reliable measurements of both optical and behavioral signals were obtained simultaneously (*26*).
18. The monkey was required to maintain fixation within 2° of a spot of light (0.1° by 0.1°) for 4 to 5 s to receive a reward (a drop of juice). Stimulation was applied in 66 to 80% of the trials. Only on stimulation trials could the monkey break fixation before the fixation spot disappeared (after stimulation onset) and still receive the reward. The fixation point disappeared immediately after the monkey broke fixation. In 77% of the stimulation sites the fixation point was positioned at the center of the screen; in the remaining sites it was positioned between 5° and 13° from the center of the screen toward the ipsilateral direction. Eye position was monitored by an infrared eye tracker (provided by Dr. Bouis Devices, Karlsruhe, Germany) and sampled at 250 Hz.
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23. The fast signals that we measure (both depolarization and hyperpolarization) are present only in the wavelength of fluorescence emission. Thus, these signals are not likely to be contaminated by mechanical artifacts or fast intrinsic signals. In addition, it has been shown recently, using intracellular recording in vivo, that the dye signal measures membrane-potential changes precisely [see figure 23 in (*15*); similar results were obtained in a recent in vitro study using the same dye (*34*)]. Furthermore, an in vitro study had previously demonstrated that microstimulation-evoked activity measured using real-time optical imaging is highly correlated with neural activity measured intracellularly (*32*).
24. Eye movements that had the typical bell-shaped velocity profile and peak velocity above 100°/s were considered saccades. In most stimulation sites the major component of the evoked saccade was horizontal; we therefore focused our quantitative analysis on the horizontal component of the evoked saccades (but see supplementary figures). To combine saccade peak velocity measurements across experiments (Fig. 4), we first normalized the peak velocity of each saccade relative to the maximal peak velocity obtained under all stimulation conditions at that site. The peak velocity of saccades in the contralateral direction was defined as positive, and in the ipsilateral direction as negative. Similarly, the average optical signal for each stimulation site was first normalized relative to the maximal depolarization observed at this site across all stimulation conditions before being combined to yield the grand mean shown in Fig. 4.
25. The properties of stimulation-evoked saccades observed here are consistent with those observed in previous studies (*6–8*, *31*, *35*). In particular, Stanford *et al.* (*35*) found that, following a short stimulation train in the superior colliculus, saccade amplitude becomes shorter with increasing saccade latency. No other study, to our knowledge, reported the existence of short ipsilateral saccades that occur during early hyperpolarization. This may not be surprising given that these saccades are comparatively rare (5% of the first saccades in Fig. 4) and, therefore, could easily have been overlooked by researchers who did not have access to the time course of depolarization and hyperpolarization. FEF stimulation can evoke ipsilateral saccades in the "colliding saccade" paradigm (*36*), when microstimulation is applied during an ongoing natural saccade. It remains to be determined whether the observed hyperpolarization plays a role also in the "colliding saccade" effect.
26. A correlation was computed between the normalized peak horizontal velocity for individual saccades (red and cyan x's in Fig. 4) and the mean normalized optical response at the time of saccade initiation (green curve in Fig. 4). To compute the correlation coefficient (Pearson's *r*), we paired each saccade peak velocity with the level of the average optical signal at the time of saccade initiation. The statistical significance of each correlation coefficient was assessed using an *F* test with 1 and $(n - 2)$ degrees of freedom, where *n* is the number of evoked saccades. The high correlation observed in Fig. 4 remains unaffected if we (i) select only the stimulation sites for which both behavioral and optical measurements were obtained simultaneously (*17*); (ii) select only stimulation sites in which stimulation current was ≤50 μA; (iii) select only stimulation sites that were likely to fall within the FEF; (iv) compute the correlation between the optical signals and the normalized saccade amplitude (see supplementary figures); or (v) compute the correlation between the optical signals and the normalized peak vertical eye velocity for saccades with a large vertical component (*37*).
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38. All experimental procedures were in accordance with institutional and NIH regulations. We thank E. Ahisar, S. Barash, R. Born, C. J. Bruce, U. Zohary, M. E. Goldberg, and D. L. Sparks for critical comments on earlier versions of this manuscript and E. Tsabary, T. Eliahu, and D. Ettner for technical assistance. E.S. was supported by a Koshland Scholar award. This work was supported by grants from Abisch-Frankel (E.S. and A.G.) and the Gorodetsky Center and by the Koerber, Margaret Enoch, and Goldsmith foundations.

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Chaperone Suppression of α-Synuclein Toxicity in a *Drosophila* Model for Parkinson's Disease

Pavan K. Auluck,[1] H. Y. Edwin Chan,[2]* John Q. Trojanowski,[3] Virginia M.-Y. Lee,[3] Nancy M. Bonini[1,2,4]†

Parkinson's disease is a movement disorder characterized by degeneration of dopaminergic neurons in the substantia nigra pars compacta. Dopaminergic neuronal loss also occurs in *Drosophila melanogaster* upon directed expression of α-synuclein, a protein implicated in the pathogenesis of Parkinson's disease and a major component of proteinaceous Lewy bodies. We report that directed expression of the molecular chaperone Hsp70 prevented dopaminergic neuronal loss associated with α-synuclein in *Drosophila* and that interference with endogenous chaperone activity accelerated α-synuclein toxicity. Furthermore, Lewy bodies in human postmortem tissue immunostained for molecular chaperones, also suggesting that chaperones may play a role in Parkinson's disease progression.

Parkinson's disease (PD) is the second most common neurodegenerative disorder and is associated with resting tremor, postural rigidity, and progressive degeneration of dopaminergic neurons in the substantia nigra pars compacta (SNpc). Characteristic pathological features of PD include Lewy bodies (LBs), which are juxtanuclear ubiquitinated proteinaceous inclusions in neuronal perikarya, and Lewy neurites (LNs), which are similar protein aggregates found in neuronal processes (*1*). LBs and LNs are also characteristic of other neurodegenerative diseases, including the LB variant of Alzheimer's disease (LBVAD) and dementia with LBs (DLB). α-synuclein is a major constituent of inclusions found in these disorders, known as synucleinopathies (*2–4*). Moreover, two missense mutations in the gene encoding α-synuclein are linked to dominantly inherited PD, thereby directly implicating α-synuclein in disease pathogenesis (*5, 6*).

In *Drosophila*, directed expression of α-synuclein induces selective and progressive loss of dopaminergic neurons, as well as formation of α-synuclein–positive perinuclear and neuritic filamentous inclusions similar to LBs and LNs (*7*). Inclusion

[1]Department of Neuroscience, [2]Department of Biology, [3]Center for Neurodegenerative Disease Research and Department of Pathology and Laboratory Medicine, and [4]Howard Hughes Medical Institute, University of Pennsylvania and University of Pennsylvania School of Medicine, Philadelphia, PA 19104, USA.

*Present address: Department of Biochemistry, The Chinese University of Hong Kong, Shatin, Hong Kong.
†To whom correspondence should be addressed. E-mail: nbonini@sas.upenn.edu

formation and progressive neuronal degeneration have also been demonstrated in *Drosophila* models of polyglutamine disease through directed expression of pathogenic human proteins with expanded polyglutamine stretches (*8–10*). The neuronal toxicity of abnormal polyglutamine proteins is suppressed by Hsp70 (*11*), a chaperone up-regulated in stress responses that refolds misfolded protein (*12–14*). Given the pathological similarities between polyglutamine- and α-synuclein–mediated neurodegeneration, we investigated whether Hsp70 could mitigate dopaminergic neuron loss induced by α-synuclein. Such a finding would also address whether modifiers of neurodegeneration in one context (polyglutamine toxicity) might be of therapeutic benefit in an unrelated context (Parkinson's disease).

We used the GAL4/UAS expression system (*15*) to direct transgene expression. Expression was targeted to dopaminergic neurons using a driver line with the 3,4-dihydroxyphenylalanine (DOPA) decarboxylase gene promoter (*Ddc-GAL4*) (*7, 16*). Neurons were counted in the dorsomedial (DM) and dorsolateral-1 (DL-1) dopaminergic neuronal clusters following immunolabeling for tyrosine hydroxylase, which specifically identifies dopaminergic neurons (*17*).

Flies expressing wild-type α-synuclein, or the two mutant forms pathogenic for familial PD (A30P and A53T α-synuclein), in dopaminergic neurons consistently exhibited marked neuronal loss (~50% of normal) in the DM clusters in flies aged to 20 days [Fig. 1 and Web table 1 (*18*)]. Neuron loss did not progress further in older flies (*19*). In DL-1 clusters, dopaminergic neuron loss was more variable, such that in some experiments no degeneration occurred, whereas in others as many as 50% of the neurons were lost [Web table 2 (*18*)]. No differences were detected in toxicity conferred by normal α-synuclein compared to the mutant forms [Fig. 1 and Web table 1 (*18*)]. We also confirmed that LB- and LN-like inclusions formed in flies expressing α-synuclein (see below).

We then addressed whether expression of the molecular chaperone Hsp70 could alter the neurotoxicity of α-synuclein. We used a transgenic line encoding human Hsp70 (*11*) to coexpress Hsp70 with α-synuclein. We then reexamined dopaminergic neuron loss in the DM and DL-1 clusters. Upon coexpression of Hsp70, we found complete maintenance of normal numbers of dopaminergic neurons in aged flies. Although α-synuclein expression in the absence of Hsp70 resulted in a 50% loss of these neurons in the DM clusters by 20 days, in the presence of added Hsp70, the same number of dopaminergic neurons were present at 20 days as were present at 1 day [Fig. 1 and Web table 1 (*18*)]. Protection was specific to Hsp70: coexpression of a control protein, β-galactosidase, together with α-synuclein did not mitigate neuronal loss. Immunoblot analysis confirmed that levels of α-synuclein were not altered upon added expression of Hsp70 [Web fig. 1 (*18*)]. Thus, Hsp70 ameliorated the toxicity of α-synuclein to dopaminergic neurons.

To determine whether suppression of α-synuclein toxicity was accompanied by a change in inclusion formation, we examined LB-like inclusion formation over time, using antibodies directed against α-synuclein. In dopaminergic neurons of flies expressing α-synuclein, inclusions increased in size and number with age (Fig. 2, A through C). Most

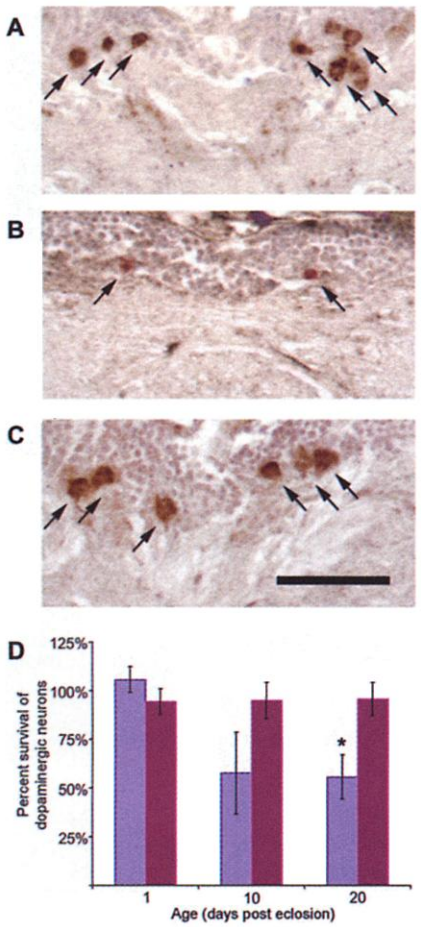

Fig. 1. Hsp70 protected against α-synuclein–induced dopaminergic neuronal degeneration. Paraffin sections of fly brains were stained with anti-TH antibody to identify dopaminergic neurons. (**A**) Control fly aged to 20 days. Many dopaminergic neurons (arrows) were visible in the DM clusters. (**B**) Fly expressing α-synuclein, aged to 20 days. Markedly fewer dopaminergic neurons (arrows) were observed in the DM clusters. (**C**) Similarly aged fly coexpressing Hsp70 with α-synuclein. Normal numbers of dopaminergic neurons were observed (arrows). Genotypes: *w;Ddc-GAL4/+*, *w;Ddc-GAL4/+;UAS-α-syn/+*, and *w;Ddc-GAL4,UAS-HspA1L/+;UAS-α-syn/+*. (**D**) Cell counts of dopaminergic neurons within the DM clusters of flies expressing α-synuclein alone or together with Hsp70. Values represent mean ± SEM of four independent experiments. Serial sections through three to five heads were examined per data point in each experiment. Blue, α-synuclein alone; red, α-synuclein coexpressed with Hsp70. Normally, no loss of dopaminergic neurons is observed in aged flies (*7, 18*). Cell numbers between α-synuclein alone at 1 day and at 20 days (blue, 1 day versus 20 days) were significantly different ($*P \leq 0.01$, Student's *t*-test). No significant difference was present in cell numbers between α-synuclein alone at 1 day (blue, 1 day) and α-synuclein with Hsp70 at 20 days (red, 20 days). Bar in (C), 32 μm.

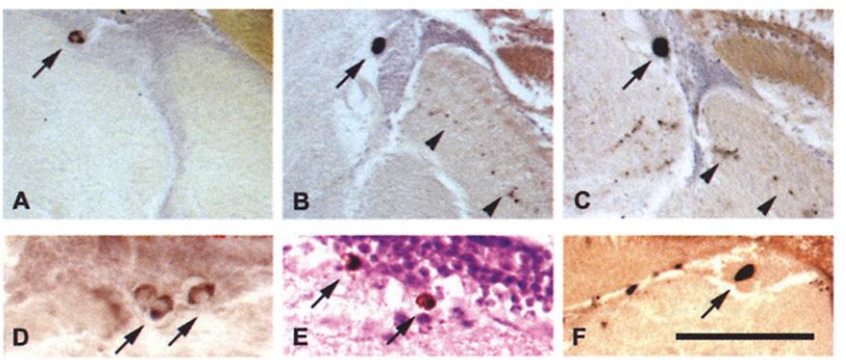

Fig. 2. Hsp70 did not alter the appearance of α-synuclein inclusions in the *Drosophila* brain. Paraffin brain sections of flies, coexpressing α-synuclein and Hsp70, were immunostained to visualize α-synuclein inclusions. Flies coexpressing α-synuclein and Hsp70, aged (**A**) 1 day and (**B**) 20 days. (A) Inclusions (arrows) appeared initially small and weakly stained. (B) Over time, inclusions became progressively larger and more immunoreactive for α-synuclein, and pathology in the neuropil (arrowheads) became evident. (**C**) Fly expressing α-synuclein alone, aged to 20 days. Genotypes: *w;Ddc-GAL4/+;UAS-α-syn,UAS-HspA1L/+* [(A) and (B)], *w;Ddc-GAL4/+;UAS-α-syn/+* (C). (**D** through **F**) LB-like inclusions were immunoreactive for Hsp70 and ubiquitin. Paraffin brain sections of 20-day-old flies expressing α-synuclein were immunostained with (D) 7FB antibody [specific to the stress-induced form of Hsp70 (*21*)] and (E) ubiquitin. (F) Coexpressed human Hsp70 protein also localized to inclusions. Genotypes: *w;Ddc-GAL4/+;UAS-α-syn/+* (D and E) and *w;Ddc-GAL4/+;UAS-α-syn,UAS-HspA1L/+* (F). Bar, 63 μm in (A) to (C) and (F), 50 μm in (D), and 40 μm in (E).

inclusions were in DL-1 and DL-2 clusters and only rarely in DM neurons (*20*). Upon coexpression of Hsp70 with α-synuclein, no change in the number, morphology, or distribution of the perinuclear protein inclusions was discernible (Fig. 2, A through C) (*20*). Thus, Hsp70 protected against the toxicity of α-synuclein, despite the continued presence of aggregate pathology. The aggregates, however, immunostained for the exogenous Hsp70 (Fig. 2F).

The fact that human Hsp70 protected *Drosophila* neurons from α-synuclein toxicity, and was present in the inclusions, raised the possibility of an interaction between endogenous chaperone activity and α-synuclein. To address this, we immunostained brain sections with an antibody specific for the stress-induced form of *Drosophila* Hsp70 (*21*). Hsp70 immunoreactivity was seen in the LB-like inclusions of flies expressing α-synuclein (Fig. 2D). As in human disease, the LB-like inclusions in flies were also immunoreactive for ubiquitin (Fig. 2E), further emphasizing the conservation of additional pathways involved in α-synuclein–related pathology between flies and humans.

We further addressed the role of Hsp70 in α-synuclein toxicity by interfering with endogenous activity of the major constitutively expressed fly Hsp70 protein, Hsc4. We coexpressed with α-synuclein a mutant form of Hsc4 bearing an amino acid substitution in the adenosine triphosphate–binding domain (Hsc4.K71S) (*22*). This transgene produces a protein that interferes with normal Hsc4 chaperone activity in a dominant-negative manner (*22*). Upon coexpression of Hsc4.K71S with α-synuclein, dopaminergic neuronal loss in flies was accelerated. Whereas neuronal loss induced by α-synuclein alone occurred by 10 to 20 days, neuronal loss was now apparent in 1-day-old flies (Table 1). In older flies, degeneration was similar to that induced by α-synuclein alone. However, some dopaminergic cell loss was evident in the DM clusters in aged flies expressing the Hsc4.K71S transgene alone (Table 1), suggesting that survival of these neurons was sensitive to the levels of endogenous chaperones. These studies emphasize the sensitivity of dopaminergic neurons to chaperone levels and suggest that endogenous chaperones may normally protect against α-synuclein toxicity by delaying the onset of degeneration. These results also support an alternate hypothesis whereby α-synuclein mediates neuronal toxicity by interfering with endogenous chaperone activity.

A role for Hsp70 has been largely unexplored in PD; however, stress responses may be critical to dopaminergic neuronal integrity in recessive forms of PD (*23*), which may also involve α-synuclein toxicity (*24*). We therefore investigated whether there was evidence to suggest a role for chaperones in tissue from patients with PD. To do this, we immunostained brain sections for Hsp70, as well as its co-chaperone Hsp40 proteins, from patients with PD (*25*).

Staining of postmortem PD brain tissue from several patients revealed LBs and LNs that were immunopositive for Hsp70 and Hsp40 chaperones (Fig. 3, A through C). These data suggest that altered chaperone activity may be involved in progression of PD. We next investigated whether this finding was a common feature of human synucleinopathies by examining tissue from patients with DLB, LBVAD, and neurodegeneration with brain iron accumulation type 1 (NBIA1; formerly known as Hallervorden-Spatz syndrome). As in PD brains, inclusions in these other diseases showed immunoreactivity for Hsp70 and Hsp40 (Fig. 3, D through I). Overall, the number of immunoreactive lesions was 1 to 5% for Hsp70; in separate experiments, a similar percentage was immunopositive for

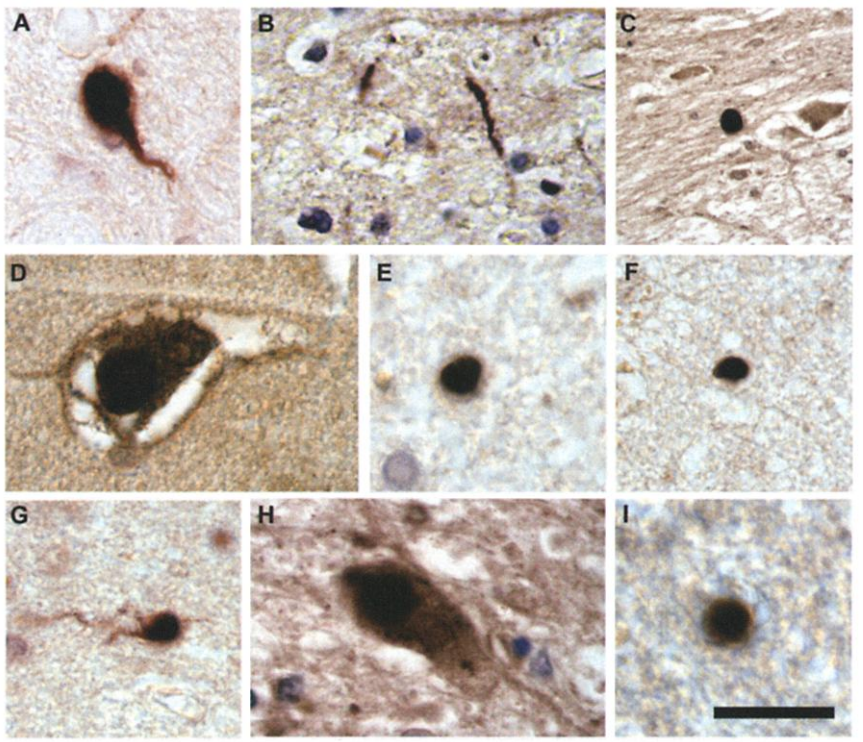

Fig. 3. Lesions from tissue of human patients with Parkinson's disease, and other synucleinopathies, were immunopositive for the molecular chaperone Hsp70 and co-chaperone Hsp40. Paraffin-embedded brain sections from human patients immunostained for Hsp70 (A, B, D, E, and G) or Hsp40 (C, F, H, and I). (**A** to **C**) LBs (A and C) and LNs (B) in the SNpc of brain tissue from PD patients. (**D**) LB in the amygdala from a patient with DLB. (**E** and **F**) LBs in the SNpc (E) and cinglate cortex (F) of patients with LBVAD. (**G** through **I**) LBs from the amygdala (G), SNpc (H), and globus pallidus (I) of a patient with NBIA1. Bar, 25 μm in (A), (D) to (G), and (I); 32 μm in (B) and (H); and 50 μm in (C).

Table 1. Inhibition of constitutive Hsp70 activity enhanced α-synuclein–induced loss of dopaminergic neurons in the DM clusters. The *UAS-Hsc4.K71S* transgene encodes a dominant-negative form of the constitutive *Drosophila* Hsp70 protein, Hsc4 (*22*). Dopaminergic neurons were visualized by immunostaining for tyrosine hydroxylase. Serial sections through three to five fly heads were examined for each time point.

Condition	Mean number of dopaminergic neurons ± SEM	
	1 day	20 days
Control (*w;Ddc-GAL4/+*)	16.5 ± 2.5	16.3 ± 0.3
Synuclein (*w;Ddc-GAL4/+;UAS-α-syn/+*)	15.5 ± 3.5	8.0 ± 0.6
Hsc4.K71S alone (*w;Ddc-GAL4,UAS-Hsc4.K71S/+*)	19.5 ± 0.5	9.7 ± 1.8
Synuclein and Hsc4.K71S (*w; Ddc-GAL4, UAS-Hsc4.K71S/+;UAS-α-syn/+*)	6.7 ± 2.3	6.3 ± 1.7

Hsp40. These data suggest a role for chaperones in pathologies involving α-synuclein in humans, such that Hsp70 may be a critical part of the neuronal arsenal that mitigates α-synuclein toxicity. An alternative interpretation is that the presence of chaperones in aggregates results in their cellular depletion, due to sequestration, and this loss of chaperone function leads to degeneration.

We present data that implicates the molecular chaperone machinery in the pathogenesis of PD using a *Drosophila* model. Augmentation of Hsp70 activity in vivo suppresses α-synuclein neurotoxicity, whereas compromising chaperone function enhances α-synuclein–induced dopaminergic neuronal loss. Thus, chaperone machinery in flies helps to protect dopaminergic neurons against degeneration and attenuates the neurotoxic consequences of α-synuclein expression. Hsp70 may mitigate α-synuclein toxicity by influencing the conformation of α-synuclein in ways that are not revealed by the morphology of aggregates in *Drosophila*. Alternatively, α-synuclein may be toxic because it interferes with chaperone activity, possibly by their sequestration, and it is this effect that is mitigated by added Hsp70. Our findings suggest a role for chaperones in human pathology, because human LBs and LNs in PD and other human synucleinopathies immunostain for Hsp70 and Hsp40. Chaperones may thus play a role in α-synuclein toxicity, such that augmentation of chaperone stress pathways may be an effective approach in the treatment of several human neurodegenerative diseases including PD.

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17. *Drosophila* were grown under standard conditions at 25°C. Transgenes used included *Ddc-GAL4* (*16*); *UAS-lacZ*; *UAS-HspA1L* encoding human Hsp70 (*11*). *UAS-α-syn*, *UAS-A30P*, and *UAS-A53T* were generated for α-synuclein; additional lines are described in (*7*). Male and female flies were aged to the indicated time, then heads were fixed in 10% neutral-buffered formalin (NBF) and embedded in paraffin. Serial sections (8 μm thickness) through the entire brain were prepared for immunostaining. Antibodies used were: TH (1:150, Pelfreez, Rogers, AR), human Hsp70 (SC-24 1:100, Santa Cruz Biotechnology, Santa Cruz, CA), *Drosophila* Hsp70 [7FB (*21*), 1:500], ubiquitin (MAB 1510, 1:2000, Chemicon, Temecula, CA), and α-synuclein [syn303 (*26*), 1:100 and 1:1000, at the latter dilution, syn303 selectively detects aggregated α-synuclein; syn514 (*26*), 1:5, which only detects aggregated α-synuclein]. Sections were incubated overnight with primary antibody at 4°C, followed by secondary antibody, Avidin-Biotin Complex incubation (Vectastain ABC Elite Kit, Vector Laboratories, Burlingame, CA), and developing with 3,3′-diaminobenzidine. For each data point, complete serial sections from three to five individual brains were examined. Similar results were seen in at least five independent experiments. No sex differences were noted. The extent of neuron loss was less than previously reported (*7*), even using the same transgenic lines. We did not see effects of α-synuclein on climbing behavior.
18. Supplementary data are available at *Science* Online at www.sciencemag.org/cgi/content/full/1067389/DC1.
19. Dopaminergic neuronal loss at 30 days was the same as at 20 days.
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25. Blocks of cingulate cortex, amygdala, and midbrain from postmortem PD, LBVAD, DLB, and NBIA1 brains were fixed in 70% ethanol/150 mM NaCl or 10% NBF and embedded in paraffin. sections (6 μm thickness) were cut and stained (*17*). Immunostaining was with α-synuclein antibodies as in (*17*), and human Hsp70 (SC-24, Santa Cruz Biotechnologies; SPA-810, StressGen Biotechnologies), Hsp40 (SC-1801, Santa Cruz Biotechnologies), and HDJ-2 (KA2A5.6, NeoMarkers, Fremont, CA).
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Germline Transmission and Tissue-Specific Expression of Transgenes Delivered by Lentiviral Vectors

Carlos Lois,* Elizabeth J. Hong,* Shirley Pease, Eric J. Brown, David Baltimore†

Single-cell mouse embryos were infected in vitro with recombinant lentiviral vectors to generate transgenic mice carrying the green fluorescent protein (GFP) gene driven by a ubiquitously expressing promoter. Eighty percent of founder mice carried at least one copy of the transgene, and 90% of these expressed GFP at high levels. Progeny inherited the transgene(s) and displayed green fluorescence. Mice generated using lentiviral vectors with muscle-specific and T lymphocyte–specific promoters expressed high levels of GFP only in the appropriate cell types. We have also generated transgenic rats that express GFP at high levels, suggesting that this technique can be used to produce other transgenic animal species.

The ability to introduce and express exogenous genes of interest in animals has become an indispensable tool to modern biologists (*1*). Transgenic mice are currently generated by pronuclear injection; however, this technique is still relatively inefficient, technically demanding, costly, and impractical in most other animal species. Another approach to transgenesis is to use retroviruses as gene delivery vehicles because they are able to stably integrate into the genome of cells. However, the generation of transgenic animals with oncoretroviruses such as the Moloney murine leukemia virus (MoMLV) is impractical because silencing of the provirus during development results in low to undetectable levels of transgene expression (*2*, *3*).

Lentiviruses are a class of retroviruses that cause chronic illnesses in the host organisms they infect. Among retroviruses, lentiviruses have the distinguishing property of being able to infect both dividing and nondividing cells, and this ability has led to their development as gene delivery vehicles (*4*).

Division of Biology, California Institute of Technology, Pasadena, CA 91125, USA.

*These authors contributed equally to this work.
†To whom correspondence should be addressed. E-mail: baltimo@caltech.edu