Molecular Therapy
Letter to the Editor

Alpha-Synuclein mRNA Is Not Increased in Sporadic PD and Alpha-Synuclein Accumulation Does Not Block GDNF Signaling in Parkinson's Disease and Disease Models

Glial cell line-derived neurotrophic factor (GDNF) protein and gene therapy are currently under clinical investigation in Parkinson's disease (PD) patients. GDNF has profound protective effects on dopamine neurons in numerous neurotoxicant PD models in rats and non-human primates.[1,2] However, in another PD model generated by recombinant adeno-associated virus (rAAV) vector transduction of human alpha-synuclein (α-syn) into the midbrain of the rat GDNF reportedly failed to exert robust neuroprotection.[3] The marked discrepancy in preclinical therapeutic efficacy between these models of PD may have relevance to the ongoing clinical studies in sporadic PD.

In a recent rat study, Decressac et al.[4] tested whether nigrostriatal overexpression of α-syn impairs GDNF signaling, rendering nigral dopamine (DA) neurons insensitive to its trophic effect. The authors used a rAAV rat model overexpressing human wild-type α-syn to levels at least 4-fold higher than endogenous rat α-syn levels.[4,5] The authors reported that the intracellular signaling response to GDNF was blocked in nigral DA neurons. The transcription factor nuclear receptor related 1 (Nurr1) and downstream target GDNF receptor tyrosine kinase (RET) were downregulated at both the transcriptional and translational levels in the rat midbrain. Overall, these data suggest that markedly increased α-syn in the rat is toxic and may result in the disruption of GDNF signaling.

Caution must be used in translating these results to clinical PD studies. The predictive value of the rAAV α-syn overexpression rat model to sporadic PD is unclear. For example, the marked overexpression of α-syn in the rAAV model fails to reproduce the pathological state of sporadic PD. Indeed, two studies of human tissue reported that α-syn mRNA expression was *decreased* in sporadic PD patients compared to age-matched controls.[6,7] The decrease of α-syn mRNA expression was due to lower mRNA expression in individual nigral DA neurons rather than to reduction in nigral cell numbers. Kinsbury et al.[6] further showed that α-syn expression decreased gradually as the disease progressed. Further, preclinical models in which synucleinopathy is induced by intracerebral injection of pre-formed α-syn fibrils similarly report a decrease in soluble α-syn in neurons possessing Lewy body (LB)-like α-syn inclusions.[8] There is also precedence for discordant observations to be made when modeling PD in rats versus primates. Viral delivery of GDNF to the nigrostriatal pathway *decreases* tyrosine hydroxylase (TH) gene expression in rats[9] but *increases* TH in primates.[1]

Herein we evaluate the association of α-syn gene SNCA with GDNF signaling molecules in PD patient brain samples, α-syn transgenic mice, and AAV-mediated α-syn transgenic rats. We demonstrate that α-syn mRNA is not increased in sporadic PD and α-syn accumulation does not block expression of GDNF signaling molecules in PD and disease models.

We observe no increase in α-syn gene (*SNCA*) expression in DA neurons laser-captured from the substantia nigra (SN) of 10 PD subjects and 8 age-matched control subjects (Gene Expression Omnibus [GEO]: GSE20141) (Figure 1A, left panel). Laser-captured samples eliminate the differences in DA neuron numbers between patients and controls. In this microarray dataset, α-syn gene (*SNCA*) expression (column ID: 236081; Figure 1B, left table) was significantly downregulated in PD subjects compared to controls ($p = 0.035$, false discovery rate [FDR] = 0.369, fold change = −4.4).

To assess whether SN neurons with high α-syn expression might selectively degenerate in the early stages of the disease compared to neurons with reduced expression represent, we analyzed another set of microarray data with samples from early preclinical PD subjects (GEO: GSE20159). These SN samples consisted of 16 cases with subclinical, PD-related, α-syn-positive, incidental LB disease and 17 age-, sex-, and postmortem interval-matched controls. In this dataset, we again failed to detect an increase in α-syn gene expression in the subclinical PD group compared to control (Figures 1A, right panel, and 1B, right table). The subclinical PD microarray analysis indicates that SN neurons with high α-syn gene expression are not present in the early stages of the disease.

Decressac et al.[4] showed a downregulation of Nurr1 and RET at the transcriptional and translational level in the rAAV-α-syn-transduced rats, which contributed to the disruption of GDNF signaling. However, by analyzing the microarray data (GEO: GSE20159 and GSE20141), which covered subclinical and clinical stages of diseases; separately, we failed to detect any significant decrease in gene expression levels for Nurr1, RET, and other associated genes (PARK7, SLC18A2, BDNF, DDC, TH, MEF2D, and PITX3) in the sporadic PD patients (Figures 1A and 1B). Furthermore, we analyzed the association between NR4A2 (Nurr1) and SNCA gene expression in individual PD patients and did not find any significant correlation between the two genes (Figure 1C), suggesting that SNCA may not affect NR4A2 expression at the transcriptional level.

To extend our analysis, we examined transgenic α-syn mice. We previously published microarray data from synuclein transgenic mice wherein a TH promoter drove overexpression of either wild-type α-syn (THsynWT) or doubly mutated (A30P and A53T) synuclein (THsynDM).[10] We observed no significant downregulation in gene expression for Nurr1 and downstream target genes, including RET, PARK7, SLC18A2, BDNF, DDC, MEF2D, and PITX3, in the transgenic mice. We further validated the gene expression for Nurr1 and RET by RT-PCR and protein expression for

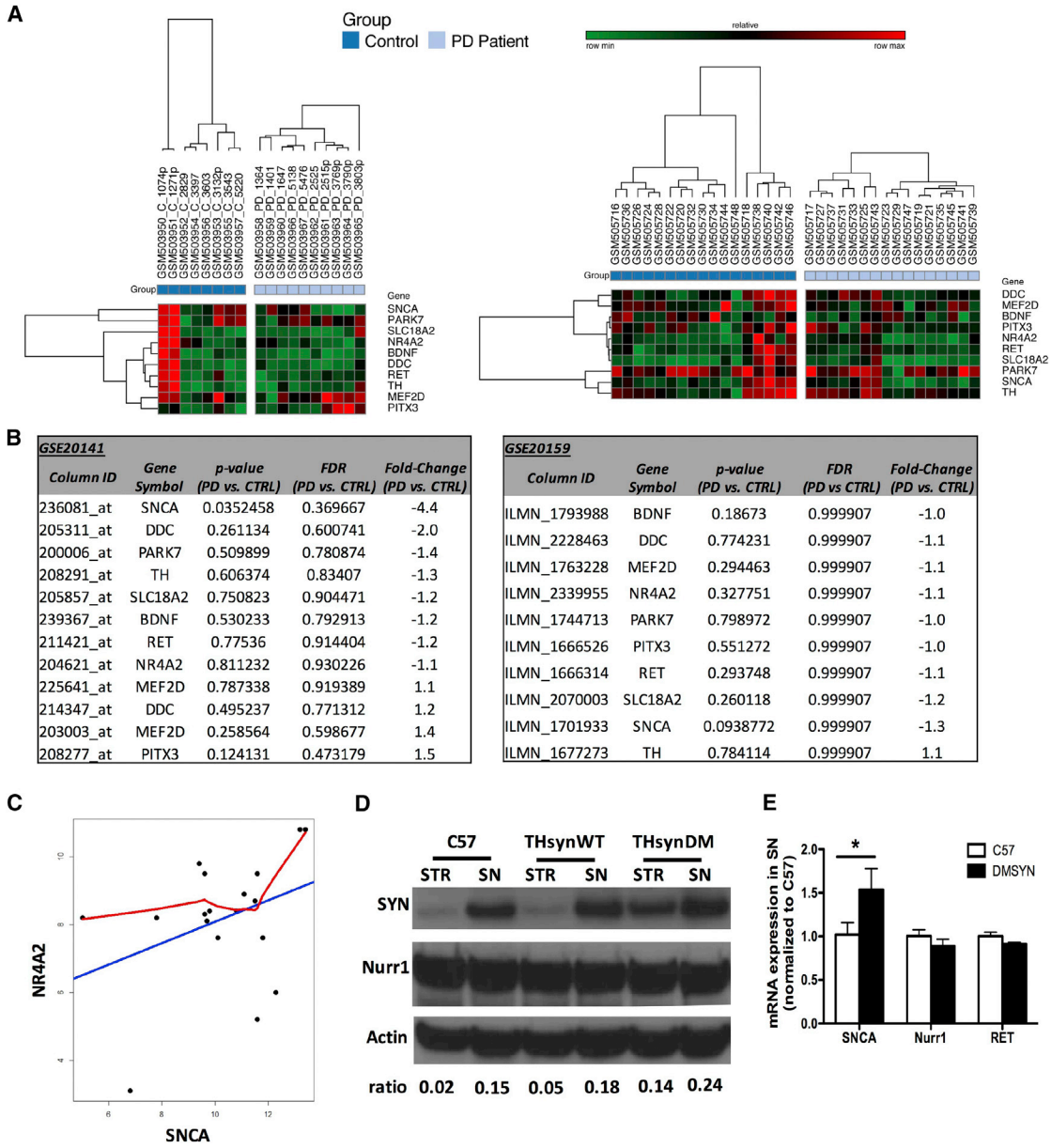

Figure 1. Nurr1 and Associated Genes Expression in Sporadic PD Patients and α-syn Transgenic Mice

(A) Hierarchical clustering heatmaps for microarray datasets GEO: GSE20141 (left panel) and GSE20159 (right panel). (B) Expression of genes of interest in the microarray datasets (GEO: GSE20141, left table; GEO: GSE20159, right table). The p values as well as Benjamini-Hochberg false discovery rates (FDRs) and fold change were reported for every single gene. (C) The scatterplot was drawn from the robust multi-array average (RMA)-processed and logarithm 2-transformed microarray intensity data of 18 samples. The x axis represents SNCA as an independent variable, and the y axis represents NR4A2 as a dependent variable. The straight blue line is the linear regression result between the two genes SNCA and NR4A2, and the red line is gained by the local weighted scatterplot smoothing (LOWESS) regression analysis. The correlation coefficient is 0.4084 with p = 0.1442. (D) α-syn and Nurr1 protein expression in the striatum and nigra of C57, THsynWT, and THsynDM mice. Striatum (STR) and nigra (SN) tissues were microdissected from 6-month-old transgenic mice with expression of wild-type synuclein (THsynWT) or doubly mutated α-syn (A30P & A53T; THsynDM) or age-controlled C57. The expression of α-syn and Nurr1 was determined by western blot analysis. The signal density ratio of α-syn over action was quantified. (E) α-syn, Nurr1, and RET gene expression in the SN were determined by qRT-PCR.

Nurr1 by western blotting and again failed to detect significant decreases for Nurr1 and RET at the transcript level and Nurr1 at the protein level in the α-syn transgenic mice compared to wild-type controls (Figures 1D and 1E).

Finally, we also used rAAV to overexpress α-syn in a manner very similar to Decressac et al.[4] rAAV expressing human wild-type

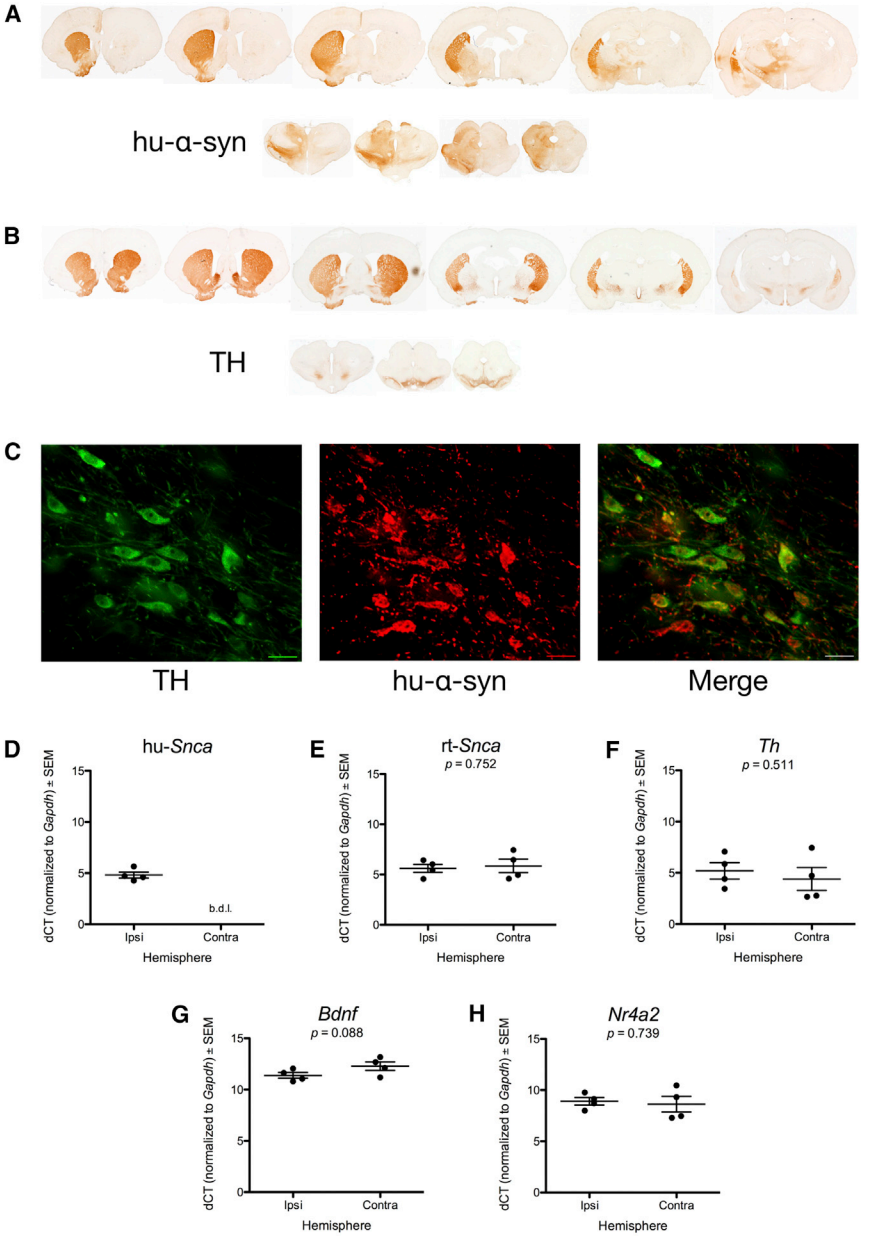

Figure 2. Moderate Viral Vector-Mediated α-syn Overexpression in the Rat Nigrostriatal System Does Not Decrease rat α-syn, BDNF, TH, or Nurr1 in the SN

Unilateral intranigral injections of recombinant rAAV2/5-α-syn results in human wild-type α-syn immunoreactivity in the nigrostriatal system. (A) Representative coronal sections demonstrating immmunoreactivity to human wild-type α-syn in the nigrostriatal hemisphere ipsilateral to injection. (B) Both hemispheres were labeled with antisera to tyrosine hydroxylase (TH). (C) Dual-label immunofluorescence identifies THir neurons of the SNpc that co-express human α-syn. A non-significant ~10% loss of SNpc neurons was observed at 8 weeks following injection. (D) SN tissue punches analyzed using qPCR demonstrate human α-syn transgene (hu-*Scna*) in the ipsilateral, but not the contralateral, SN. (E–H) No differences between the ipsilateral and contralateral SN were detected for rat α-syn (rt-*Snca*) (E), BDNF (*Bdnf*) (F), TH (*Th*) (G), or Nurr1 (*Nr4a2*) (H). Scale bars, 25 μm.

α-syn (rAAV2/5-α-syn, 2.2 × 10^{12} genome copies per ml) was injected unilaterally into the SN as described previously.[11] Eight weeks after nigral injection, brains were processed for immunohistochemistry for human wild-type α-syn (Figures 2A and 2C) or TH (Figures 2B and 2C). Stereological analysis of TH immunoreactive (THir) neurons in the SN pars compacta (SNpc) revealed a non-significant ~10% loss of SNpc neurons at 8 weeks following injection. The ipsilateral SNpc possessed an estimated 12,626 ± 411 THir neurons compared to 11,401 ± 561 remaining THir neurons in the contralateral SNpc ($t_{(8)} = 1.764$, $p > 0.05$). This modest level of degeneration is associated with an ~50% increase in human α-syn protein in the striatum.[11,12] In contrast, Decressac et al.[5] report an ~80% SNpc loss with transduction parameters, which resulted in an ~8-fold increase in α-syn protein in the striatum.

In a separate cohort of identical rAAV2/5-α-syn-injected rats, SN tissue ipsilateral and contralateral to rAAV2/5-α-syn were examined for human wild-type α-syn transcript levels (hu-*Snca*). Expression of human wild-type α-syn transcript was only evident in the ipsilateral SN, with none detected in the contralateral SN (Figure 2D). The SN was also examined for levels of endogenous rat wild-type α-syn (rt-*Snca*), brain-derived neurotrophic factor (*Bdnf*), TH (*Th*) and Nurr1 (NR4A2). No statistically significant differences were observed due to α-syn transduction and overexpression compared to the contralateral, non-transduced, control hemisphere.

The analysis of PD brain tissue, transgenic mouse, and rAAV-transduced rat data indicate that expression of the α-syn gene (*SNCA*) is not increased in sporadic PD and α-syn accumulation does not block GDNF signaling in PD and disease models.

The analysis of public human datasets indicates that expression of the α-syn gene (*SNCA*) is not increased in the nigral dopaminergic neurons in patients with sporadic PD, which aligns with the previous studies showing no increase of *SNCA* gene expression in sporadic PD enteric neurons,[13] CSF,[14] and blood.[15] Thus, accumulation of aggregated α-syn protein in sporadic PD is unlikely due to the enhanced *SNCA* gene expression, but, rather, is mediated by downstream failure to clear the protein, owing to either a breakdown in normal protein degradation processes and/or aberrant protein

misfolding/post-translational processes that render these conformers resistant to normal degradation processes. The analysis of public human datasets also shows no change in the transcription level of Nurr1, RET, and other associated genes (PARK7, SLC18A2, BDNF, DDC, TH, MEF2Ds and PITX3) in the sporadic PD patients as well as no positive correlation of expression of SNCA and the Nurr1 gene, NR4A2. These findings at least suggest that, at the transcriptional level, GDNF signaling molecules Nurr1, RET, and other associated genes are not affected in sporadic PD.

It is also important to assess the GDNF signaling molecule Nurr1 expression at the translational level in PD patients. Chu et al.[16] reported that SN neurons lacking α-syn inclusions from sporadic PD subjects displayed Nurr1 immunofluorescence optical density (OD) measurements that were similar to age-matched controls, whereas nigral neurons with α-syn LBs exhibited significantly decreased Nurr1 measurements. However those LB-bearing neurons were only, on average, representing 3.5%–15% of total SN neurons in sporadic PD patients, as shown by recent studies.[17] It therefore appears that the majority (>85%) of SN neurons from sporadic PD contain normal protein levels of Nurr1.

The variability of rAAV α-syn gene transfer to the rat nigrostriatal pathway to elicit changes in SN dopamine neuron numbers, alterations in signaling molecules and neurobehavioral changes suggests that vector type, particle number, packaging methods, and purifications are potential contributors. Systematic evaluation of each is required to delineate which of these may be the most important determinant of the observed variability. Because these studies all require forced expression of a gene product from a virus vector administrated intracerebrally, the clinical relevance of any such models to sporadic PD is limited, if at all relevant.

Based on our PD human data, the gene expression of GDNF signaling molecules, including RET and NUR1, are not downregulated disregarding α-syn accumulation. It should be noted that Hadaczek et al.[18] reported attenuated GNDF signaling as demonstrated by decreased phosphorylated RET (pRET) in neuronal cell lines and animals deficient in ganglio-series gangliosides. Whether pRET was decreased in PD brain remains to be investigated. Interestingly, AAV2-GDNF treatment was able to restore nigral TH-positive neurons and improve behavioral dysfunction in animals with ganglio-series gangliosides deficiency, suggesting that excess GDNF may suffice to maintain effective neuroprotective signaling, regardless of decreased pRET.

In summary, there are several important conclusions from these human transgenic mice and rAAV-transduced rat data. First, α-syn gene expression levels are not increased in the early stage of PD or in association with disease progression. Second, the majority of SN neurons in PD contain normal levels of Nurr1. Third, transgenic overexpression of human α-syn in mice did not result in downregulation of Nurr1 or RET. Fourth, rAAV transduction of rat SN producing a moderate increase in human α-syn did not result in downregulation of Nurr1, TH, or BDNF. We conclude that forced and marked overexpression of α-syn, as described in the rat rAAV model by Decressac et al.,[4] is not a relevant model for human sporadic PD. Given there is no evidence to indicate that patients with sporadic PD will be refractory to GDNF therapy, clinical equipoise is warranted for ongoing GDNF therapeutic trials.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Materials and Methods and can be found with this article online at http://dx.doi.org/10.1016/j.ymthe.2017.04.018.

Xiaomin Su,[1] D. Luke Fischer,[5] Xin Li,[2] Krystof Bankiewicz,[4] Caryl E. Sortwell,[5] and Howard J. Federoff[3]

[1]Department of Neuroscience, Georgetown University Medical Center, Washington DC 20057, USA; [2]Department of Biostatistics, Bioinformatics and Biomathematics, Georgetown University Medical Center, Washington, DC 20057, USA; [3]Department of Neurology, University of California at Irvine, Irvine, CA 92697, USA; [4]Department of Neurosurgery, University of California at San Francisco, San Francisco, CA 94143, USA; [5]Translational Science and Molecular Medicine, College of Human Medicine, Michigan State University, Grand Rapids, MI 48824, USA

http://dx.doi.org/10.1016/j.ymthe.2017.04.018

Correspondence: Howard J. Federoff, Department of Neurology, University of California at Irvine, Irvine, CA 92697, USA.
E-mail: federoff@uci.edu

REFERENCES

1. Kells, A.P., Eberling, J., Su, X., Pivirotto, P., Bringas, J., Hadaczek, P., Narrow, W.C., Bowers, W.J., Federoff, H.J., Forsayeth, J., and Bankiewicz, K.S. (2010). Regeneration of the MPTP-lesioned dopaminergic system after convection-enhanced delivery of AAV2-GDNF. J. Neurosci. *30*, 9567–9577.
2. Kirik, D., Rosenblad, C., Bjorklund, A., and Mandel, R.J. (2000). Long-term rAAV-mediated gene transfer of GDNF in the rat Parkinson's model: intrastriatal but not intranigral transduction promotes functional regeneration in the lesioned nigrostriatal system. J. Neurosci. *20*, 4686–4700.
3. Decressac, M., Ulusoy, A., Mattsson, B., Georgievska, B., Romero-Ramos, M., Kirik, D., and Björklund, A. (2011). GDNF fails to exert neuroprotection in a rat α-synuclein model of Parkinson's disease. Brain *134*, 2302–2311.
4. Decressac, M., Kadkhodaei, B., Mattsson, B., Laguna, A., Perlmann, T., and Björklund, A. (2012). α-Synuclein-induced down-regulation of Nurr1 disrupts GDNF signaling in nigral dopamine neurons. Sci. Transl. Med. *4*, 163ra156.
5. Decressac, M., Mattsson, B., Lundblad, M., Weikop, P., and Björklund, A. (2012). Progressive neurodegenerative and behavioural changes induced by AAV-mediated overexpression of α-synuclein in midbrain dopamine neurons. Neurobiol. Dis. *45*, 939–953.
6. Kingsbury, A.E., Daniel, S.E., Sangha, H., Eisen, S., Lees, A.J., and Foster, O.J. (2004). Alteration in alpha-synuclein mRNA expression in Parkinson's disease. Mov. Disord. *19*, 162–170.
7. Neystat, M., Lynch, T., Przedborski, S., Kholodilov, N., Rzhetskaya, M., and Burke, R.E. (1999). Alpha-synuclein expression in substantia nigra and cortex in Parkinson's disease. Mov. Disord. *14*, 417–422.
8. Osterberg, V.R., Spinelli, K.J., Weston, L.J., Luk, K.C., Woltjer, R.L., and Unni, V.K. (2015). Progressive aggregation of alpha-synuclein and selective degeneration of lewy inclusion-bearing neurons in a mouse model of parkinsonism. Cell Rep. *10*, 1252–1260.
9. Georgievska, B., Kirik, D., and Björklund, A. (2004). Overexpression of glial cell line-derived neurotrophic factor using a lentiviral vector induces time- and dose-dependent downregulation of tyrosine hydroxylase in the intact nigrostriatal dopamine system. J. Neurosci. *24*, 6437–6445.
10. Miller, R.M., Kiser, G.L., Kaysser-Kranich, T., Casaceli, C., Colla, E., Lee, M.K., Palaniappan, C., and Federoff, H.J. (2007). Wild-type and mutant alpha-synuclein induce a multi-component gene expression profile consistent with shared pathophysiology in different transgenic mouse models of PD. Exp. Neurol. *204*, 421–432.

11. Gombash, S.E., Manfredsson, F.P., Kemp, C.J., Kuhn, N.C., Fleming, S.M., Egan, A.E., Grant, L.M., Ciucci, M.R., MacKeigan, J.P., and Sortwell, C.E. (2013). Morphological and behavioral impact of AAV2/5-mediated overexpression of human wildtype alpha-synuclein in the rat nigrostriatal system. PLoS One. *8*, e81426.

12. Polinski, N.K., Gombash, S.E., Manfredsson, F.P., Lipton, J.W., Kemp, C.J., Cole-Strauss, A., Kanaan, N.M., Steece-Collier, K., Kuhn, N.C., Wohlgenant, S.L., and Sortwell, C.E. (2015). Recombinant adenoassociated virus 2/5-mediated gene transfer is reduced in the aged rat midbrain. Neurobiol. Aging *36*, 1110–1120.

13. Barrenschee, M., Zorenkov, D., Böttner, M., Lange, C., Cossais, F., Scharf, A.B., Deuschl, G., Schneider, S.A., Ellrichmann, M., Fritscher-Ravens, A., and Wedel, T. (2017). Distinct pattern of enteric phospho-alpha-synuclein aggregates and gene expression profiles in patients with Parkinson's disease. Acta Neuropathol. Commun. *5*, 1.

14. Mollenhauer, B., Locascio, J.J., Schulz-Schaeffer, W., Sixel-Döring, F., Trenkwalder, C., and Schlossmacher, M.G. (2011). α-Synuclein and tau concentrations in cerebrospinal fluid of patients presenting with parkinsonism: a cohort study. Lancet Neurol. *10*, 230–240.

15. Tan, E.K., Chandran, V.R., Fook-Chong, S., Shen, H., Yew, K., Teoh, M.L., Yuen, Y., and Zhao, Y. (2005). Alpha-synuclein mRNA expression in sporadic Parkinson's disease. Mov. Disord. *20*, 620–623.

16. Chu, Y., Le, W., Kompoliti, K., Jankovic, J., Mufson, E.J., and Kordower, J.H. (2006). Nurr1 in Parkinson's disease and related disorders. J. Comp. Neurol. *494*, 495–514.

17. Parkkinen, L., O'Sullivan, S.S., Collins, C., Petrie, A., Holton, J.L., Revesz, T., and Lees, A.J. (2011). Disentangling the relationship between lewy bodies and nigral neuronal loss in Parkinson's disease. J. Parkinsons Dis. *1*, 277–286.

18. Hadaczek, P., Wu, G., Sharma, N., Ciesielska, A., Bankiewicz, K., Davidow, A.L., Lu, Z.H., Forsayeth, J., and Ledeen, R.W. (2015). GDNF signaling implemented by GM1 ganglioside; failure in Parkinson's disease and GM1-deficient murine model. Exp Neurol. *263*, 177–189.

Unfounded Claims of Improved Functional Outcomes Attributed to Follistatin Gene Therapy in Inclusion Body Myositis

A recent study by Mendell et al.[1] in *Molecular Therapy* claims to have demonstrated clinical and biomarker efficacy for inclusion body myositis (IBM) from follistatin gene therapy. Although the authors are to be congratulated for performing a long and difficult study, its design could not possibly support this claim. Additionally, the publication reports a different primary outcome measure than the ClinicalTrials.gov registered primary outcome measure, uses post hoc analyses that bias efficacy evidence, presents safety data in a confusing manner, and misrepresents published IBM literature.

The study is an analysis of selected data obtained in one clinical trial[2] combined with data obtained from a neuromuscular clinical practice and analyzed using a post hoc-defined primary outcome measure (Figure 1A). The clinical trial is a phase 1A, open-label, single group assignment (there was no comparator or "control" group) study of 15 patients, 9 with IBM and 6 with Becker muscular dystrophy (BMD). Three IBM patients received unilateral quadriceps dosing and are not discussed. Analysis of 6 patients with BMD participating in the trial was previously published in *Molecular Therapy*.[3]

This new study reports on the remaining 6 IBM patients.[1] They received at least 4 potentially therapeutic interventions: follistatin gene therapy (AAV-FS344) into bilateral quadriceps muscles, high-dose prednisone for approximately 60 days, a prescribed and monitored exercise program, and the well-known placebo effects that come from both participation in a clinical study alone and the receipt of open-label candidate therapies with intended clinical efficacy. In addition, the authors use aggregate 6 min walk test data from 8 IBM patients drawn from a neuromuscular clinic as a comparator to make the claim that follistatin gene therapy has clinical efficacy.

However, it is impossible for the authors to make that conclusion. Because the "treated" group received 4 possibly therapeutic interventions and the "control" patients were not matched for any of these interventions, it is impossible to attribute any outcome differences between the two groups to any specific intervention. A hypothetical design that might have allowed such a conclusion is outlined in Figure 1B; such a design is typically reserved for phase 2 studies. The authors present circumstantial arguments as to why they attribute the apparent clinical efficacy to follistatin gene therapy rather than prednisone or exercise therapy: "A question could be raised regarding efficacy entirely related to exercise, but we believe this to be highly unlikely given the failure of exercise alone (including 10-m and 30-m walk, timed-up-and-go, stair climbing) to improve function in the absence of follistatin therapy." However, they neglected to cite a study[4] that did show statistically significant benefits from exercise for patients with IBM using 30-min walk time and stair climbing outcome measures.

Furthermore, the study did not control for placebo and related effects. Participants (patients and investigators) in this clinical trial were aware of the use of an intended therapeutic candidate based on cutting-edge science in an otherwise relentless progressive disease. Patient performance and its measurement, theoretically enhanced by placebo effects, was compared with patients from a neuromuscular clinic who had expectations of continued decline and whose performance and its measurement were theoretically reduced by nocebo responses. Empirically, placebo responses in IBM are readily apparent in several published IBM double-blind randomized clinical trials, and their magnitiude may exceed that seen in the current study (A.A. Amato et al., 2016, American College of Rheumatology Annual Meeting, abstract).[5,6]

The publication states that "The primary outcome for this trial was distance traveled for the 6-min walk test", yet the trial registration indicates its primary outcome measure is "Safety trial based on development of unacceptable toxicity defined as the occurrence of any Grade III or higher treatment-related toxicities Time Frame: 2 years." Another