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 (αSyn)-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 αSyn-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 αSyn-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 αSyn and only very few screens investigated wild-type αSyn. Particularly, no genome-wide αSyn toxicity screen in human dopaminergic neurons has been published so far.

Summary

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

Introduction

Alpha-synuclein (αSyn) is a small 140 amino acids long protein whose physiological function is not fully understood in spite of a myriad of publications on this topic. It is thought that αSyn carries out many functions [1]. Pathological aggregates of αSyn in the brain define a group of neurodegenerative disorders termed synucleinopathies [2]. The term synucleinopathies emerged when αSyn 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 αSyn 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 αSyn in the etiology of PD is emphasized by genetic findings. Mutations [6] and multiplications [7, 8] of *SNCA*, the gene coding for αSyn, 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 options addressing the pathophysiology of neurodegenerative synucleinopathies. αSyn is an aggregation prone protein and even though the exact nature of the toxic αSyn species is still

unknown, it is a well-established concept that oligomeric intermediates between αSyn monomers and larger aggregates, as found in Lewy bodies, confer toxicity [16]. Previous, hypothesis-based approaches led to the identification of mechanisms involved in degradation of αSyn 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 αSyn 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 αSyn, which are currently investigated in clinical trials (e.g. NCT03100149, NCT03318523). Thus, targeting αSyn, 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 αSyn toxicity are a possibility to identify such new ways to counteract the pathological process in neurodegenerative synucleinopathies. Modifier screens for αSyn can be roughly divided into two categories: genetic modifier screening and compound modifier screening. Every screening approach has its advantages and disadvantages, which will be discussed giving examples of published screens in αSyn toxicity models. We focused on unbiased screens for modifiers of αSyn toxicity. However, it should be mentioned that many screens targeted a desired mechanism of action such as αSyn aggregation or its degradation as readout in the primary screen. Many of these screens could confirm a protective effect on αSyn 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 organism 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 [18] 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 6,000 genes are available [19]. Additionally, overexpression plasmid libraries such as the Yeasts FLEXGene open reading frame (ORF) library consisting of 5,500 yeast ORFs are available [20]. These libraries allow genome-wide deletion or overexpression screens. Even though yeast does not encode αSyn, yeast cells expressing human αSyn under the control of the galactose promoter show growth inhibition upon addition of galactose [21].

Genetic modifier screens

Table 1 presents an overview of modifier screens of αSyn-induced toxicity. One of the first screens was published in 2003 [22]. The authors determined cell growth of 4,850 deletion strains overexpressing human αSyn. 86 of the deletions reproducibly increased toxicity of human αSyn. Of the genes that could be assigned to a function, roughly one third was involved in lipid metabolism and vesicular transport [22]. 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 [22].

Another genetic modifier screen was performed in a yeast strain expressing human αSyn coupled to yellow fluorescent protein (YFP) under a galactose promoter [23]. The authors

screened a library of 3,000 randomly selected ORFs also under the control of the galactose promoter and monitored the growth rate. 34 genes suppressed and 20 enhanced αSyn-induced toxicity. Many suppressors of αSyn toxicity were in involved in ER-Golgi trafficking and the strongest suppressor of αSyn toxicity was Rab1. This finding was validated both in *Caenorhabditis elegans* and in *Drosophila melanogaster*. Like yeast, *C. elegans* does not have αSyn, but expression of human αSyn under the DAT1-promoter in dopaminergic neurons leads to degeneration of this cell population [24]. Expression of Rab1 in *C. elegans* rescued αSyn induced dopaminergic neurons cell death. They further investigated the effects of Rab1 in primary rat midbrain cultures transduced with lentiviral vectors expressing human A53T-mutated αSyn, 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 [23].

Investigating the transcriptional response to αSyn overexpression is another promising avenue for finding modifiers. In a paper by Yeger-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 [25]. They concluded that genetic screens identify in general genes involved in regulatory processes, whereas expression analyses rather identify metabolic aspects of cellular processes. The authors then developed a computational approach (ResponseNet, [26, 27]) to identify cellular processes that bridge the gap between these two approaches. They then applied this approach to compare the hits of the same αSyn–YFP yeast model as used in the previous screen [23], but expanded to encompass 5,500 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 αSyn overexpression [25]. 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 [25]. 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 αSyn toxicity in yeast [28]. The authors confirmed their finding in *C. elegans* expressing αSyn-GFP in body wall cells and showed that knockdown of the *C. elegans* ortholog to ATP13A2 led to enhanced misfolding of αSyn. 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- αSyn [28]. 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-αSyn identified genes that blocked reactive oxygen species (ROS) production and were protective against αSyn-induced toxicity [29, 30]. More recently, another genetic modifier screen using αSyn-YFP expressed under a galactose promoter was carried out using randomized CRISPR-Cas9-based transcription factors to perturb expression profiles [31]. A transcription analysis was performed to determine which genes were differentially regulated and suppressed αSyn-induced toxicity. They identified 114 genes that were differentially expressed of which 93% were not previously identified in overexpression or knockdown screens [31]. 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) [31]. These cells were differentiated into a dopaminergic phenotype, in which overexpression of wild-type αSyn leads to degeneration [32]. The expression of the four genes reduced αSyn induced toxicity [31]. 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 αSyn. Since the authors did not observe toxicity from αSyn 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 querticin and (-)epigallocatechin-3-gallate (EGCG) reduced αSyn-induced growth inhibition [33]. 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 αSyn-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 αSyn-induced toxicity [34]. This screen demonstrated the feasibility of the screening of natural products to identify compounds that protect against αSyn-induced toxicity.

In another screen, 115,000 compounds from various collections were tested in the yeast αSyn-YFP model at 15 μM [35]. 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 compound. As top hits the authors identified four 1,2,3,4,-tetrahydrochionolinones. They confirmed that the compounds did not change αSyn expression levels. Furthermore, as in previous screens, the hits were validated in *C. elegans* expressing αSyn in dopaminergic cells as well as in rat primary neurons transduced with A53T- αSyn. All four compounds protected in *C. elegans*, three of them also led to restoration of the number of dopaminergic neurons in rat primary cultures [35]. In another screen, the hypothesized pore-formation ability of αSyn oligomers was screened. An assay was developed which monitored pore formation upon treatment of human osteoblastoma cells with

C-terminally truncated αSyn (αSyn119) by measuring leakage of a fluorescent agent ((bis-(1,3-dibutylbarbituric acid)-trimethine oxonol; DiBAC4(3)) [36]. While the authors did not perform a high throughput screen, they showed as proof of concept that preincubation of αSyn119 with known anti-aggregatory compounds (e.g. EGCG) reduced pore formation as measure for toxicity [36]. Their model was only tested by preincubation of compounds with αSyn119 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 [37]. After addition of tetracycline to the culture medium, the cells can be differentiated to human postmitotic midbrain neurons [38]. In these cells, moderate overexpression of human wild-type αSyn leads to half-maximal cell death [39]. We screened 1,600 FDA-approved drugs in this model and identified an unspecific PDE inhibitor as protective against αSyn-induced toxicity. We then investigated more specific PDE inhibitors and found that specific inhibition of PDE1 with vinpocetine protected against αSyn 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 from degeneration induced by local overexpression of human wild-type αSyn after injection of adenovirus associated viral vectors [40]. 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 αSyn 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 αSyn-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 αSyn-YFP and 96 clones were randomly chosen for validation. Of these 31 were reproducibly protective and 2 best hits were chosen for validation, which were then confirmed to be protective against αSyn 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 [41]. 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 αSyn could reduce toxicity [42]. Since most known αSyn mutations are located between amino acids 46 and 53 they reasoned that this segment of αSyn might be most relevant for αSyn induced toxicity. They overexpressed wild-type αSyn 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 αSyn, presuming that these might interact with αSyn 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 αSyn together with the decapeptides and only 200 colonies survived, suggesting that only these 200 decapeptides could reduce αSyn toxicity. After six passaging steps, they identified one decapeptide that was most efficient in reducing αSyn induced toxicity. They confirmed that this decapeptide was inhibiting αSyn aggregation in a thioflavin T assay [42]. This screen demonstrated that peptides designed to interact with αSyn can prevent αSyn toxicity.

Another recent screen followed a totally different approach and gained much attention due to its possible clinical implication [43]. The authors did not overexpress αSyn or looked for toxicity in the primary screen. Instead, they supposed that high αSyn 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 1,126 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 6 related compounds (hit expansion) were confirmed to also affect αSyn protein levels. β2-receptor agonists were overrepresented and in follow-up experiments they showed that transfection of their cell line with β2-receptors reduced αSyn levels, whereas treatment with β2-receptor blockers increased αSyn levels. Moreover, they showed that β2-receptor agonists reduced αSyn expression in induced pluripotent stem cells from patients with a SNCA triplication and reduced mitochondrial superoxide production [43]. The finding is particularly interesting, because β2-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 β2-receptor blockage can increase αSyn expression in patients, one would have to be much more cautious in prescribing β2-receptor blockers in patients with tremor.

Screen that primarily addressed αSyn oligomerization

Many screens addressing the neurobiology of αSyn have been performed in models in which a different phenotypic readout (e.g. αSyn oligomerization or aggregation) other than αSyn-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 folled up by investigating αSyn toxicity. Other screens

that did not look into modulation of αSyn-induced toxicity in primary or secondary analyses were not considered at all in this review.

Conclusion

Unbiased genetic screens for modifiers of αSyn-induced toxicity can deepen the understanding of the pathophysiological processes involved in synucleinopathies by identifying pathways that had not been previous identified by hypothesis-driven approaches (e.g. ER-Golgi trafficking) [23]. 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 was 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 αSyn with unknown mechanisms, the post-screening phase needs to include a target-deconvolution or target-identification step. When the mechanism of action (MOA) of hits are identified, knockdown experiments can be used to confirm the MOA [40]. However, especially when the MOA of the screened modifiers is unknown, target deconvolution can be difficult [41]. One possibility is transcriptional profiling. The transcriptional changes within the cells in presence of individual compounds can reveal information about the mechanism of action [35, 44]. Other methods are target-based approaches testing possible targets in a hypothesis-driven manner [35, 45], or affinity-based assays, in which the interaction between the compounds and different proteins are determined [45]. 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 αSyn screens reported to date were performed in yeast. This fact is surprising since the yeast genome comprises roughly 6,000 genes [46] and does not encode an ortholog of αSyn. Nevertheless, researchers have taken advantage of the fact that αSyn 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 [18] 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 [28]. In spite of these encouraging results, it is likely that some of the cellular processes involved in the neurobiology of αSyn cannot be recapitulated in this simple model. In spite of these shortcomings of the yeast model, our literature search did not identify a single genetic screen addressing toxicity induced by wild-type αSyn in human cells. We have performed a compound screen in a human cell line that can be differentiated into dopaminergic neurons [38]. In this model, dopaminergic neurons show half-maximal cell death over a course of six days upon moderate overexpression of human wild-type αSyn [39, 40]. 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 αSyn biology.

Shortcomings of previously reported screens

In most previously reported screens the cells were challenged with αSyn fused to a fluorescent protein such as GFP or YFP. However, αSyn 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 αSyn coupled to halves of GFP was actually representing the pathophysiological process of oligomerization [47]. Other models study the neurobiology of αSyn containing rare genetic mutants leading to autosomal dominant PD. These are much more toxic to cells than wild-type αSyn [39], suggesting that other cellular processes may be involved in cell death induced by mutant αSyn compared to the wild-type protein. Furthermore, wild-type αSyn is responsible for the vast majority of sporadic synucleinopathies and thus findings with mutant forms of αSyn might have limited significance.

Many biased αSyn screens have been reported, monitoring αSyn aggregation, degradation by the proteasome [48], chaperone-mediated autophagy, or macroautophagy. Such screens are based on hypotheses that are not necessarily true, since we understand so little of αSyn function and pathology. Even though we did not focus on these screens in the current review, examples are presented in table 2.

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 αSyn [23, 28], suggesting that different types of neurons will yield different insights into the mechanisms of αSyn-induced toxicity. There is therefore a need to develop various human dopaminergic neuronal models to better capture the complexity of αSyn 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 αSyn 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 [49] will facilitate conduction of genetic modifier screen in human cells in the future.

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Figure Legend:

Figure 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 easy be translated into clinical application, since data for pharmacokinetics, pharmacodynamics, dosage, and molecular mechanism of action 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.