the production process are efficient in eliminating contaminations, such as excess empty capsid particles and other proteins that may cause non-specific toxic effects or immune reactions. In addition, the salt content of the concentrated vector solution should be within the physiological range, since high concentration of salts or other chemicals may impact the outcome. Since production, purification, and titration methods vary between laboratories, it is often difficult to compare results between centers, and even between different vector preparations generated at different occasions within the same production unit.

Selection of working titers and non-specific toxicity

Improvements in the techniques for AAV production and purification have made it possible to generate high-titer AAV vector stocks of high purity. Direct administration of such undiluted vector stocks in the brain may result in excessively high expression of the transgenic protein product, which might have unwanted consequences. This deserves careful consideration as optimal working dilution of vectors encoding for functional proteins (e.g., cytoplasmic enzymes or secreted factors), non-mammalian proteins, typically used as markers, or RNA interference constructs may well be different from each other. Even the most commonly used control constructs expressing the green fluorescent protein (GFP) marker protein may become toxic at high concentration. For example, we have recently reported a dose-dependent toxicity following AAV-mediated GFP expression, showing that the very high levels of GFP expressed after injection of very high-titer AAV vectors may induce significant

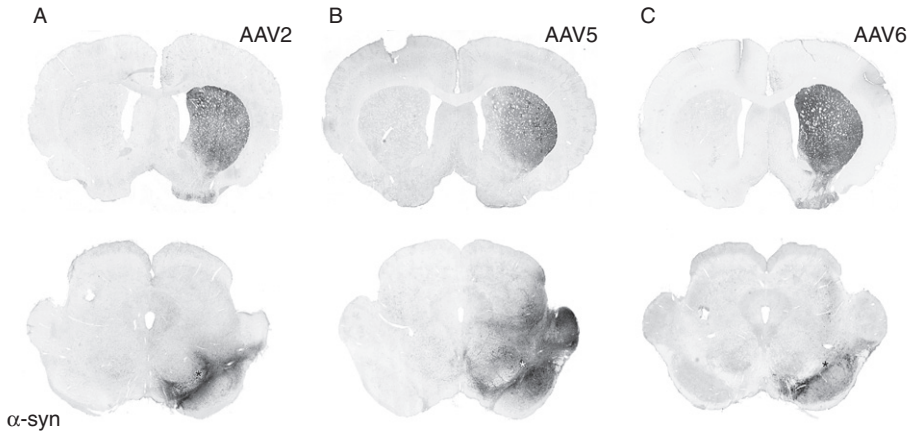

Fig. 7. Transduction of AAV-α-syn vectors of different capsid serotypes in the midbrain. The figure illustrates the expression of human wild-type α-syn in the midbrain (lower panels) and striatum (upper panels) delivered by AAV2 (a), AAV5 (b) and AAV6 (c) vectors injected to SN. Note that α-syn expression mediated by AAV2 and AAV6 vectors have distinct specificity for the cells located in SN and ventral tegmental area, whereas AAV5-mediated transgene expression has a diffuse pattern in the midbrain without any apparent specificity. All serotypes illustrated in this figure result in efficient transduction of DAergic neurons as assessed by the expression of α-syn in the striatal terminals. Asterisks denote the approximate site of vector injection.

non-specific damage and cell death (Ulusoy et al., 2009). In this experiment, injection of AAV5-GFP vectors at titers between 3×10^{12} and 3×10^{13} genome copies/ml in the SN resulted in >30% reduction in TH-positive cell numbers, which was associated with pronounced microglial activiation. Both the cell death and the microglial activity disappeared after diluting the vector stock to between 2×10^{10} and 3×10^{12} genome copies/ml while GFP expression remained robust and covered the whole nigra (Ulusoy et al., 2009). In light of these observations, we recommend that an optimal titer range for each vector construct is defined in order to achieve an expression of the transduced protein at sufficient levels, without causing non-specific toxicity. For each new batch we suggest that prior to use in actual experiments the vector is tested *in vivo* at three different dilutions (e.g., undiluted, 3× diluted and 10× diluted) in order to assess the optimal working titer for use in subsequent studies. In case of AAV-α-syn it is important to make sure that the vector is used at the right dilution, i.e., at a titer that allows wide-spread transduction in the SN and VTA without excessive spread to areas outside the SN-VTA region, as illustrated in Fig. 7 for three representative cases, given single intranigral deposits of AAV-α-syn of serotypes 2, 5, and 6. In most experiments an AAV-GFP vector is used to control for non-specific damage. Since GFP is potentially toxic to the cells, many investigators in the field agree that GFP protein does not provide an optimal control in viral vector experiments; however, a consensus on the use of a better alternative has not been reached yet.

Immune reactions

Although initial studies suggested that intracerebral injections of AAV vectors do not trigger any significant immune reactions, more recent work showed that a humoral immune response is activated against the AAV capsid proteins following injections in the central nervous system (CNS), and that re-administration of the same vector leads to a robust inflammatory response (Brockstedt et al., 1999; Chirmule et al., 2000; Manning et al., 1997;

Peden et al., 2004, 2009). As a consequence, the expression of the second vector injection is significantly reduced. The immune response directed toward the second vector deposit, however, is serotype specific and can be avoided by using a different AAV serotype for the second injection. In experiments where repeated injections of vectors are needed, therefore, one should select a vector of one serotype for the first injection (e.g., AAV2) and a different serotype vector for the second injection (e.g., AAV5).

Depending on the dose used, a transient glial reaction may be observed at the injection site even after the first administration, but if it occurs, it will subside over time. Nevertheless, the experimental design should include appropriate controls to rule out the possibility that this vector-dependent glial response can affect the results (Peden et al., 2004; Ulusoy et al., 2009). Empty viral particles should, in principle, be possible to use as a control for the immune response elicited by the capsid proteins. However, the detection of transduction efficiency of empty viral particles *in vivo* is problematic, and the use of empty virus does not make it possible to control for non-specific effects related to the expression of a protein in the target cell.

Assessment of levels of AAV-mediated protein expression

In most studies using AAV vectors, the outcome of the experiment requires comparison of different vector injection groups. For example, we usually compare our experimental groups with control groups at different time points in cohorts of animals that have received injections of the same vector batches. At the same time, we expect that the results obtained in one set of experiments should be possible to compare with the results obtained with the same vector constructs in other experiments performed either in our own laboratories or by investigators at other centers. This raises the important issue of how to compare the efficiency of AAV-mediated α-syn overexpression between different batches of vector. Since the level of AAV-mediated transgene expression is directly related to the number of viable AAV vector particles injected, the method used to assess AAV vector titers becomes important. Infectious AAV vector particles can be quantified by several means, such as determination of total particle numbers and amount of capsid protein, or by assessment of the number of functional genome copies in the vector batch. Currently, the most common method to quantify AAV vectors is to measure the quantity of genome copies per volume using quantitative polymerase chain reaction (qPCR). The values obtained with this technique are quite standardized and comparable. However, the vector preparations contain not only infective particles but also inactive (e.g., defective) viral particles and their relative proportion can differ significantly from one production site/method to another. Therefore, the genome copy titration method does not provide accurate information on the amount of the infectious AAV particles in a vector batch. Biological assays, such as the replication center assay (Shabram and Aguilar-Cordova, 2000; Yakobson et al., 1987) or the qPCR-based infectious titration assay (Rohr et al., 2005), provide a more meaningful infectious titer estimation, but on the other hand, they are poor predictors of *in vivo* efficacy and have an intrinsic variability due to experimental conditions such as the cell types used for the assay, cell confluency, and variability in *in vitro* infection efficiency.

A second possibility for defining the efficacy of a viral vector batch is the level of gene or protein expression *in vivo*. This can be done either by measuring the level of mRNA expressed by the vector or by determining the level of protein expression *in vivo*. Although there are good examples where the level of transgene expression is very well matched to the injected AAV genome titers (see, e.g., Bjorklund et al., 2009), it is clear that the correlation between the AAV vector titer (genome copies determined by qPCR) and the protein expression levels may not always be

linear. We have noted that intracerebral injections of vectors that differ two- to threefold in titer may not result in readily measureable differences in the protein product *in vivo*. Conversely, exact titer-matched vectors (by qPCR) may not lead to precisely the same expression levels in the brain. As the half-life of proteins can be very different, and especially when the expression is expected to induce neurotoxicity, leading to cell death and loss of the transgene product (as is the case for α-syn), one cannot rely only on the level of protein expression for assessment of the *in vivo* efficacy of the vector used.

Factors related to efficiency of targeting midbrain DA neurons

Since the transduction efficacy of different AAV vector serotypes differs between regions and between different animal species for the same target, it is important to carry out *in vivo* tests to identify most suited AAV serotype for a given *in vivo* application/target nucleus in the brain. In the rat brain, AAV vectors based on different capsid serotypes have been utilized. Figure 7 illustrates the expression of human wild-type α-syn obtained in the rat ventral midbrain after injection of three different AAV serotypes: AAV2 (Fig. 7a), AAV5 (Fig. 7b), and AAV6 (Fig. 7c). Note also that the projections in the striatal target areas are filled with the transgene product, showing that the α-syn protein is efficiently transported along the nigrostriatal axons.

A major variability factor in AAV-mediated gene delivery is the targeting accuracy in the surgical procedure. The delivery of the vector to the appropriate site using a vector batch with suitable titer should result in transduction of the whole SN and in α-syn expression throughout the striatal axonal terminal network. Although, in our hands, the success rate for correct targeting of the SN-VTA area in rats is high (>90%), the situation is different in mice. Targeting the ventral midbrain in the mouse can be difficult both due to the fact that the brain size is smaller than the rat and the head positioning in the stereotaxic frame is less accurate. These factors lead to a higher variability (relative to the size of the nucleus) in the position of the tip of the needle or the glass

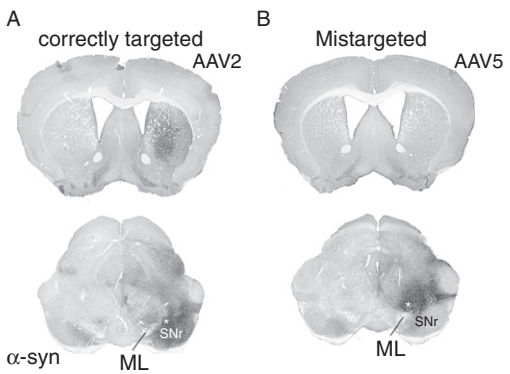

Fig. 8. AAV-mediated expression of human α-syn and targeting in mouse SN. Accurate targeting of the vector injection in the SN is critical, and also more challenging, when applied in mice. The figure gives examples of nigral AAV-α-syn injections in two different mice. The injection in panel a is correctly placed, resulting in a robust nigral transduction (lower panel) and expression at the terminal level (upper panel). Panel b illustrates a mistargeted vector injection due to the misplacement of the glass capillary tip that failed to transduce the nigral DA neurons (lower panel) and therefore the striatal projections (upper panel). In this latter case the medial lemniscus has served as a barrier for diffusion of the vector ventrally toward the SN. Asterisks mark the approximate site of vector injection. ML, medial lemniscus; SNr, SN pars reticulata.

capillary used for the delivery. In cases where the injection is misplaced dorsally, the surrounding white matter tracks (the medial lemniscus in particular) can act as a physical barrier for the vector solution to diffuse into the SN and lead to partial or no transduction of DA neurons, while other populations in the thalamus express the trangene (compare Fig. 8a with b). Thus, not only accurate coordinates need to be determined but also precise microscope-aided surgical techniques should be utilized and post-mortem histological analysis of gene transduction should be used as means to screen the variability in targeting AAV vectors to the area of interest, in order to allow exclusion of injection failures in the experimental groups.

Concluding remarks

The viral vector model of α-syn overexpression has been slow in gaining wide acceptance. One reason for this is probably that models that involve more precise stereotaxic surgery are cumbersome to apply in routine screening work. In addition, the access to high-quality AAV vectors has been a limiting factor, and the work with viral vectors for *in vivo* delivery in the brain has so far been a technique that has been developed in a limited number of specialized laboratories. The situation is changing, however. Standardized and validated AAV vectors are now readily available commercially for routine use, and the basic procedures for AAV-α-syn delivery to the SN in mice and rats are now well worked out. The model offers unique opportunities to induce and study the development of PD-like functional and neurodegenerative changes in midbrain DA neurons, in a wide variety of species, including both rats and primates, and is so far the only model that replicates the profound, progressive α-syn-related neuropathology in nigrostriatal neurons that develops over time in PD patients.

The progressive feature of this model provides an interesting new tool for the study neuroprotective and disease-modifying therapeutic inventions. As discussed above, models replicating the progressive α-syn-related changes characteristic for human PD will be an essential complement to the toxin-based models currently used in such studies. AAV-α-syn delivery offers the opportunity to overexpress α-syn not only in the nigrostriatal projection neurons but also in the possibility to target other, non-DAergic systems in the brain. This property allows us to replicate selected aspects of the more widespread synucleinopathy seen in more advanced cases of PD, or so-called PD-plus syndromes. For good and consistent results in the AAV-α-syn model, however, it is necessary to pay attention to a number of technical issues, discussed above. In particular, it is essential to check the quality (and possible toxicity) of the vector batches prior to use, and to make separate tests to select the optimal working titers of both the AAV-α-syn and the control vectors. In mice, in particular, precise and reproducible targeting of the vector deposits in the SN has to be ensured and checked in post-mortem immunostained sections.

A presently unsolved shortcoming of the AAV-α-syn model is that the extent of behavioral deficits is quite variable. Only a fraction of the AAV-α-syn-treated animals show significant long-term motor deficits (usually in the order of 25% in AAV-α-syn-treated rats, and even less in mice). This limits the use of the model for studies where functional recovery or functional sparing is the main focus. In AAV-α-syn-treated rats the magnitude of DA neuron cell loss is on average about 50–60%, which we know is a borderline for induction of significant impairments in the standard drug-induced and spontaneous motor tests. Efforts are now being made to combine α-syn overexpression with "a second hit" that will increase α-syn-mediated neurodegeneration and induce more robust behavioral deficits. Such second hits may include the use of more toxic variants of α-syn or the induction of a more prominent or long-lasting inflammatory response. Application of AAV-α-syn overexpression in transgenic mice may also offer interesting opportunities for more refined disease modeling.

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