



Unbiased Screens for Modifiers of Alpha-Synuclein Toxicity

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Abstract

Purpose of Review We provide an overview about unbiased screens to identify modifiers of alpha-synuclein (α Syn)-induced toxicity, present the models and the libraries that have been used for screening, and describe how hits from primary screens were selected and validated.

Recent Findings Screens can be classified as either genetic or chemical compound modifier screens, but a few screens do not fit this classification. Most screens addressing α Syn-induced toxicity, including genome-wide overexpressing and deletion, were performed in yeast. More recently, newer methods such as CRISPR-Cas9 became available and were used for screening purposes. Paradoxically, given that α Syn-induced toxicity plays a role in neurological diseases, there is a shortage of human cell-based models for screening. Moreover, most screens used mutant or fluorescently tagged forms of α Syn and only very few screens investigated wild-type α Syn. Particularly, no genome-wide α Syn toxicity screen in human dopaminergic neurons has been published so far.

Summary Most unbiased screens for modifiers of α Syn toxicity were performed in yeast, and there is a lack of screens performed in human and particularly dopaminergic cells.

Keywords Screen · Alpha-synuclein · Toxicity · Parkinson's disease

Introduction

Alpha-synuclein (α Syn) is a small 140 amino acid long protein whose physiological function is not fully understood in spite of a myriad of publications on this topic. It is thought that α Syn carries out many functions [1••]. Pathological aggregates of α Syn in the brain define a group of neurodegenerative disorders termed synucleinopathies [2]. The term synucleinopathies emerged when α Syn was identified as being the main component of Lewy bodies, which are intracellular inclusions that are the histopathological hallmark of

Parkinson's disease (PD) [3], and α Syn was also identified in cellular deposits in multiple system atrophy (MSA) and dementia with Lewy bodies [4]. PD is the most common synucleinopathy and the second most common neurodegenerative disorder after Alzheimer's disease [5]. The importance of α Syn in the etiology of PD is emphasized by genetic findings. Mutations [6] and multiplications [7, 8] of *SNCA*, the gene coding for α Syn, cause autosomal-dominantly inherited Parkinson's syndromes. Moreover, results from genome-wide association studies reproducibly found that single-nucleotide polymorphisms in *SNCA* are risk factors for the sporadic form of PD [9]. Like other neurodegenerative disorders, synucleinopathies are progressive, and no treatment with proven disease-modifying efficacy exists [10]. In PD, motor symptoms are caused by the death of dopaminergic neurons in the *substantia nigra pars compacta* [11]. The main therapeutic treatments for PD and related disorders are dopamine replacement or the inhibition of dopamine degradation. These treatments are not curative but only alleviate symptoms [12]. Additionally, there are no effective therapeutic options available for many non-motor symptoms [13]. Furthermore, in other synucleinopathies, the dopaminergic therapy is much less effective and side effects limit its usability [14, 15]. Therefore, there is an unmet medical need for therapeutic

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options addressing the pathophysiology of neurodegenerative synucleinopathies. α Syn is an aggregation-prone protein and even though the exact nature of the toxic α Syn species is still unknown, it is a well-established concept that oligomeric intermediates between α Syn monomers and larger aggregates, as found in Lewy bodies, confer toxicity [16]. Previously, hypothesis-based approaches led to the identification of mechanisms involved in degradation of α Syn aggregates (e.g., chaperone-mediated autophagy or macroautophagy) and the identification of compounds with anti-aggregatory properties, such as (-)epigallocatechin-3-gallate (EGCG). EGCG was recently investigated in a clinical trial in patients with MSA [17]. The finding that α Syn enters the extracellular space and is taken up by neighboring cells thereby leading to a cell-to-cell spread of the pathology throughout the brain led to the development of neutralizing antibodies directed against α Syn, which are currently investigated in clinical trials (e.g., NCT03100149, NCT03318523). Thus, targeting α Syn, its degradation, aggregation, post-translational modifications, or the mechanisms involved in cell-to-cell spreading are rational approaches in the development of a disease modifying therapy for neurodegenerative synucleinopathies. However, as long as there is no proven disease-modifying therapy available, there is still need for the discovery of new approaches to slow or halt disease progression. Unbiased, hypothesis-free screens for modifiers of α Syn toxicity are a possibility to identify such new ways to counteract the pathological process in neurodegenerative synucleinopathies. Modifier screens for α Syn can be roughly divided into two categories: genetic modifier screens and compound modifier screens. Every screening approach has its advantages and disadvantages, which will be discussed giving examples of published screens in α Syn toxicity models. We focused on unbiased screens for modifiers of α Syn toxicity. However, it should be mentioned that many screens targeted a desired mechanism of action such as α Syn aggregation or its degradation as readout in the primary screen. Many of these screens could confirm a protective effect on α Syn-induced toxicity in the follow-up phase. Table 2 shows an overview of these screens.

Yeast as Model Organism

Most of the genetic modifier screens were performed in yeast. These are small eukaryotic organisms, and many aspects of cell biology are well conserved from yeast to mammals. Human orthologs exist for roughly one third of the genes in the yeast genome [23], and yeast cells are suitable for genetic editing. Genome-wide collections of yeast strains containing knockouts of single genes are available in large libraries (e.g., Yeast Knockdown Collection). Currently, about 21,000 mutant strains for over 6000 genes are available [24]. Additionally, overexpression plasmid libraries such as the

Yeasts FLEXGene open reading frame (ORF) library consisting of 5500 yeast ORFs are available [25]. These libraries allow genome-wide deletion or overexpression screens. Even though yeast does not encode α Syn, yeast cells expressing human α Syn under the control of the galactose promoter show growth inhibition upon addition of galactose [26].

Genetic Modifier Screens

Table 1 presents an overview of modifier screens of α Syn-induced toxicity. One of the first screens was published in 2003 [27]. The authors determined cell growth of 4850 yeast deletion strains overexpressing human α Syn. Eighty-six of the deletions reproducibly increased toxicity of human α Syn. Of the genes that could be assigned to a function, roughly one third was involved in lipid metabolism and vesicular transport [27]. This screen was simultaneously performed in yeast expressing a human mutant huntingtin fragment. Only very little overlap between the two screens was found, suggesting that the hits were specific to the biology of the two different proteins [27].

Another genetic modifier screen was performed in a yeast strain expressing human α Syn coupled to yellow fluorescent protein (YFP) under a galactose promoter [28]. The authors screened a library of 3000 randomly selected ORFs also under the control of the galactose promoter and monitored the growth rate. Thirty-four genes suppressed and 20 enhanced α Syn-induced toxicity. Many suppressors of α Syn toxicity were involved in ER-Golgi trafficking, and the strongest suppressor of α Syn toxicity was Rab1. This finding was validated in both *Caenorhabditis elegans* and in *Drosophila melanogaster*. Like yeast, *C. elegans* does not have α Syn, but expression of human α Syn under the DAT1-promoter in dopaminergic neurons leads to degeneration of this cell population [42]. Expression of Rab1 in *C. elegans* rescued α Syn-induced cell death of dopaminergic neurons. They further investigated the effects of Rab1 in primary rat midbrain cultures transduced with lentiviral vectors expressing human A53T-mutated α Syn, which leads to a marked decrease of dopaminergic neurons. Rab1 expression rescued cell death of dopaminergic neurons in this model, demonstrating that results obtained in yeast can be translated to in vivo and mammalian cell models [28].

Investigating the transcriptional response to α Syn overexpression is another promising avenue for finding modifiers. In a paper by Yeager-Lotem et al., a method to compare data from genetic screens to expression analyses in response to various cellular stressors was published. The authors show that genetic hit lists overlap poorly with lists of differentially expressed genes [29]. They concluded that genetic screens identify in general genes involved in regulatory processes, whereas

Table 1 Modifier screens for αSyn toxicity

Genetic screens	Library	Read-out	Follow-up	Result	Reference
Model organism Yeast overexpressing human WT or A53T-mutated αSyn	Selection of 4850 deletion mutation strands	Cell growth/deterioration of growth inhibition by αSyn expression	Replication to eliminate false-positives	18 genes functionally related to lipid or vesicle-transport mechanism aggravated αSyn toxicity	[27]
Yeast expressing αSyn-YFP under a galactose promoter	Overexpression of 3000 randomly selected ORFs from a genome-wide collection	Cell growth/amelioration of growth inhibition by αSyn expression	Validation in drosophila, <i>C. elegans</i> and rat midbrain primary cultures	YPT1 (ortholog of Rab1) expression mitigates αSyn-induced cell death, Rab1 involved in ER-Golgi transport	[28]
Yeast expressing αSyn-YFP under a galactose promoter (same model as used in [28])	5500 (85% of the genome) overexpressing yeast strains	Cell growth/amelioration of growth inhibition by αSyn expression	Investigation of synergistic effect of 1 hit (YPK9) with YPT1 (hit from previous screen, Cooper et al., 2006) Confirmation in <i>C. elegans</i> expressing αSyn under DAT1-promoter Confirmation in rat midbrain primary cultures	YPK9 (yeast ortholog of ATP13A2/PARK9) protects from αSyn-induced toxicity synergistically with YPT1, both converging on vesicular transport Expression of <i>c. elegans</i> ortholog of ATP13A2 protects DA neurons from αSyn-induced cell death	[29, 30]
Yeast expressing αSyn-YFP under a galactose promoter	Combination of genetic hits with transcriptional data	Computational approach to identify connectors between the two datasets		Identification of 106 proteins that were linked between genetic hits and differentially expressed genes, not identified by either of the individual approaches	[29]
Yeast expressing A30P mutated αSyn, challenged with H ₂ O ₂	High copy yeast genomic library	Cell growth after additional challenging with H ₂ O ₂	Testing in WT-αSyn and A53T-αSyn yeast	YPP1 protects A30P-αSyn, but not WT-αSyn or A53T-αSyn expressing yeast from ROS production	[31]
Yeast expressing WT- αSyn, challenged with H ₂ O ₂	High copy yeast genomic library	Cell growth after additional challenging with H ₂ O ₂	Validation of hits with deletion strains Overexpression of ENT3 (strongest suppressor) in αSyn-GFP yeast model	Identification of genes involved in ROS production ENT3 led to intracellular redistribution of αSyn, mediates transport of αSyn to the vacuole (yeast equivalent to lysosome) for degradation	[32]
Yeast expressing αSyn-YFP under a galactose promoter (same model as used in Cooper et al., 2006)	CRISPR-Cas9 based transcription factors for randomized perturbation of transcriptional networks	Cell growth	Expression analysis Validation of four top hits (DJ-1, ALS2, GGA1, and DNAJB1) in SH-SY5Y cells expressing human WT αSyn	Identification of 114 differentially expressed genes that were not found by overexpression or knockdown screens DJ-1, ALS2, GGA1, and DNAJB1 also protected human neuroblastoma (SH-SY5Y) cells from αSyn-induced toxicity	[33•]
Compound screens					
Model organism Yeast expressing human WT-αSyn plus challenge with FeCl ₃	Library 10,022 diverse compounds	Readout Cell growth	Follow-up Dose-response investigation of two hit compounds	Result Protection from αSyn-induced growth inhibition by quercetin and EGCG	Reference [34]
Yeast expressing human WT-αSyn-GFP	Natural products	Cell growth	Fractionation of protective green tea extract	EGCG was isolated and confirmed as being protective against αSyn toxicity	[35]
Yeast expressing αSyn-YFP	115,000 compounds	Cell growth	Dose-response investigation Confirmation that four top hits did not change αSyn expression Testing in <i>E. elegans</i> expressing αSyn in DA neurons and in rat primary	Identification of four 1,2,3, 4,-tetrahydrochionones All four protected in <i>C. elegans</i> Three also protected in rat primary midbrain cultures	[36]

Table 1 (continued)

		midbrain cultures			
Human osteoblastoma cells (U2-OS cell) challenged with C-terminally truncated α Syn (α Syn119)	Proof of concept	Leakage of DiBAC4(3) as fluorescent agent as measure for toxic pore formation by α Syn oligomers		Preincubation of α Syn119 with four known anti-aggregatory compounds reduced their ability to form toxic pores	[37]
LUHMES cells overexpressing human WT- α Syn	1600 FDA approved drugs	Cell death measured by PI incorporation		Identification of PDE1 inhibition as mechanism to protect human dopaminergic neurons against α Syn-induced toxicity. PDE1 inhibition also protected dopaminergic neurons from α Syn-induced toxicity in the SN of mice	[38••]
Other α Syn toxicity modifier screens					
Model organism	Library	Readout	Follow-up	Result	Reference
Yeast expressing α Syn-YFP	Library of 50,000,000 cyclic peptides (CP)	Cell growth	Picking of 96 random clones, each expressing one CP	Identification of cyclic peptides that protected from α Syn-induced toxicity in yeast and in dopaminergic neurons on <i>C. elegans</i>	[39]
<i>E. coli</i> expressing WT α Syn	~200,000 decapeptides based on the α Syn sequence between AA 45 and 54 with exchanges of 2–6 AA at each position	Survival of <i>e. coli</i> colonies Subtle changes in growth rates	Investigation of vesicle trafficking as possible mechanism Confirmation of two top hits in <i>C. elegans</i> expressing α Syn-GFP in DA neurons	Mechanism independent of membrane trafficking	
Expression levels of α Syn in human SK-N-MC neuroblastoma cells	1126 approved drugs	Expression level of SNCA mRNA	Six passaging steps were performed to amplify subtle differences in the growth rate of <i>E. coli</i> colonies expressing α Syn and the different decapeptides	Initial survival of 200 colonies After six passages one peptide expressing colony showed the best survival Identification of a decapeptide that interacted with α Syn and reduced α Syn-induced growth inhibition	[40]
			Quantification of α Syn protein levels with ELISA Overexpression of β 2-receptors in SK-N-MC neuroblastoma cells Testing of β 2-agonists in iPSC cells from patients with α Syn triplications	β 2-antagonist led to increased α Syn mRNA and protein levels β 2-receptor overexpression reduced mitochondrial superoxide production as measure for toxicity	[41•]

AA amino acid, α Syn alpha-synuclein, DA dopamine, EGCG (-) (-)epigallocatechin-3-gallate, ELISA enzyme-linked immunosorbent assay, GFP green fluorescent protein, iPSC induced pluripotent stem cells, PI propidium iodide, RNAi RNA interference, SN substantia nigra, WT wild-type, YFP yellow fluorescent protein

expression analyses rather identify metabolic aspects of cellular processes. The authors then developed a computational approach (ResponseNet [43, 44]) to identify cellular processes that bridge the gap between these two approaches. They then applied this approach to compare the hits of the same α Syn-YFP yeast model as used in the previous screen [28] but expanded to encompass 5500 genes (85% of yeast genome) with mRNA transcriptional profiles in this model. They again found little overlap between genetic hits and genes whose expression in yeast cells was altered due to α Syn overexpression [29]. Their computational approach then identified 44% of the genetic hits to be linked to 27% of differentially expressed genes by 106 intermediary proteins that had not been previously discovered by any of the approaches. Moreover, some of these proteins were part of pathways that were eligible for pharmacological modulation [29]. This demonstrates the necessity of using computational approaches to combine large datasets and broaden the understanding of cellular processes beyond the scope of a single dataset. In the same primary overexpression screen, also the ortholog to human ATP13A2 or PARK9 was identified as being protective against α Syn toxicity in yeast [30]. The authors confirmed their finding in *C. elegans* expressing α Syn-GFP in body wall cells and showed that knockdown of the *C. elegans* ortholog to ATP13A2 led to enhanced misfolding of α Syn. Furthermore, they showed that co-expressing of human ATP13A2 protected dopaminergic neurons in primary rat midbrain cultures from degeneration induced by lentiviral transduction-mediated overexpression of human A53T- α Syn [30]. This further demonstrates that findings obtained in yeast primary screens may translate to mammalian models.

Other genome-wide overexpression screens in yeast overexpressing human wild-type or human A30P- α Syn identified genes that blocked reactive oxygen species (ROS) production and were protective against α Syn-induced toxicity [31, 32]. More recently, another genetic modifier screen using α Syn-YFP expressed under a galactose promoter was carried out using randomized CRISPR-Cas9-based transcription factors to perturb expression profiles [33•]. A transcription analysis was performed to determine which genes were differentially regulated and suppressed α Syn-induced toxicity. They identified 114 genes that were differentially expressed of which 93% were not previously identified in overexpression or knockdown screens [33•]. Many of the top hits were related to chaperone function. Four of these genes (DJ-1, ALS2, GGA1, and DNAJB1) were validated in human neuroblastoma cells (SH-SY5Y) [33•]. These cells were differentiated into a dopaminergic phenotype, in which overexpression of wild-type α Syn leads to degeneration [45]. The expression of the four genes reduced α Syn-induced toxicity [33•]. This shows that the more global modulation of transcriptional networks can lead to identification of genes that were not

identified by single-gene knockdown or overexpression approaches.

Compound Modifier Screens

One compound screen was performed in yeast cells expressing human wild-type α Syn. Since the authors did not observe toxicity from α Syn alone, they additionally challenged the cell with FeCl_3 , which led to growth inhibition of yeast. They screened a library of 10,022 diverse compounds and found that quercetin and (-)epigallocatechin-3-gallate (EGCG) reduced α Syn-induced growth inhibition [34]. Since their work focused on presenting the model, not much information was given about the hit selection and follow-up. Even though the neuroprotective properties of EGCG were known, the authors demonstrated the feasibility of large compound screens in transgenic yeast. Interestingly, another group also found EGCG, when they screened a library of natural products. They found that green-tea extract was protective against α Syn-induced growth inhibition. In the follow-up, they isolated multiple compounds from the green-tea extract and showed that several compounds, including EGCG, could protect yeast from α Syn-induced toxicity [35]. This screen demonstrated the feasibility of the screening of natural products to identify compounds that protect against α Syn-induced toxicity.

In another screen, 115,000 compounds from various collections were tested in the yeast α Syn-YFP model at 15 μM [36]. As follow-up, a selection of hits was tested in a dose-response manner from 0.1 to 10 μM in order to identify the most potent compounds. As top hits, the authors identified four 1,2,3,4,-tetrahydrochionolinones. They confirmed that the compounds did not change α Syn expression levels. Furthermore, as in previous screens, the hits were validated in *C. elegans* expressing α Syn in dopaminergic cells as well as in rat primary neurons transduced with A53T- α Syn. All four compounds protected in *C. elegans*, and three of them also led to restoration of the number of dopaminergic neurons in rat primary cultures [36]. In another screen, the hypothesized pore-formation ability of α Syn oligomers was screened. An assay was developed which monitored pore formation upon treatment of human osteoblastoma cells with C-terminally truncated α Syn (α Syn119) by measuring leakage of a fluorescent agent ((bis-(1,3-dibutylbarbituric acid)-trimethine oxonol; DiBAC4(3)) [37]. While the authors did not perform a high-throughput screen, they showed as proof of concept that preincubation of α Syn119 with known anti-aggregatory compounds (e.g., EGCG) reduced pore formation as measure for toxicity [37]. Their model was only tested by preincubation of compounds with α Syn119 before addition to cells. It is therefore unclear whether compounds could be identified that would reduce also intracellular aggregation.

We recently performed a compound screen in LUHMES cells, derived from human embryonic midbrain that were immortalized by cloning-in v-myc under control of a tet-off system [46]. After addition of tetracycline to the culture medium, the cells can be differentiated to human postmitotic midbrain neurons [47]. In these cells, moderate overexpression of human wild-type α Syn leads to half-maximal cell death [48]. We screened 1600 FDA-approved drugs in this model and identified an unspecific PDE inhibitor as protective against α Syn-induced toxicity. We then investigated more specific PDE inhibitors and found that specific inhibition of PDE1 with vinpocetine protected against α Syn-induced toxicity. This finding was confirmed by RNAi in the LUHMES cell model. Inhibition of PDE1 as potential therapeutic target was further validated in a mouse model, where the PDE1 inhibitor vinpocetine protected dopaminergic neurons in the substantia nigra pars compacta from degeneration induced by local overexpression of human wild-type α Syn after injection of adenovirus-associated viral vectors [38••]. Therefore, we could show that compound screens in human dopaminergic cell lines are feasible, and findings obtained in our cell model also translate to mammalian in vivo models.

Other α Syn Toxicity Modifier Screens

One screen published in 2009 is neither a genetic modifier screen nor a compound screen in the general sense. The authors used a PCR cloning technique to express randomized inserts in yeast leading to the expression of cyclic peptides within the cells. They applied this technique to the yeast α Syn-YFP model and produced a library of roughly 50,000,000 octamer cyclic peptides (CPs) of which roughly 70% were expressed in the cells. Many of these CPs led to better survival of yeast expressing α Syn-YFP, and 96 clones were randomly chosen for validation. Of these, 31 were reproducibly protective and the two best hits were chosen for validation, which were then confirmed to be protective against α Syn-induced toxicity in *C. elegans*. Moreover, they excluded an effect of the protective CPs on vesicle trafficking, and therefore, supposed that the CPs must act independently of already identified pathways [39]. The possibility to screen millions of different genetically engineered peptides shows the value of their approach. However, they were not able to identify a distinct mechanism of action for their best CPs demonstrating that target deconvolution in screens investigating libraries with items of unknown mechanisms of action can be extremely challenging, especially when the primary readout is rather unspecific such as cytotoxicity.

Another group hypothesized that peptides interacting with α Syn could reduce toxicity [40]. Since most known α Syn mutations are located between amino acids 46 and 53, they reasoned that this segment of α Syn might be most relevant for α Syn-

induced toxicity. They overexpressed wild-type α Syn in *Escherichia coli*, which led to growth inhibition. They then randomly expressed peptides of 10 amino acids (AAs) length corresponding to residues 45 to 54 of α Syn, presuming that these might interact with α Syn and reduce toxicity. Using a mutational approach, they randomly exchanged 2 to 6 amino acids between position 45 and 54, generating a total of 209,952 decapeptides. They then screened *E. coli* expressing α Syn together with the decapeptides, and only 200 colonies survived, suggesting that only these 200 decapeptides could reduce α Syn toxicity. After six passaging steps, they identified one decapeptide that was most efficient in reducing α Syn-induced toxicity. They confirmed that this decapeptide was inhibiting α Syn aggregation in a thioflavin T assay [40]. This screen demonstrated that peptides designed to interact with α Syn can prevent α Syn toxicity.

Another recent screen followed a totally different approach and gained much attention due to its possible clinical implication [41•]. The authors did not overexpress α Syn or looked for toxicity in the primary screen. Instead, they supposed that high α Syn levels are a risk factor for dopaminergic cell death per se, which is confirmed by observations in patients with *SNCA* multiplications leading to autosomal dominant PD. Therefore, they treated human SK-N-MC neuroblastoma cells with 1126 small molecule compounds from different collections and investigated changes in endogenous *SNCA* mRNA levels. They identified 41 compounds that changed expression levels of *SNCA*. These and six related compounds (hit expansion) were confirmed to also affect α Syn protein levels. β 2-receptor agonists were overrepresented, and in follow-up experiments, they showed that transfection of their cell line with β 2-receptors reduced α Syn levels, whereas treatment with β 2-receptor blockers increased α Syn levels. Moreover, they showed that β 2-receptor agonists reduced α Syn expression in induced pluripotent stem cells from patients with a *SNCA* triplication and reduced mitochondrial superoxide production [41•]. The finding is particularly interesting, because β 2-receptor blockers are first-line therapy for essential tremor, one of the most important differential diagnoses of early tremor-dominant Parkinsonism. If it holds true that β 2-receptor blockage can increase α Syn expression in patients, one would have to be much more cautious in prescribing β 2-receptor blockers in patients with tremor.

Screens That Primarily Addressed α Syn Oligomerization

Many screens addressing the neurobiology of α Syn have been performed in models in which a different phenotypic readout (e.g., α Syn oligomerization or aggregation) rather than α Syn-induced toxicity was used. While the current review focuses primarily on screens that measured cytotoxicity as readout (e.g., growth inhibition or cell death), Table 2 provides

examples of other screening modalities, which were then followed up by investigating α Syn toxicity. Other screens that did not look into modulation of α Syn-induced toxicity in primary or secondary analyses were not considered at all in this review.

Conclusion

Unbiased genetic screens for modifiers of α Syn-induced toxicity can deepen the understanding of the pathophysiological processes involved in synucleinopathies by identifying pathways that had not been previously identified by hypothesis-driven approaches (e.g., ER-Golgi trafficking) [28]. These cellular processes are potential therapeutic targets for disease-modifying therapies for synucleinopathies. In a similar way, screens with compounds of known mechanisms of action can identify potential pharmacological targets for neuroprotective therapies. Screens with compounds of unknown mechanism, however, can reveal lead structures for the design of the new drugs for these diseases.

Post-screening Phase and Target Deconvolution

In most screens, the findings from the primary screen were followed by a validation phase, in which the primary observations were validated in different experimental settings or in more complex models than the ones that had been used in the primary screen. Additionally, for compound screens or screens for modifiers of α Syn toxicity with unknown mechanisms, the post-screening phase needs to include a target-deconvolution or target-identification step. When the mechanism of actions (MOA) of hits are identified, knockdown experiments can be used to confirm the MOA [38•]. However, especially when the MOA of the screened modifiers is unknown, target deconvolution can be difficult [39]. One possibility is transcriptional profiling. The transcriptional changes within the cells in the presence of individual compounds can reveal information about the mechanism of action [36, 49]. Other methods are target-based approaches testing possible targets in a hypothesis-driven manner [36, 50], or affinity-based assays, in which the interaction between the compounds and different proteins is determined [50]. Figure 1 illustrates the duration needed for therapy development, the effort needed to identify the mechanism of action, and the degree of innovation that is relevant to develop intellectual property.

Yeast as a Model System for Synucleinopathies

Most α Syn screens reported to date were performed in yeast. This fact is surprising since the yeast genome comprises roughly 6000 genes [20] and does not encode an ortholog of

α Syn. Nevertheless, researchers have taken advantage of the fact that α Syn is toxic in yeast to devise survival screens in this powerful experimental organism. There is a human ortholog for roughly one third of the yeast genes [23], and many findings in yeast proved to be also valid in mammals. This was demonstrated by showing that five out of six genes identified in an overexpression yeast screen could be validated in rat primary neurons [30]. In spite of these encouraging results, it is likely that some of the cellular processes involved in the neurobiology of α Syn cannot be recapitulated in this simple model. These shortcomings of the yeast model notwithstanding, our literature search did not identify a single genetic screen addressing toxicity induced by wild-type α Syn in human cells. We have performed a compound screen in a human cell line that can be differentiated into dopaminergic neurons [47]. In this model, dopaminergic neurons show half-maximal cell death over a course of six days upon moderate overexpression of human wild-type α Syn [38•, 48]. This screen demonstrates the feasibility of large screens in human dopaminergic neurons, which are physiologically relevant to the cells that are affected in human patients and are responsible for the motor symptoms of PD. This model could be used for a genetic screen. Such a screen would be of great interest to uncover novel cellular processes involved in α Syn biology.

Shortcomings of Previously Reported Screens

In most previously reported screens, the cells were challenged with α Syn fused to a fluorescent protein such as GFP or YFP. However, α Syn is a small protein of 14 kDa, and it seems unlikely that its functions are not altered by coupling such a large tag to it. The ability to directly monitor the oligomerization process is appealing. However, it was recently questioned whether the dimerization process of α Syn coupled to halves of GFP was actually representing the pathophysiological process of oligomerization [51]. Other models study the neurobiology of α Syn containing rare genetic mutants leading to autosomal dominant PD. These are much more toxic to cells than wild-type α Syn [48], suggesting that other cellular processes may be involved in cell death induced by mutant α Syn compared to the wild-type protein. Furthermore, wild-type α Syn is responsible for the vast majority of sporadic synucleinopathies, and thus, findings with mutant forms of α Syn might have limited significance.

Many biased α Syn screens have been reported, monitoring α Syn aggregation, degradation by the proteasome [52], chaperone-mediated autophagy, or macroautophagy. Such screens are based on hypotheses that are not necessarily true, since we understand so little of α Syn function and pathology. Even though we did not focus on these screens in the current review, examples are presented in Table 2.

Table 2 Screens that used α Syn-induced toxicity as follow-up but not for primary screening

Model	Library	Read-out	Follow-up	Result	Reference
Screens with alpha-synuclein aggregation as primary readout					
<i>C. elegans</i> expressing α Syn-GFP in body wall cells	RNAi of 868 genes associated with PD, involved in protein degradation and interactors of these	Aggregation of α Syn-GFP in body wall cells	Overexpression of seven hits, survival of DA neurons in <i>C. elegans</i>	Two genes protected DA neurons from α Syn toxicity, one (Erv29p) involved in ER-Golgi transport	[18]
Human H4 neuroglioma cells expressing N-terminal GFP and C-terminal GFP halves linked to α Syn	Lentiviral mediated RNAi library of 1387 genes involved in membrane trafficking and signal transduction related pathways	Bimolecular fluorescence complementation as measure for α Syn oligomerization	Cell-to-cell trafficking by mixture of cells expressing either half of GFP coupled to α Syn H4 cells with transient overexpression of selected hits	Silencing of four Rab proteins and five kinases or signal transduction proteins modulated α Syn oligomerization Silencing of RAB8B, TAB13, and SYTL5 increased cell-to-cell transfer of α Syn Overexpression of selected hits protected from α Syn-induced toxicity measured by LDH release	[19]
Screens with a distinct mechanism of action as readout					
HEK293 cells	2750 compounds (Lopac: 1261, John Hopkins Chemical Compound Library: 1514)	FACS with fluorescent proteasome activity reporter	Confirmation in other cell lines Knockdown of p38 α with siRNAs Effect of p38 inhibition on α Syn aggregation as measured by BiFC Effect of p38 inhibition on endogenous α Syn levels in primary mouse neurons	11 compounds increased proteasome activity Identification of p38 MAPK kinase inhibitors Reduction of endogenous α Syn level in primary mouse neurons by p38 inhibition	[20]
Screens with aggregation of a different protein in the primary screen					
Fluorescently labeled prion protein	20,000 chemical diverse compounds	SIFT	Prion and α Syn oligomer formation of a focused library of 150 DPP Validation in TG-A30P- α Syn mice, Rotarod test, immune-histochemistry	Identification of di-phenyl-pyrazole (DPP) as lead structure Identification of Anle138b (one DPP) Anle138b improved motor performance, increased survival, and reduced α Syn deposits in TG-A30P- α Syn Group of six 8-hydroxyquinolines (8-OHQ) improved growth two protective compounds also protected yeast and dopaminergic neurons in <i>C. elegans</i> from α Syn-induced toxicity	[21]
Yeast expressing TDP-43-GFP under galactose promoter	200,000 compounds from various sources	Growth rate	Validation of two protective compounds in yeast expressing α Syn Validation of two protective compounds in <i>C. elegans</i> expressing α Syn in dopaminergic cells		[22]

α Syn alpha-synuclein, *BiFC* bimolecular fluorescence complementation, *DPP* of di-phenyl-pyrazole, *GFP* green fluorescent protein, *RNAi* RNA interference *SIFT* scanning for intensely fluorescent targets, *siRNA* small interfering ribonucleic acid

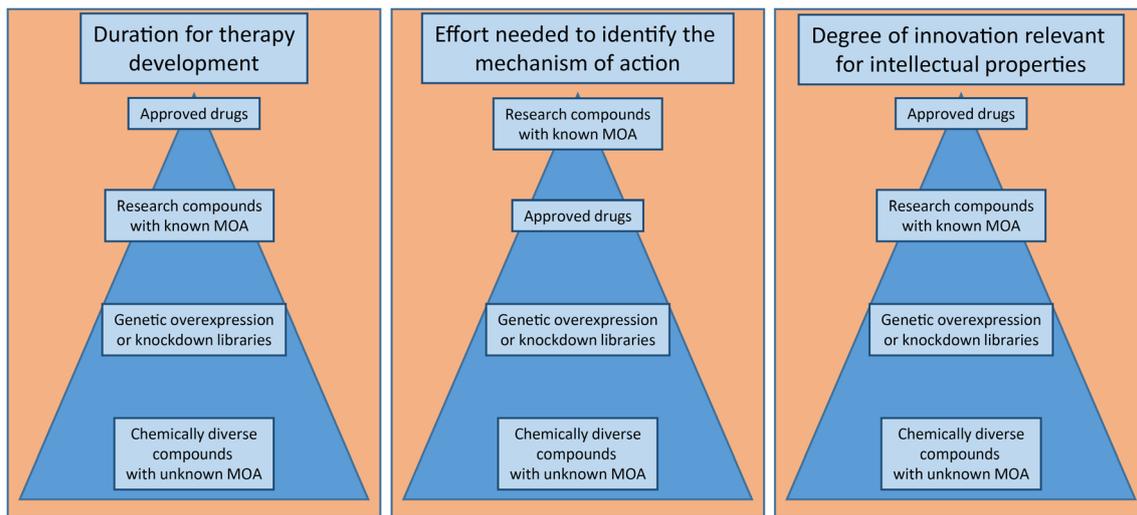


Fig. 1 Categorization of different high-throughput screening libraries regarding duration for therapy development, effort needed to identify the mechanism of action, and degree of innovation relevant for intellectual property. Approved drugs may relatively easily be translated into clinical application, since data for pharmacokinetics, pharmacodynamics, dosage, and molecular mechanism of actions (MOA) are available. However, the likeliness to obtain novel

intellectual property rights with patent-protected approved drugs in new indications is rather small. Compounds of unknown mechanism have high innovative potential, but the duration and effort needed to identify the mechanism of action and to develop an investigational new drug on their basis may take a long time. Screens with compounds with known mechanism of action and genetic libraries require intermediate effort to develop proprietary drugs

Needs for Future Screens

There is a need for screens in further mammalian and particularly human models. Different populations of neurons have different susceptibility to α Syn [28, 30], suggesting that different types of neurons will yield different insights into the mechanisms of α Syn-induced toxicity. There is therefore a need to develop various human dopaminergic neuronal models to better capture the complexity of α Syn biology and pathology. There is much room for novel insights with innovative compound libraries to identify further therapeutic targets. Finally, there is still an unmet need for genetic modifier screens, such as overexpression or knock-down or knock-out screens in α Syn toxicity model in human dopaminergic neurons. Improvement of techniques of genome-wide editing of mammalian cells, e.g., CRISPR-Cas9, which became available in a genome-wide basis in human cells [53] will facilitate conduction of genetic modifier screen in human cells in the future.

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Compliance with Ethical Standards

Conflict of Interest Günter Höglinger and Matthias Höllerhage each declare no potential conflict of interest.

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