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Transcriptional Dysregulation in a Transgenic Model of Parkinson's Disease

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Abstract

Alpha-synuclein has been implicated in Parkinson's disease, yet the mechanism by which alpha-synuclein causes cell injury is not understood. Using a transgenic mouse model, we evaluated the effect of alpha-synuclein overexpression on gene expression in the substantia nigra. Nigral mRNA from wildtype and alpha-synuclein transgenic mice was analyzed using Affymetrix gene arrays. At three months, before pathological changes are apparent, we observed modest alterations in gene expression. However, nearly 200 genes were altered in expression at nine months, when degenerative changes are more apparent. Functional genomic analysis revealed that the genes altered at nine months were predominantly involved in gene transcription. As in human Parkinson's disease, gene expression changes in the transgenic model were also modulated by gender. These data demonstrate that alterations of gene expression are widespread in this animal model, and suggest that transcriptional dysregulation may be a disease mechanism that can be targeted therapeutically.

Keywords

Parkinson's disease; alpha-synuclein; microarray; substantia nigra; laser capture microdissection; mouse; transcription

Introduction

Parkinson's disease (PD) is a debilitating neurological disorder associated with dopaminergic cell loss in the substantia nigra (SN). Familial PD cases have been linked to several genes, including α -synuclein (α -syn), a 140-amino-acid protein that may function in neurotransmitter release (Abeliovich et al., 2000; Murphy et al., 2000). Families with mutated α -syn exhibit autosomal dominant PD (Athanasiadou et al., 1999; Kruger et al., 1998; Polymeropoulos et

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al., 1997; Zarranz et al., 2004), and gene multiplication leading to increased wildtype α -syn levels also causes disease (Singleton et al., 2003). Certain α -syn promoter polymorphisms are PD risk factors, suggesting that even modest increases in expression can predispose to disease (Farrer et al., 2001; Kruger et al., 1999; Pals et al., 2004; Tan et al., 2000; Wang et al., 2006). In patients without genetic mutations, α -syn is found in Lewy bodies and degenerating neurites (Irizarry et al., 1998; Spillantini et al., 1997).

Alpha-syn overexpression has been used to generate cellular and animal models of PD. Mutant α -syn or overexpression of wildtype α -syn induces cell death in dopaminergic cell lines and in primary dopaminergic cultures (Oluwatosin-Chigbu et al., 2003; Xu et al., 2002; Zhou et al., 2000; Zhou et al., 2002). Transgenic mice expressing mutant or wildtype α -syn show motor deficits, alterations in dopamine levels, and α -syn-positive inclusions (Hashimoto et al., 2003; Maries et al., 2003). Alpha-syn is also involved in the action of neurotoxins, such as MPTP and rotenone, used to model PD (Dauer et al., 2002; Betarbet et al., 2000).

The mechanism by which α -syn injures dopaminergic neurons remains unclear. A potential mechanism not closely examined is transcriptional dysregulation, in which interference with normal gene expression patterns leads to cellular dysfunction. Transcriptional dysregulation has been implicated in Huntington's and Alzheimer's diseases (Cha, 2000; Robakis, 2003). Using microarray methods, we have observed that PD causes gene expression alterations in human dopaminergic neurons (Cantuti-Castelvetri et al., 2007). Alpha-syn can also inhibit histone acetylation, a potent mechanism for altering gene expression (Kontopoulos et al., 2006).

Here we examined gene expression patterns in SN cells isolated from transgenic mice in which human wildtype α -syn is overexpressed under the platelet-derived growth factor β promoter. These mice develop neuronal α -syn inclusions in the SN and other brain regions (Masliah et al., 2000). At twelve months, they show motor impairments and decreased striatal dopaminergic terminals and tyrosine hydroxylase activity (Masliah et al., 2000). We used laser capture microdissection to isolate nigral cell RNA from transgenic and control mice, and microarray analysis to evaluate for alterations in gene expression induced by α -syn overexpression at two time points: 1) three months, when pathological changes are few, and 2) nine months, when α -syn inclusions are more widespread. Our data reveal the early effects of α -syn overexpression are modest. At the later time point, changes in gene expression are more prominent in these transgenic mice, and many of the altered genes have functions related to transcription.

Methods

Animals

α -synuclein mice were originally generated by Masliah et al. (2000). A breeding colony from the Masliah D line was set up at Charles River Laboratories to generate transgenic and wildtype littermates. The use of mice was supervised by the Massachusetts General Hospital Animal Resources Program in accordance with the PHS policy on Humane Care and Use of Laboratory Animals. Prior to sacrifice, animals were euthanized by CO₂ inhalation. Six gender-matched wildtype and six transgenic littermates were sacrificed at three months of age, and another six control and six transgenic mice were sacrificed at nine months of age. Brains were immediately frozen in isopentane and stored at -80°C. Brains were sectioned at eight μ m by cryostat, mounted on uncoated glass slides, and stored at -80°C.

Laser capture microdissection (LCM)

All of the following procedures were done under strict RNase-free conditions. Eight μm sections through the midbrain were first thawed and fixed with acetone for 40 seconds. To identify nigral neurons, these sections were immunostained using a primary mouse monoclonal antibody against tyrosine hydroxylase (TH; 5 minutes at 1:1500; Sigma) and a cy3-conjugated goat anti-mouse secondary antibody (5 minutes at 1:200; Jackson ImmunoResearch Labs, West Grove, PA) and then dehydrated (50, 70, 95, 100% ethanol, 5 seconds at each concentration, followed by xylene for several minutes). TH-stained substantia nigra pars compacta (SNpc) were laser captured from each section with an Arcturus PixCell II instrument (Mountain View, CA). Because the SNpc in mice is very densely packed with cells, we were unable to accurately count the number of captured TH-positive neurons and likely also captured some other cell types. To minimize differences in the amount of captured RNA, we attempted to capture cells from about eight nigral sections of comparable size for each animal. Global normalization of microarray data and normalization of quantitative PCR data with “housekeeping” genes were performed to correct for differences in the number of captured cells among animals.

Amplification and gene expression microarray

RNA from laser-captured nigral cells was extracted using the Picopure™ RNA isolation kit (Arcturus) according to manufacturer's specifications. Purified RNA was then amplified two rounds using the RiboAmp™ RNA amplification kit (Arcturus). This kit is based on a T7-RNA polymerase method that can linearly amplify total RNA quantities as small as 1 ng. After amplification, mRNA quality was assessed by an Agilent Bioanalyzer (Agilent, Palo Alto, CA). Selected samples had similar product sizes averaging approximately 500-1000 bp. Those samples with smaller average product sizes were discarded. Appropriate amplified RNA (aRNA) samples were sent to the Harvard Partners Center for Genetics and Genomics (HPCGG) facility to generate biotinylated aRNA that was then hybridized to Mouse Expression 430 2.0 arrays (Affymetrix, Santa Clara, CA). Each biotinylated aRNA sample from an individual animal was hybridized to one array, for a total of 24 arrays.

Microarray validation

Validation PCRs were performed on a separate set of nigral neurons laser captured from wildtype and transgenic mice using the Arcturus Veritas system. RNA from these cells was extracted using the Picopure™ RNA isolation kit. The extracted RNA was reverse transcribed into first-strand cDNA using the SuperScript™ II reverse transcriptase kit (Invitrogen, Carlsbad, CA). cDNA was then precipitated using sodium acetate and ethanol with 1 μg of glycogen as a carrier.

For each age group, two genes whose levels were altered in the microarray analysis were selected to validate microarray data. The two genes selected for validation were altered at both time points in the microarray analysis and were expressed at high intensities. 20mer, synthetic PCR primers were designed using Primer3 (<http://frodo.wi.mit.edu>). Primers against cyclin D (**NM_007631**) were 5' tgaaccaaggaggagatcag 3' (forward) and 5' gaagcccaaatccacaaac 3' (reverse; product size 256 bp). Primers against TCDD-inducible poly (ADP-ribose) polymerase (**NM_178892**) were 5' ctggaaccctgagatccttg 3' (forward) and 5' gacacgatgggttgatttc 3' (reverse; product size 153 bp). For real-time quantitative PCR (QPCR), first strand cDNA created from extracted RNA was incubated with appropriate forward and reverse primers and SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) in a 96-well plate. QPCR was performed using an iQ5Cycler (BioRad, Hercules, CA) set to the following protocol: 1 cycle of denaturation at 95°C for 10 minutes; 50 cycles of denaturation at 95°C for 30 seconds, annealing at 57°C for 30 seconds, and polymerization at 72°C for 45 seconds; and finally, 80 0.5°C increases in temperature (starting at 55°C) to collect melting curve data. For each primer set, we used a standard curve with known concentrations of cDNA

to calculate primer efficiency and to quantitate PCR products. The quantity of each PCR sample was calculated using the $\Delta\Delta C_t$ method (Fink et al., 1998; Livak and Schmittgen, 2001). We used both pyruvate decarboxylase (forward primer 5' gctggagaaggacctgattg 3'; reverse primer 5' ccagctcaacctcaaacctcc 3'; product size 191 bp) and hypoxanthine phosphoribosyltransferase (forward primer 5' tgtgttgatgccccttg 3'; reverse primer 5' tgcgctcatcttagccttg 3'; product size 107 bp) levels to normalize PCR results, as both genes were unchanged between transgenic and wildtype mice.

Microarray analysis

Microarray analysis was performed as previously described (Cantuti-Castelvetri et al., 2007). Chips were developed, scanned, and normalized using global scaling. All quality control parameters calculated by Affymetrix GCOS Software were monitored. The images of the chips were analyzed to find spotted or damaged array regions, and the graphical analysis of all chips through this step provided evidence that the data was of good quality (Quackenbush, 2002). All data were normalized using the GeneChip Robust MultiChips Analysis (GCRMA) algorithm (Cope et al., 2004) performed with ArrayAssistLite (Stratagene, La Jolla, CA). Normalized data was then statistically analyzed with multiclass analysis followed by two-tailed unpaired t-tests using the Significance Analysis of Microarrays (SAM) algorithm with SAM 2.0 plug-in for Excel (Efron and Tibshirani, 2002; Larsson et al., 2005; Tusher et al., 2001).

Any probes associated with transcripts that were no longer listed in the EntrezGene or Unigene databases were excluded, as were probes which were present for less than half the animals in a given experimental group (transgenic, wildtype, female transgenic, female nontransgenic, etc). Probes with intensities lower than background in all samples were also filtered out ($\log_2$ intensity <3). Average expression for each probe/gene was calculated for each of the following groups of animals: 1) all three-month-old transgenic animals, 2) all three-month-old wildtype animals, 3) all nine-month-old transgenic animals, 4) all nine-month-old wildtype animals, 5) three-month-old female transgenic mice, 6) three-month-old female wildtype mice, 7) three-month-old male transgenic mice, 8) three-month-old male wildtype mice, 9) nine-month-old female transgenic mice, 10) nine-month-old female wildtype mice, 11) nine-month-old male transgenic mice, and 12) nine-month-old male wildtype mice. Average expression for a given probe was calculated for all transgenic mice and for all wildtype mice, and the ratio of average transgenic mouse expression to average wildtype mouse expression was calculated for each probe. This ratio was expressed as a $\log_2$ ratio in our computer analyses programs. Probes were considered to be different between two groups if the $\log_2$ ratio between the two groups was greater than 0.5 (equivalent to 1.4-fold difference) or less than -0.5 (equivalent to 0.7-fold difference) and if the p value was less than 0.05 for the unpaired t-test. To reduce the possibility of a type I statistical error and to control for the variance within each probe set considered in the analysis, we also estimated the false discovery rates (represented by q values) using SAM and limited the final lists of differentially expressed genes to those probes with q values $\leq$ 20%.

Clustering

Hierarchical clustering on median normalized samples using cosine correlation with complete linkage was performed for each selected probe sets lists on all three-month samples and on all nine-month samples using SpotFire DecisionSite for Functional Genomics 8.0 (Fig. 1; Eisen et al., 1998).

Functional profiling

To analyze the biological roles of the differentially expressed genes, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID) 2007 (Dennis et al., 2003;

Hosack et al., 2003). DAVID 2007 is a freely-available, NIH-sponsored data-mining tool that uses several databases containing gene-specific functional data, such as Gene Ontology (GO), Kyoto Encyclopedia of Genes and Genomes (KEGG), the Protein Information Resources (PIR), and others. DAVID calculates the probability that particular functional categories are overrepresented in a given data set using a one-tailed Fisher exact probability for overrepresentation (or EASE score) using a Gaussian hypergeometric probability distribution. This distribution describes sampling without replacement from a finite population made of two types of elements: genes that belong to a particular functional category and genes that do not belong to that particular category.

For the functional analysis, we used only those differentially expressed probe sets that had a q value $\leq 20\%$. First, the DAVID functional annotation tool was used to generate those categories defined by GO database annotations that were overrepresented. All GO categories that had a p value ≤ 0.05 and which contained at least 3% of all regulated genes were considered significant, regardless of how broad or specific the categories were. The functional annotation clustering tool in DAVID was then used as a second level of functional analysis. This tool evaluated each annotation term by the genes that it was comprised of from a given data set and then clustered that term with similar annotations from other ontology databases based on the amount of genes that were co-associated. Only those functional clusters that gave a median p value ≤ 0.05 were considered significant.

Results

Isolation and analysis of mRNA from cells collected by laser capture microdissection

We used laser capture microdissection to isolate nigral cell RNA from both α -syn transgenic and wildtype mice. After amplification, RNA quality was assessed by an Agilent Bioanalyzer. Samples selected for microarray hybridization had similar product sizes averaging 500-1000 base pairs. Those samples with smaller-than-expected average product sizes were discarded, and more nigral cell RNA was captured and amplified. For each age group, we isolated and amplified RNA from gender-matched groups of six transgenic and six wildtype mice. The resultant aRNA samples were labeled and hybridized to Affymetrix Mouse Expression microarrays. Each aRNA sample from an individual animal was hybridized to one array, for a total of 24 arrays. Background intensity levels for all arrays were similar with a mean 58.9 ± 6.8 (SEM). Average signal for present probes across all arrays was 388.6 ± 22.2 (SEM). As a group, the 24 arrays had a mean $42.0 \pm 0.8\%$ (SEM) of probes classified as “present.”

α -syn overexpression leads to early alterations in gene expression

We initially examined nigral neurons from mice at three months of age, a point at which there is no apparent histological abnormality in the SN. Data normalized by the GCRMA algorithm (Cope et al., 2004) was statistically analyzed with multiclass analysis followed by two-tailed unpaired t-tests using the SAM algorithm (Efron and Tibshirani, 2002; Larsson et al., 2005; Tusher et al., 2001). We considered probes to be different between wildtype and transgenic mice if the $\log_2$ ratio between the groups was greater than 0.5 (equivalent to 1.4-fold difference compared to wildtype) or less than -0.5 (equivalent to 0.7-fold difference compared to wildtype) and if the p value was less than 0.05 for the unpaired t-test. 237 genes were differentially expressed between transgenic and wildtype littermates (Supp. Table 1). 180 of these genes have been identified in the Entrez Gene and Unigene databases with names instead of expressed sequence or RIKEN numbers. Hierarchical clustering performed using the data from these differentially expressed genes appropriately classified all of the samples from three-month-old mice, although it was clear that there was some variation within the groups in the strength of the phenotype defined by this set of genes (Fig. 1a).

Among this list of genes, we validated two genes by quantitative PCR. The two genes chosen were cyclin D and TCDD-inducible poly (ADP-ribose) polymerase (Tiparp), which were both expressed at high intensities at three months. We collected SNpc by laser capture microdissection from six transgenic and six wildtype mice. Expression levels were determined in cDNA samples produced from the isolated neurons without any intervening amplification steps. The expression levels of these two genes as determined by QPCR were similar to the microarray results (Table 1).

To reduce the possibility of a type I statistical error and to control for the variance within each probe set considered in the analysis, we also estimated the false discovery rate for each gene (represented by the q value) altered at three months and eliminated those genes that had a q value > 20%. By this more conservative statistical approach, we reduced the initial list of 237 genes to 29 (Table 2). Six of these genes were downregulated in the transgenic mice, while 23 were upregulated. 22 of the 29 genes were associated with gene names other than expressed sequence or RIKEN numbers. None of the altered genes in this list include molecules previously linked to PD. All upregulated genes from the initial 237 gene list were found in this more conservative list, while only six of the initial 214 downregulated genes had q values $\leq$ 20%. Because of the small number of genes modulated at this early time point, further classification by ontological analysis was not informative.

Late effects of α -syn overexpression on gene expression

We performed similar laser capture microdissection and array experiments with nine-month-old mice, which have widespread α -syn inclusions in the brain, to evaluate gene expression changes at a later point in disease pathogenesis. We found that the magnitude of the transcriptional dysregulation, as measured by the number of altered genes, was much larger at this late stage of the disease model, and the identities of the genes modified were different than those observed to be altered at the earlier time point.

As with the three month data, we performed an initial analysis using criteria of p value $\leq$ 0.05 and a log₂ ratio of at least $\pm$ 0.5. Using these filters, we found that 278 genes were altered between nine-month-old α -syn and wildtype mice (Supp. Table 2). 211 of these genes have been identified in the Entrez Gene and Unigene databases with names other than expressed sequence or RIKEN numbers. Hierarchical clustering using this gene set revealed that the data readily classified the transgenic and control animals (Fig. 1b). Indeed, the distinctions between the groups were much more striking than at three months, suggesting that at a later stage in the model the effect of α -syn overexpression is more uniform across the population of animals studied. Among the nine-month list of genes, we validated cyclin D and Tiparp by QPCR. The expression levels of these two genes as determined by QPCR were similar to the microarray results (Table 1).

As with the three-month microarray data, we also estimated the false discovery rate for each gene altered at nine months and eliminated those genes that had a q value > 20%. This conservative statistical approach reduced the initial list of 278 genes to 179 (Table 3). In contrast to the three-month time point, the predominant effect at nine months was a reduction of gene expression, with more genes downregulated than upregulated in the transgenic mice. The mRNA levels of 165 genes were decreased, while the mRNA levels of 14 genes were elevated in the nine-month-old α -syn mice. Some of the genes that were altered in the nine-month-old group included an E3 ubiquitin protein ligase (Cbl), a proteosomal subunit (Psm14), and other molecules linked to PD, including the neurotrophin receptor TrkB (von Bohlen und Halbach et al., 2005), and 14-3-3 σ (Berg et al., 2003; Kawamoto et al., 2002). Interestingly, among the downregulated genes was ceruloplasmin, which is decreased in Wilson's disease (Scheinberg and Gitlin, 1952), a genetic disorder with Parkinsonian symptoms.

We next compared the identity of the genes altered in the three-month-old and nine-month-old groups. Surprisingly, we found no genes that overlapped between the three-month and nine-month groups when analyzing those lists limited by q value $\leq 20\%$. When looking at the less conservative gene lists (in which genes were not eliminated by q value criterion), nine genes showed alterations in expression level at both time points. These genes included calmodulin 1, cyclin D2, disheveled associated activator of morphogenesis 1, fibronectin type III domain containing 4 (Fndc4), hematological and neurological expressed sequence 1 (Hn1), nucleoporin 62, PCTAIRE-motif protein kinase 2 (RIKEN 6430598J10), Tiparp, and expressed sequence AI55357. Seven of these genes showed concordant downregulation at both three and nine months, while Fndc4 and Hn1 showed alterations in opposing directions at the two time points.

Functional genomic analysis of the data from the nine-month-old mice was performed to evaluate whether the nine-month data set was enriched in any particular functional categories. We used the DAVID 2007 functional annotation tool (<http://david.abcc.ncifcrf.gov/home.jsp>; Dennis et al., 2003; Hosack et al., 2003), which classifies each gene into one or more functional categories using annotations from over 40 ontological databases, including the GO database, and then determines which functional categories are represented in the data set more frequently than expected by chance alone. We used the more conservative nine-month list of 179 genes with upregulated and downregulated genes combined to perform this analysis. We considered significantly overrepresented those functional categories in which the number of genes altered exceeded the number expected with a p value ≤ 0.05 . We limited the functional annotation chart generated by DAVID to those categories defined by GO database annotations (<http://www.geneontology.org/>). Table 4 reveals 39 GO ontological categories that contain at least 3% of the regulated genes. We did not exclude broad categories from this ontology table and instead included all parent and children categories that revealed a p value ≤ 0.05 and contained at least 3% of all regulated genes. Categories that were enriched in our data set included GTPase regulator activity, cytoskeletal protein binding, ubiquitin cycle, protein amino acid phosphorylation, transcription, and signal transduction.

We next performed a higher order functional analysis using the functional annotation clustering tool in DAVID, which evaluates each annotation term by the genes that it is comprised of from a given data set and then clusters that term with similar annotations from other ontology databases based on the amount of genes that are co-associated. By this clustering method, we found six functional clustering groups that had a median p value ≤ 0.05 (Table 5). Five of these six functional clusters were associated with DNA transcription and related categories, and one was associated with signaling. Among these co-clustering functional categories, 14.89% of genes altered at nine months were involved in transcription, 13.62% bind DNA, 12.34% bind zinc ions, 4.26% comprise transcription factor complexes, and 8.09% have transcription factor activity. Interestingly, 2.13% show steroid hormone receptor activity. Based on this functional annotation clustering, transcription appeared to be the predominant biological function that was altered in the nine-month group.

Gender modifies the effect of α -syn on gene expression

Because gender has recently been found to play an important role in transcriptional changes in human PD (Cantuti-Castelvetri et al., 2007), we evaluated whether gender modified the gene expression changes in the α -syn transgenic mice. The primary analysis described above was conducted in a group of animals where the number of mice of each gender was balanced. To analyze the influence of gender on gene expression patterns between wildtype and transgenic mice, we compared the average gene expression of female α -syn mice to female wildtype littermates and did a similar comparison of male transgenic to male wildtype mice. The group sizes in the gender analysis were necessarily smaller than in the primary analysis, and therefore,

may lack statistical power to detect gene expression alterations. For this analysis, we limited the gender-specific data sets by the following criteria: 1) p value ≤ 0.05 , and 2) $\log_2$ ratio of at least ± 0.5 . At both time points, females showed a greater dysregulation in gene expression than males did. 226 genes were altered in three-month-old transgenic females compared to wildtype females, and 332 genes were altered in nine-month-old transgenic females by at least a $\log_2$ ratio of ± 0.5 . The majority of the genes were downregulated in the transgenic female mice compared to gender-matched controls. Expression of 191 genes was decreased, while 35 genes showed increased expression at three months. At nine months, 193 genes showed decreased expression, and 85 had elevated levels in the transgenic females.

In contrast, fewer genes showed alterations in expression in the transgenic males compared to male controls. In three-month-old transgenic males, alterations in gene expression were apparent in 173 genes, while in nine-month-old transgenic males, 188 genes were altered by at least a $\log_2$ ratio of ± 0.5 . Unlike the females, transgenic males had more genes that increased in expression. At three months, 73 genes had decreased expression levels, and 100 had increased levels in transgenic males. At nine months, a decrease in expression was found in 77 genes, and an increase was seen in 111 genes in transgenic males.

A comparison of the identities of the genes regulated in the two genders revealed that the difference between the males and females was not simply the result of a difference in magnitude of regulation. Few of the same genes were altered in both genders at either age. Six genes were altered in both male and female transgenic mice at three months, and 20 were altered in both genders at nine months. Because our group sizes in the gender analysis were small, interpretation of our gender-based findings is limited. While we cannot conclude which genes are necessarily crucial to gender-related differences in α -syn toxicity, this gender analysis does point to the general conclusion that gender impacts the gene expression changes observed in α -syn mice.

Discussion

In this study, we examined the effect of α -syn overexpression in mice on gene expression in the SNpc. Our data showed that α -syn overexpression caused alterations in a small number of genes early in the pathological process, before any cellular or behavioral changes were apparent in the transgenic mice. Alterations in gene expression were more pronounced at a later stage of disease when pathological changes were apparent, and these late changes affected different genes than those modified early. These late gene expression changes were focused around transcription-related processes. Our analysis also suggested that gender modified the alterations of gene expression in this model.

We found striking differences between early changes in gene expression and those observed after pathological changes had clearly developed in the α -syn model. Prior to the onset of pathological changes, gene expression changes in the α -syn transgenic mice were modest. By conservative statistical criteria, we found only 29 genes to be altered. In contrast, 179 genes were altered in older transgenic mice. The larger gene list from the nine-month-old mice analysis reflects in part a reduction in variability among the animals, which presumably is a result of a more stable state of α -syn-related pathology at this later stage. When limiting our data sets by false discovery rates, we found no genes altered at both early and late time periods. Only nine genes were found altered in transgenic mice at both time periods when we evaluated our less stringent gene lists not limited by false discovery rate. Two of these nine genes are implicated with cell cycle regulation – calmodulin 1 (Rasmussen and Means, 1989) and cyclin D2 (Jena et al., 2002). The other seven genes do not seem to share any particular biological processes, with their functions still largely unknown.

The most striking result of our study is that many of the genes altered later in the disease model were directly related to transcriptional regulation, nuclear function, and DNA binding. These data support the view that transcriptional dysregulation is an important consequence of α -syn overexpression and, therefore, may be critical to the pathogenesis of synucleinopathies. Alpha-syn has been identified within cell nuclei and has been shown to associate with histones *in vitro* (Goers et al., 2003; Maroteaux et al., 1988). Studies by Kontopoulos et al. (2006) have provided direct evidence that α -syn can inhibit histone acetylation in both mammalian cell culture models and transgenic *Drosophila*. The effect of α -syn on histone acetylation appears critical to α -syn-induced toxicity, as treatment with histone deacetylase inhibitors rescues against α -syn-induced toxicity (Kontopoulos et al., 2006). The present study confirms the transcriptional effect of α -syn in a mouse model for PD.

It is important to recognize that microarray studies do not directly assess transcription. The parameter measured is the abundance of specific mRNAs, which can be affected not only by transcription but also by alterations in RNA half-lives. While we cannot exclude an effect of α -syn on the stability of some transcripts, the large number of alterations as well as the observed changes in mRNAs for many components of the transcriptional process suggests that transcriptional dysregulation is the predominant effect underlying the changes.

Another significant finding is the confirmation in this mouse model that gender influences gene expression changes induced by α -syn overexpression. This analysis was prompted by the recent observation that gender has a marked effect on gene expression in human dopaminergic SN neurons, influencing the patterns of gene expression in both normal brain and the response to PD (Cantuti-Castelvetri et al., 2007). Other microarray studies have also revealed gender differences in gene expression in normal human brain (Vawter et al., 2004), but there is little additional data available on the interaction of gender and disease state on neuronal gene expression in either humans or animal models. The importance of gender is emphasized by the fact that the incidence of PD is nearly twice that in men as in women (Baldereschi et al., 2000; Van Den Eeden et al., 2003). Since our study was originally designed with gender-matched animal groups, we were able to examine the effect of gender directly, although the number of animals in the subgroup analysis is necessarily smaller and lacks statistical power compared to the primary analysis. Nevertheless, we found substantial gender-based differences in the response to α -syn overexpression. We observed that α -syn caused more gene changes among female mice, and most altered genes were different between genders. At present it is not known whether mice exhibit gender-based differences in vulnerability to α -syn toxicity, yet in other animal PD models, males have exhibited increased susceptibility to MPTP (Dluzen and McDermott, 2000; Freyaldenhoven et al., 1996; Miller et al., 1998) and 6-hydroxydopamine (Murray et al., 2003; Tamas et al., 2005). Our findings suggest that issues of gender should be carefully considered in the design of experimental studies using animal models of PD.

The mouse model used here was selected because it demonstrates progressive dopaminergic dysfunction with a motor phenotype (Masliah et al., 2000), but the nature of the model imposes some limitations on our study. Because the mouse SNpc is much more densely packed with cells than in the human SNpc, we dissected the entire SNpc region for this study, and not individual neurons. Thus, the transcriptional changes may reflect alterations in glia as well as neurons. It is also important to note that this transgenic α -syn mouse does not fully recapitulate all pathological aspects of PD. This mouse does show evidence of dopaminergic dysfunction and mild motor impairment, but it does not show loss of dopaminergic nigral neurons (Masliah et al., 2000). Another pathological difference is that α -syn inclusions are much more widespread in Masliah mouse brains than are Lewy bodies found in PD brains. Therefore, many of the expression changes that we saw may be more broadly applicable to other synucleinopathies, such as dementia with Lewy Bodies. All other published α -syn transgenic

mice also fail to show dopaminergic neuron loss, yet these models vary in the appearance of α -syn-positive inclusions and severity of motor disability (Hashimoto et al., 2003; Maries et al., 2003). Transgenic mice expressing the A53T α -syn mutant show more severe motor impairments (Giasson et al., 2002; Lee et al., 2002), and some of the other transgenic mice have α -syn inclusions more typical of α -syn aggregates found in PD (Kahle et al., 2000; Lee et al., 2002).

While there are accepted principles for microarray analyses, different approaches to normalization and selection may lead to different outcomes regarding which genes are defined as differentially expressed (Hoffmann et al., 2002). Significance Analysis of Microarrays (SAM) is currently the method most commonly used that corrects for the large number of comparisons inherent in microarray experiments by estimating false discovery rate (Efron and Tibshirani, 2002; Larsson et al., 2005; Tusher et al., 2001). We limited false positives by excluding those genes whose q values were $> 20\%$. However, by using this conservative statistical method, we likely eliminated some genes that are truly altered between wildtype and transgenic animals. Indeed, by QPCR we did confirm alterations in two genes identified by less stringent criteria, Tiparp and cyclin D2, yet both of these genes had high q values in the three-month animals and were thus eliminated from the more stringent three-month list. Attempts to validate directly individual genes introduce additional issues, especially with regard to the selection of genes for normalization. Although genes such as beta-actin and GAPDH have often been used for this purpose, expression of such “housekeeping” genes can vary, especially in neurodegenerative disease (Gutala and Reddy, 2004; Radonic et al., 2004; Vandesompele et al., 2002). Indeed, our array data does show that GAPDH levels are altered in the nine-month-old α -syn transgenic mice. We chose pyruvate decarboxylase and hypoxanthine phosphoribosyltransferase, both of which were unchanged in our microarray lists, to verify that similar results were obtained regardless of the gene chosen for normalization.

Given these considerations, we have decided to publish two sets of gene lists in this paper: 1) the three-month and nine-month tables (Tables 2 & 3) restricted by q values in addition to fold-change and p value criteria; and 2) the supplementary tables (Supp. Tables 1 & 2) restricted only by fold-change and p value criteria. In addition, the full gene array dataset from this study is publicly available on the Array Express site and permits further analysis and comparison with other studies.

In conclusion, we have explored the molecular changes induced by α -syn at a transcriptional level by comparing α -syn transgenic mice to wildtype mice by microarray analysis. Significant transcriptional changes occur in the SNpc of older transgenic mice, and these changes are influenced by gender. A large proportion of the altered genes are involved in transcriptional regulation. The next step is to evaluate whether some of the differentially expressed genes are protective or, alternatively, can magnify α -syn toxicity in cellular and animal models. This study reinforces the concept that targeting transcriptional dysregulation as a mechanism may be a useful therapeutic approach for PD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Hierarchical clustering on median normalized samples using cosine correlation with complete linkage was performed on all samples from a) three-month-old mice and from b) nine-month-old mice. Green lines reflect decreased expression of probes, and red lines reflect increased expression of probes. This method appropriately sorts wildtype from transgenic mice at both ages.

Table 1

Quantitative PCR validation.

Age	Gene	TG ± SEM*	WT ± SEM*	QPCR TG/WT	Array TG/WT
3 month	Cyclin D2	0.179±0.016	0.222±0.031	0.806	0.556
	Tiparp	0.255±0.011	0.363±0.027	0.702	0.509
9 month	Cyclin D2	0.180±0.017	0.363±0.072	0.496	0.444
	Tiparp	0.471±0.088	0.844±0.105	0.558	0.493

Comparison of microarray results with QPCR results for two genes, cyclin D2 and TCDD-inducible poly(ADP-ribose) polymerase (Tiparp), which were altered at both three and nine months.
* QPCR results shown in table were normalized to pyruvate decarboxylase.

Table 2

Probes whose expressions are altered by at least a $\log_2$ ratio of ± 0.5 in three-month-old transgenic α -syn mice. P value for each probe is ≤ 0.05 and q-value is $\leq 20\%$.

Probe Set ID	Genbank ID	Gene Name	$\log_2$ (TG/WT)	q-value
1451127_at	BC024822	EXPRESSED SEQUENCE AW146242	-1.79	0
1460037_at	BF303035	RIKEN CDNA 2610510H03 GENE	-0.91	0
1434735_at	BB744589	HEPATIC LEUKEMIA FACTOR	-0.75	0
1448101_s_at	NM_009054	TRIPARTITE MOTIF PROTEIN 27	-0.74	0
1429097_at	BB090042	RIKEN CDNA C030044C12 GENE	-0.67	0
1451240_a_at	BC024663	RIKEN CDNA 1110008E19 GENE	-0.52	0
1426421_s_at	AK005802	RIKEN CDNA 1700009P03 GENE	0.51	0
1441807_s_at	AI851474	RIKEN CDNA A830093I24 GENE	0.53	0
1420296_at	T25545	CHLORIDE CHANNEL 5	0.53	0
1452975_at	AK005060	ALANINE-GLYOXYLATE AMINOTRANSFERASE 2-LIKE 1	0.56	0
1444064_at	AV376100	SAM DOMAIN AND HD DOMAIN, 1	0.56	0
1450688_at	NM_009059	RAL GUANINE NUCLEOTIDE DISSOCIATION STIMULATOR-LIKE 2	0.56	0
1416055_at	NM_009669	AMYLASE 2, PANCREATIC	0.57	0
1457443_at	BB311369	G PATCH DOMAIN CONTAINING 2	0.57	0
1443430_at	BM234069	CASP2 AND RIPK1 DOMAIN CONTAINING ADAPTOR WITH DEATH DOMAIN	0.58	0
1420115_at	C80158	EXOSOME COMPONENT 8	0.59	0
1441266_at	BM238307	STRIATIN, CALMODULIN BINDING PROTEIN 3	0.60	0
1439205_at	BM122872	NUCLEAR FACTOR OF ACTIVATED T-CELLS, CYTOPLASMIC, CALCINEURIN-DEPENDENT 2	0.63	0
1435453_at	AV343709	RIKEN CDNA A930011O12 GENE	0.70	0
1418539_a_at	U35368	PROTEIN TYROSINE PHOSPHATASE, RECEPTOR TYPE, E	0.78	0
1452831_s_at	AV305746	PHOSPHORIBOSYL PYROPHOSPHATE AMIDOTRANSFERASE	0.79	0
1418264_at	NM_021790	SOXLZ/SOX6 LEUCINE ZIPPER BINDING PROTEIN IN TESTIS	0.82	0
1445718_at	BM237480	WD REPEAT AND FYVE DOMAIN CONTAINING 3	0.88	0
1434585_at	BB667130	TUBBY LIKE PROTEIN 4	0.95	0
1427822_a_at	U20265	COATOMER PROTEIN COMPLEX, SUBUNIT GAMMA 2, ANTISENSE 2	0.98	0
1457634_at	BG093687	RIKEN CDNA 4930488E11 GENE	0.99	0
1426113_x_at	U07662	T-CELL RECEPTOR ALPHA CHAIN	0.99	0
1457455_at	BB430212	SUPPRESSOR OF HAIRY WING HOMOLOG 4 (DROSOPHILA)	1.05	0
1440764_at	AI552435	V-RAF MURINE SARCOMA 3611 VIRAL ONCOGENE HOMOLOG	1.05	0

Probes whose expressions are altered by at least a log₂ ratio of ± 0.5 in nine-month-old transgenic α -syn mice. P value for each probe is ≤ 0.05 and q-value is $\leq 20\%$.

Table 3

Probe Set ID	Genbank ID	Gene Name	log ₂ (TG/WT)	q-value
I436964_at	BB314814	DNA SEGMENT, CHR 7, ERATO DOI 715, EXPRESSED	-2.78	18.07
I442568_at	BM213120	RIKEN CDNA 3110039L19 GENE	-1.98	18.07
I443281_at	BB297094	RIKEN CDNA C030017F07 GENE	-1.61	8.66
I442742_at	BE994454	RIKEN CDNA 1700121J11 GENE	-1.55	17.17
I440773_at	BB209878	F-BOX ONLY PROTEIN 8	-1.37	8.51
I440163_at	BB392503	RIKEN CDNA 6030490B17 GENE	-1.33	8.51
I430660_at	AV291471	RIKEN CDNA 2610100B20 GENE	-1.31	11.70
I431248_at	BB637274	RIKEN CDNA 5031426D15 GENE	-1.26	8.66
I426413_at	BM116592	NEUROGENIC DIFFERENTIATION 1	-1.22	17.17
I45512_at	AI852151	GENE MODEL 879, (NCBI)	-1.20	8.66
I455956_x_at	AV310588	CYCLIN D2	-1.17	10.02
I429769_at	BI107300	PROTEIN GERANYLGERANYLTRANSFERASE TYPE I, BETA SUBUNIT	-1.12	8.66
I451301_at	BB633110	TROPOMODULIN 2	-1.06	8.51
I426721_s_at	BB707122	TCDD-INDUCIBLE POLY(ADP-RIBOSE) POLYMERASE	-1.02	0.00
I440304_at	BB085100	EXPRESSED SEQUENCE BB214985	-0.99	8.51
I455393_at	BB090937	CERULOPLASMIN	-0.95	17.17
I422795_at	AV273804	CULLIN 3	-0.95	12.54
I434216_a_at	BG070689	NUDIX (NUCLEOSIDE DIPHOSPHATE LINKED MOIETY X)-TYPE MOTIF 19	-0.95	18.07
I432944_at	AK013658	RIKEN CDNA 2900046L07 GENE	-0.94	0.00
I450061_at	BM120053	ECTODERMAL-NEURAL CORTEX 1	-0.93	8.66
I440534_at	BB177862	RIKEN CDNA 6330403A02 GENE	-0.93	15.94
I457198_at	AV291009	NEUROFILIN 1	-0.92	17.17
I447314_at	AI480643	TRANSFORMING GROWTH FACTOR, BETA RECEPTOR III	-0.91	10.02
I429703_at	AV154947	RIKEN CDNA 2900072G11 GENE	-0.90	0.00
I442214_at	BG173293	NUCLEAR FACTOR I/B	-0.90	8.51
I433184_at	AK020162	RIKEN CDNA 6720477C19 GENE	-0.89	15.02
I424525_at	BC024515	GASTRIN RELEASING PEPTIDE	-0.89	13.49
I454286_at	AV007471	RIKEN CDNA 1110004M10 GENE	-0.88	12.54

Probe Set ID	Genbank ID	Gene Name	log ₂ (TC/WT)	q-value
1458263_at	BE979587	CUG TRIPLET REPEAT, RNA BINDING PROTEIN 2	-0.87	0.00
1453402_at	AV338754	RIKEN CDNA 6430500C12 GENE	-0.86	10.02
1459144_at	BB518323	HYPOTHETICAL PROTEIN LOC213817	-0.85	13.49
1430808_at	AK005461	TBC1 DOMAIN FAMILY, MEMBER 5	-0.85	10.02
1420965_a_at	BM120053	ECTODERMAL-NEURAL CORTEX 1	-0.84	10.66
1445391_at	AW554652	DIAPHANOUS HOMOLOG 1 (DROSOPHILA)	-0.83	19.25
1455914_at	AW554430	EXPRESSED SEQUENCE A987944	-0.82	19.25
1435129_at	AW495875	PROTEIN TYROSINE PHOSPHATASE 4A2	-0.81	10.02
1444557_at	BG060557	SMT3 SUPPRESSOR OF MIF TWO 3 HOMOLOG 1 (YEAST)	-0.81	8.66
1439940_at	AV328280	RIKEN CDNA 2900019G14 GENE	-0.80	19.25
1443526_at	BM204661	PHD FINGER PROTEIN 21A	-0.80	0.00
1439123_at	BB367687	PHD FINGER PROTEIN 21A	-0.79	15.02
1445708_x_at	AV158652	RIKEN CDNA 3110021A11 GENE	-0.79	19.25
1453068_at	BM226301	PR DOMAIN CONTAINING 2, WITH ZNF DOMAIN	-0.78	19.25
1446953_at	BG073799	TRANSCRIPTION FACTOR 4	-0.77	8.66
1445037_at	AV339661	RIKEN CDNA 6430510B20 GENE	-0.77	10.02
1437793_at	BB711615	TANKYRASE, TRF1-INTERACTING ANKYRIN-RELATED ADP-RIBOSE POLYMERASE 2	-0.76	8.51
1446374_at	BB460605	RHO GUANINE NUCLEOTIDE EXCHANGE FACTOR 10	-0.76	12.54
1442500_at	C77406	EXPRESSED SEQUENCE C77406	-0.76	10.02
1460186_at	AV061337	RIKEN CDNA 1810073M23 GENE	-0.76	19.25
1441538_at	BB267954	RIKEN CDNA B930011P16 GENE	-0.75	17.17
1442409_at	BB166984	DNA SEGMENT, CHR 9, WAYNE STATE UNIVERSITY 90, EXPRESSED	-0.74	19.25
1429051_s_at	BE825056	SRY-BOX CONTAINING GENE 11	-0.74	13.49
1444472_at	BB154631	SNF1-LIKE KINASE 2	-0.74	13.49
1440728_at	BB283560	POTASSIUM LARGE CONDUCTANCE CALCIUM-ACTIVATED CHANNEL, SUBFAMILY M, ALPHA MEMBER 1	-0.74	18.07
1437180_at	BE824567	RIKEN CDNA 6530403A03 GENE	-0.73	0.00
1439169_at	BB471557	EUKARYOTIC TRANSLATION INITIATION FACTOR 4E NUCLEAR IMPORT FACTOR 1	-0.73	13.49
1449068_at	X98096	ZINC FINGER PROTEIN 148	-0.72	8.66
1434835_at	BM230523	CDNA SEQUENCE BC037674	-0.72	17.17
1432352_at	AK014201	RIKEN CDNA 5730405I09 GENE	-0.71	17.17
1446159_at	BB044011	P21 (CDKN1A)-ACTIVATED KINASE 7	-0.71	8.51

Probe Set ID	Genbank ID	Gene Name	log2 (TCG/WT)	q-value
1454510_at	AK013612	RIKEN CDNA 2900034C19 GENE	-0.71	8.51
1444785_at	AI503808	SLOAN-KETTERING VIRAL ONCOGENE HOMOLOG	-0.71	12.54
1442659_at	BB244656	PROTACADHERIN 9	-0.70	13.49
1423433_at	BG069917	TROVE DOMAIN FAMILY, MEMBER 2	-0.69	8.51
1448282_at	BC006750	PLEIOTROPIC REGULATOR 1, PRL1 HOMOLOG (ARABIDOPSIS)	-0.69	11.70
1460123_at	AW541072	G PROTEIN-COUPLED RECEPTOR 1	-0.69	17.17
1453864_at	AK014097	RIKEN CDNA 3110030G19 GENE	-0.69	19.25
1445847_at	BE948333	NEUROTROPHIC TYROSINE KINASE, RECEPTOR, TYPE 2	-0.69	18.07
1459219_at	BM120546	RAP GUANINE NUCLEOTIDE EXCHANGE FACTOR 2	-0.68	10.02
1458469_at	BB205662	CASITAS B-LINEAGE LYMPHOMA B	-0.68	12.54
1459238_at	AV319481	PHOSPHOLAMBAN	-0.68	8.51
1441072_at	BB353142	CUG TRIPLET REPEAT, RNA BINDING PROTEIN 2	-0.67	10.02
1416892_s_at	BC021353	RIKEN CDNA 3110001A13 GENE	-0.67	8.51
1459734_at	BB737833	PROTEASOME (PROSOME, MACROPAIN) 26S SUBUNIT, NON-ATPASE, 14	-0.67	17.17
1430675_at	BM932606	RIKEN CDNA 2900055120 GENE	-0.67	18.07
1434378_a_at	BG868949	MAX DIMERIZATION PROTEIN 4	-0.67	8.51
1436248_at	AW120749	RAS PROTEIN ACTIVATOR LIKE 2	-0.67	8.51
1441487_at	BB197646	TRIPARTITE MOTIF PROTEIN 2	-0.66	18.07
1442679_at	BM241898	MITOGEN ACTIVATED PROTEIN KINASE KINASE 4	-0.66	17.17
1451506_at	BB280300	MYOCYTE ENHANCER FACTOR 2C	-0.66	15.02
1428936_at	BI080417	ATPASE, CA++ TRANSPORTING, PLASMA MEMBRANE 1	-0.66	10.02
1438282_at	AV344830	SYNAPTOTAGMIN 1	-0.66	10.66
1434178_at	AV297525	MYELOID/LYMPHOID OR MIXED-LINEAGE LEUKEMIA 3	-0.66	13.58
1454424_at	AK011729	RIKEN CDNA 2610040L17 GENE	-0.66	8.66
1455799_at	BB751387	RAR-RELATED ORPHAN RECEPTOR BETA	-0.66	8.51
1443508_at	BB131767	EXPRESSED SEQUENCE BB075781	-0.65	17.17
1420579_s_at	NM_021050	CYSTIC FIBROSIS TRANSMEMBRANE CONDUCTANCE REGULATOR HOMOLOG	-0.65	12.54
1458376_at	BE648536	RIKEN CDNA B930025B16 GENE	-0.65	18.07
1458690_at	BB079254	MEMBRANE-ASSOCIATED RING FINGER (C3HC4) 7	-0.65	15.02
1439209_at	BB540782	TRANSCRIPTION FACTOR 12	-0.65	12.54
1416129_at	NM_133753	ERBB RECEPTOR FEEDBACK INHIBITOR 1	-0.65	8.66

Probe Set ID	Genbank ID	Gene Name	log2 (TCG/WT)	q-value
1443260_at	BB055155	MYELOID ECOTROPIC VIRAL INTEGRATION SITE 1	-0.64	11.70
1437064_at	AV232123	ANDROGEN RECEPTOR	-0.64	17.17
1418955_at	NM_009567	ZINC FINGER PROTEIN 93	-0.64	8.51
1454743_at	BQ177153	NUCLEOPORIN 205	-0.64	10.66
1435505_at	BB698273	DYSTROPHIA MYOTONICA-CONTAINING WD REPEAT MOTIF	-0.64	15.02
1445340_at	BB156822	PAM, HIGHWIRE, RPM 1	-0.64	0.00
1431482_at	AV153257	RIKEN CDNA 2900012M01 GENE	-0.63	18.07
1426546_at	BQ179435	TESTIS-SPECIFIC KINASE 2	-0.62	15.94
1444409_at	BQ176035	RABPHILIN 3A-LIKE (WITHOUT C2 DOMAINS)	-0.62	10.66
1420136_a_at	AI427540	OPIOID GROWTH FACTOR RECEPTOR-LIKE 1	-0.62	19.25
1441231_at	BB233964	GENE MODEL 1167, (NCBI)	-0.62	15.94
1456896_at	BB053380	RIKEN CDNA 6720462K09 GENE	-0.62	18.07
1454922_at	BM246326	EXPRESSED SEQUENCE AI553587	-0.62	13.49
1456413_at	BB235927	PHOSPHODIESTERASE 4D INTERACTING PROTEIN	-0.61	8.66
1441654_at	BB345762	GUANINE NUCLEOTIDE BINDING PROTEIN, ALPHA 10	-0.61	19.25
1428616_at	BG075140	ZINC FINGER PROTEIN 131	-0.61	17.17
1459947_at	BB007036	THIOREDOXIN DOMAIN CONTAINING 5	-0.61	15.02
1444516_at	AI503166	PROTEIN TYROSINE PHOSPHATASE, RECEPTOR-TYPE, F INTERACTING PROTEIN, BINDING PROTEIN 2	-0.61	13.58
1438245_at	BI664122	NUCLEAR FACTOR I/B	-0.61	8.66
1435663_at	AI646838	ESTROGEN RECEPTOR 1 (ALPHA)	-0.60	18.07
1430195_at	BF662697	RIKEN CDNA 2810043O03 GENE	-0.60	15.94
1437426_at	BM240080	RIKEN CDNA 1110067P07 GENE	-0.60	8.66
1437984_x_at	BB461609	HLA-B-ASSOCIATED TRANSCRIPT 1A	-0.59	17.17
1437339_s_at	BB241731	PROTEIN CONVERTASE SUBTILISIN/KEXIN TYPE 5	-0.59	18.07
1439106_at	AV320128	ZINC FINGER PROTEIN 462	-0.59	10.66
1457019_s_at	BB241474	RIKEN CDNA 3110030G19 GENE	-0.59	13.49
1417644_at	BC021484	SARCOSPAN	-0.59	18.07
1437784_at	AW550878	CBFA2T1 IDENTIFIED GENE HOMOLOG (HUMAN)	-0.59	17.17
1435474_at	AV117817	TAF5 RNA POLYMERASE II, TATA BOX BINDING PROTEIN (TBP)-ASSOCIATED FACTOR	-0.59	18.07
1445774_at	BB479183	POTASSIUM LARGE CONDUCTANCE CALCIUM-ACTIVATED CHANNEL, SUBFAMILY M, ALPHA MEMBER 1	-0.59	8.51
1440020_at	BB212045	EXPRESSED SEQUENCE AI195322	-0.59	10.66

Probe Set ID	Genbank ID	Gene Name	log2 (TCG/WT)	q-value
1438352_at	BM217851	MICROTUBULE-ASSOCIATED PROTEIN, RP/EB FAMILY, MEMBER 2	-0.58	18.07
1430058_at	AK016826	STEM-LOOP BINDING PROTEIN	-0.58	15.94
1430291_at	AK004325	RIKEN CDNA 1110060D06 GENE	-0.58	19.25
1452092_at	AK019474	RIKEN CDNA 4631426J05 GENE	-0.58	12.54
1446670_at	BM241206	CUG TRIPLET REPEAT, RNA BINDING PROTEIN 2	-0.57	13.58
1444186_at	BB519728	RIKEN CDNA 2900006B13 GENE	-0.57	15.02
1421948_a_at	BB042564	RIKEN CDNA 2610507L03 GENE	-0.57	18.07
1441100_at	BB496952	MBT DOMAIN CONTAINING 1	-0.57	15.94
1446272_at	BB200545	RIKEN CDNA 6430598J10 GENE	-0.57	11.70
1443161_at	BM240648	TRICHORHINOPHALANGEAL SYNDROME 1 (HUMAN)	-0.57	8.66
1446750_at	BB524087	IMPRINTED AND ANCIENT	-0.57	11.70
1439174_at	AI644595	UNC-5 HOMOLOG C (C. ELEGANS)	-0.57	10.02
1431228_s_at	AA120741	RIKEN CDNA 4930526I15 GENE	-0.56	15.02
1417818_at	BC014727	WW DOMAIN CONTAINING TRANSCRIPTION REGULATOR 1	-0.56	18.07
1444456_at	BB460570	RIKEN CDNA 9030425P06 GENE	-0.56	13.49
1435433_at	BE688580	EXPRESSED SEQUENCE R75364	-0.56	18.07
1457716_at	BM235074	ZINC FINGER, A20 DOMAIN CONTAINING 1	-0.55	18.07
1453416_at	BE199211	GROWTH ARREST-SPECIFIC 2 LIKE 3	-0.55	15.02
1443337_at	BB531021	RIKEN CDNA B130020M22 GENE	-0.55	19.25
1442587_at	BB451946	RIKEN CDNA C030017F07 GENE	-0.55	19.25
1441545_at	BM243297	RIKEN CDNA 9230115F04 GENE	-0.55	12.54
1437676_at	AV279478	SPERM ASSOCIATED ANTIGEN 9	-0.54	8.51
1431402_at	BB626088	KIN OF IRRE LIKE 3 (DROSOPHILA)	-0.54	19.25
1447204_at	BB337014	MITOGEN-ACTIVATED PROTEIN KINASE KINASE KINASE 5	-0.54	10.02
1418168_at	BB223737	ZINC FINGER, CCHC DOMAIN CONTAINING 14	-0.53	8.51
1440770_at	AI505544	B-CELL LEUKEMIA/LYMPHOMA 2	-0.53	19.25
1457781_at	BG063584	KCNQ1 OVERLAPPING TRANSCRIPT 1	-0.53	10.02
1434829_at	BB487560	CASITAS B-LINEAGE LYMPHOMA	-0.53	8.51
1442924_at	BB430910	CALSYNTENIN 2	-0.53	15.02
1422486_a_at	AK004804	MAD HOMOLOG 4 (DROSOPHILA)	-0.53	17.17
1454182_at	AK019946	RIKEN CDNA 5430417C01 GENE	-0.53	11.70

Probe Set ID	Genbank ID	Gene Name	log2 (TCG/WT)	q-value
I438535_at	BB523030	NEURONAL DIFFERENTIATION RELATED PROTEIN	-0.52	19.25
I429652_at	AK009976	RIKEN CDNA I190002C06 GENE	-0.52	8.51
I439626_at	BB209799	TROPOMODULIN 3	-0.52	15.02
I439192_at	BB524078	NEURO-ONCOLOGICAL VENTRAL ANTIGEN 2	-0.52	19.25
I415925_a_at	NM_053074	NUCLEOPORIN 62	-0.52	13.58
I454852_at	BB795189	TRANS-ACTING TRANSCRIPTION FACTOR 1	-0.52	15.94
I433543_at	BI690018	ANILLIN, ACTIN BINDING PROTEIN (SCRAPS HOMOLOG, DROSOPHILA)	-0.52	13.49
I442320_at	BB364132	HYPOTHETICAL LOC553096	-0.52	19.25
I453371_at	AK012860	RIKEN CDNA 4930535B03 GENE	-0.51	18.07
I455225_at	AV237615	SYNAPTIC NUCLEAR ENVELOPE 1	-0.51	18.07
I433641_at	AV014063	MAD HOMOLOG 5 (DROSOPHILA)	-0.51	17.17
I440311_at	BB259710	SORBIN AND SH3 DOMAIN CONTAINING 1	-0.51	19.25
I436795_at	BM247060	RIKEN CDNA 9630058J23 GENE	-0.51	10.66
I433803_at	BQ032637	JANUS KINASE 1	-0.51	18.07
I458053_at	BB051811	ABL-INTERACTOR 2	-0.51	18.07
I429918_at	AK018317	RIKEN CDNA 6530403F17 GENE	-0.51	11.70
I443020_at	BB027719	RIKEN CDNA F830020C16 GENE	-0.51	8.66
I455854_a_at	BB053082	SLINGSHOT HOMOLOG 1 (DROSOPHILA)	-0.50	19.25
I446965_at	BG067578	RHO GUANINE NUCLEOTIDE EXCHANGE FACTOR 12	-0.50	18.07
I441635_at	BM202709	NUCLEAR RECEPTOR SUBFAMILY 6, GROUP A, MEMBER 1	-0.50	18.07
I418000_a_at	NM_008410	INTEGRAL MEMBRANE PROTEIN 2B	-0.50	8.51
I428368_at	BM213829	RHO GTPASE ACTIVATING PROTEIN 21	0.51	15.94
I418506_a_at	NM_011563	PEROXIREDOXIN 2	0.55	17.17
I416028_a_at	NM_008258	HEMATOLOGICAL AND NEUROLOGICAL EXPRESSED SEQUENCE 1	0.58	15.02
I426448_at	BM199789	PRAJA1, RING-H2 MOTIF CONTAINING	0.58	15.02
I420828_s_at	NM_011739	TYROSINE 3-MONOOXYGENASE/TRYPTOPHAN 5-MONOOXYGENASE ACTIVATION PROTEIN, THETA POLYPEPTIDE	0.65	15.94
I435258_at	AW551717	DNA SEGMENT, CHR 2, ERATO DOI 217, EXPRESSED	0.66	15.02
I421873_s_at	NM_009000	RAB24, MEMBER RAS ONCOGENE FAMILY	0.67	15.02
I450243_a_at	NM_030598	DOWN SYNDROME CRITICAL REGION GENE 1-LIKE 1	0.72	15.02
I433799_at	AV366849	RETINOL DEHYDROGENASE 13 (ALL-TRANS AND 9-CIS)	0.79	10.66

Probe Set ID	Genbank ID	Gene Name	log2 (TG/WT)	q-value
1415773_at	BF118393	NUCLEOLIN	0.85	10.66
1455868_a_at	BB387906	TUBULIN, GAMMA COMPLEX ASSOCIATED PROTEIN 2	0.86	15.02
1428370_at	AK018264	RIKEN CDNA 1500011B03 GENE	1.02	7.49
1448732_at	M14222	CATHEPSIN B	1.25	15.02
AFFX-GapdhM	U00109.2	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)	1.57	15.02

Table 4Functional profiling of genes altered in nine-month-old transgenic α -syn mice.

GO annotation term	% of regulated genes	p value
protein binding	28.94%	6.81E-06
regulation of biological process	25.11%	3.05E-05
regulation of cellular process	21.70%	8.93E-04
regulation of metabolism	17.02%	0.0013
regulation of physiological process	21.28%	0.0013
transcriptional activator activity	3.40%	0.0014
biopolymer modification	12.77%	0.0018
regulation of cellular physiological process	20.43%	0.0018
transcription regulator activity	10.21%	0.0021
cytoskeletal protein binding	4.68%	0.0022
DNA binding	13.62%	0.0026
intracellular signaling cascade	8.94%	0.0031
biopolymer metabolism	17.45%	0.0034
transcription factor activity	8.09%	0.0035
regulation of transcription	14.89%	0.0039
protein modification	11.91%	0.0044
zinc ion binding	12.34%	0.0044
binding	48.09%	0.0045
regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	14.89%	0.0047
transcription	14.89%	0.0064
regulation of transcription, DNA-dependent	14.04%	0.0064
regulation of cellular metabolism	15.32%	0.0076
transcription, DNA-dependent	14.04%	0.0082
cellular physiological process	48.94%	0.0099
cellular process	54.47%	0.012
enzyme linked receptor protein signaling pathway	3.40%	0.015
nucleic acid binding	17.87%	0.017
ubiquitin cycle	4.68%	0.021
GTPase regulator activity	3.40%	0.021
transition metal ion binding	13.19%	0.025
primary metabolism	34.47%	0.026
protein amino acid phosphorylation	5.53%	0.027
nucleobase, nucleoside, nucleotide and nucleic acid metabolism	18.30%	0.029
macromolecule metabolism	22.55%	0.035
negative regulation of biological process	6.38%	0.039
signal transduction	14.47%	0.040
enzyme regulator activity	5.11%	0.040
negative regulation of cellular process	5.96%	0.043
cell communication	15.32%	0.043

To determine functional categories associated with α -syn overexpression, we used the DAVID 2007 functional annotation tool (<http://david.abcc.ncifcrf.gov/home.jsp>), which classifies each probe into one or more functional categories using annotations from over 40 ontological databases and then determines which functional categories are represented in the data set more frequently than expected by chance alone. We limited the functional annotation chart generated by DAVID by classification of genes to functional categories defined by Gene Ontology Database annotations. Only probes with a q value $\leq 20\%$ were used for the ontology analysis. We considered significant those categories with a p value ≤ 0.05 and contained at least 3% of the regulated genes. All molecular function and biological process annotation terms that fulfilled these criteria were included in the table regardless of how broad or narrow the annotation was. Therefore, we included broad categories, such as “binding,” as well as more specific and informative categories, such as “zinc ion binding.” The website QuickGO (<http://www.ebi.ac.uk/ego>) describes the GO annotation categories with their meanings and hierarchical relationships.

Table 5Functional annotation clustering in nine-month-old transgenic α -syn mice.

Functional Group 1		Median: 0.0034982 Geometric mean: 0.0022242	
Database	Term	%	p value
GOTERM_BP_ALL	regulation of biological process	25.11%	3.05E-05
GOTERM_CC_ALL	nucleus	24.26%	5.12E-05
SP_PIR_KEYWORDS	transcription regulation	10.64%	1.03E-04
SP_PIR_KEYWORDS	transcription	10.21%	2.97E-04
SP_PIR_KEYWORDS	nuclear protein	17.45%	6.26E-04
GOTERM_BP_ALL	regulation of cellular process	21.70%	8.93E-04
GOTERM_BP_ALL	regulation of metabolism	17.02%	0.0013
GOTERM_BP_ALL	regulation of physiological process	21.28%	0.0013
GOTERM_BP_ALL	regulation of cellular physiological process	20.43%	0.0018
GOTERM_MF_ALL	transcription regulator activity	10.21%	0.0021
GOTERM_MF_ALL	DNA binding	13.62%	0.0026
GOTERM_MF_ALL	transcription factor activity	8.09%	0.0035
GOTERM_BP_ALL	regulation of transcription	14.89%	0.0039
GOTERM_BP_ALL	regulation of nucleobase, nucleoside, nucleotide and nucleic acid metabolism	14.89%	0.0047
GOTERM_BP_ALL	transcription	14.89%	0.0064
GOTERM_BP_ALL	regulation of transcription, DNA-dependent	14.04%	0.0064
SP_PIR_KEYWORDS	dna-binding	8.51%	0.0067
GOTERM_BP_ALL	regulation of cellular metabolism	15.32%	0.0076
GOTERM_BP_ALL	transcription, DNA-dependent	14.04%	0.0082
GOTERM_MF_ALL	nucleic acid binding	17.87%	0.0166
GOTERM_CC_ALL	intracellular membrane-bound organelle	28.51%	0.0203
GOTERM_CC_ALL	membrane-bound organelle	28.51%	0.0213
GOTERM_BP_ALL	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	18.30%	0.0287
Functional Group 2		Median: 0.00977724 Geometric mean: 0.00506997	
Database	Term	%	p value
SP_PIR_KEYWORDS	transcription regulation	10.64%	1.03E-04
GOTERM_MF_ALL	transcription regulator activity	10.21%	0.0021
GOTERM_CC_ALL	protein complex	13.19%	0.0043
GOTERM_CC_ALL	nucleoplasm	5.53%	0.0070
GOTERM_CC_ALL	nuclear lumen	5.96%	0.0125
GOTERM_CC_ALL	organelle lumen	6.38%	0.0149
GOTERM_CC_ALL	membrane-enclosed lumen	6.38%	0.0149
GOTERM_CC_ALL	transcription factor complex	4.26%	0.0239
Functional Group 3		Median: 0.01106735 Geometric mean: 0.00732782	
Database	Term	%	p value
GOTERM_CC_ALL	nucleus	24.26%	5.12E-05
GOTERM_CC_ALL	intracellular	38.72%	5.43E-04
SP_PIR_KEYWORDS	nuclear protein	17.45%	6.26E-04

Functional Group 1		Median: 0.0034982 Geometric mean: 0.0022242	
Database	Term	%	p value
GOTERM_CC_ALL	intracellular organelle	33.19%	0.0030
GOTERM_CC_ALL	organelle	33.19%	0.0031
GOTERM_MF_ALL	binding	48.09%	0.0045
GOTERM_CC_ALL	cell	49.36%	0.0081
GOTERM_BP_ALL	cellular physiological process	48.94%	0.0099
GOTERM_BP_ALL	cellular process	54.47%	0.0123
GOTERM_CC_ALL	intracellular membrane-bound organelle	28.51%	0.0203
GOTERM_CC_ALL	membrane-bound organelle	28.51%	0.0213
GOTERM_BP_ALL	primary metabolism	34.47%	0.0264
GOTERM_BP_ALL	nucleobase, nucleoside, nucleotide and nucleic acid metabolism	18.30%	0.0287
GOTERM_BP_ALL	cellular metabolism	34.89%	0.0533
GOTERM_BP_ALL	physiological process	51.06%	0.0724
GOTERM_BP_ALL	metabolism	36.60%	0.0770
Functional Group 4		Median: 0.0397 Geometric mean: 0.0175	
Database	Term	%	p value
GOTERM_BP_ALL	intracellular signaling cascade	8.94%	0.0031
GOTERM_BP_ALL	signal transduction	14.47%	0.0397
GOTERM_BP_ALL	cell communication	15.32%	0.0435
Functional Group 5		Median: 0.023 Geometric mean: 0.0285	
Database	Term	%	p value
SP_PIR_KEYWORDS	Zinc-finger	8.94%	0.0038
GOTERM_MF_ALL	Zinc ion binding	12.34%	0.0044
SP_PIR_KEYWORDS	metal binding	11.91%	0.0115
SP_PIR_KEYWORDS	Zinc	9.36%	0.0209
GOTERM_MF_ALL	transition metal ion binding	13.19%	0.0245
GOTERM_MF_ALL	cation binding	15.74%	0.1200
GOTERM_MF_ALL	metal ion binding	16.17%	0.1915
GOTERM_MF_ALL	ion binding	16.17%	0.1915
Functional Group 6		Median: 0.027 Geometric mean: 0.033	
Database	Term	%	p value
GOTERM_MF_ALL	steroid hormone receptor activity	2.13%	0.0033
GOTERM_MF_ALL	ligand-dependent nuclear receptor activity	2.13%	0.0035
SMART_NAME	SM00399:ZnF_C4	2.13%	0.0053
SMART_NAME	SM00430:HOLI	2.13%	0.0063
INTERPRO_NAME	IPR001628:Nuclear hormone receptor, DNA-binding	1.70%	0.0093
INTERPRO_NAME	IPR000536:Nuclear hormone receptor, ligand-binding	1.70%	0.0105
UP_SEQ_FEATURE	DNA-binding region:Nuclear receptor	1.70%	0.0107
UP_SEQ_FEATURE	zinc finger region:NR C4-type	1.70%	0.0107
GOTERM_BP_ALL	steroid hormone receptor signaling pathway	1.28%	0.0270
GOTERM_BP_ALL	intracellular receptor-mediated signaling pathway	1.28%	0.0364

Functional Group 1	Median: 0.0034982 Geometric mean: 0.0022242		
Database	Term	%	p value
INTERPRO_NAME	IPR001723:Steroid hormone receptor	1.28%	0.0727
GOTERM_MF_ALL	steroid binding	1.28%	0.0922
GOTERM_MF_ALL	sequence-specific DNA binding	3.40%	0.1219
SP_PIR_KEYWORDS	zinc finger	1.28%	0.2049
SP_PIR_KEYWORDS	DNA binding	2.13%	0.2837
GOTERM_MF_ALL	lipid binding	1.70%	0.4329
SP_PIR_KEYWORDS	receptor	4.68%	0.7521