The free parameters $\alpha$, $\beta$, $\tau$, and $n$ describe the temporal dynamics of the saturating exponential function, independent of motion strength. Only the delay term, $\delta_n$, varied with motion strength.

Received 15 October 1999, accepted 26 January 2000.


Acknowledgements
We thank M. Mihali for animal training and technical support, and E. Freedman, G. Horwitz, B. Jagadeesh, T. Movshon and E. Rice for helpful comments on the manuscript. This work was supported by the NEI, NCRR and the McKnight Foundation.

Correspondence and requests for materials should be addressed to M.N.S. (e-mail: shadlen@uwashington.edu).
always represented in well oriented sections by four or five robustly staining cells (Fig. 1c). These cells are present in one-day-old control flies and are not lost with age, being readily identified in 60-day-old flies. The lifespan of control flies and α-synuclein transgenic animals is about 60 days under our 25°C culture conditions.

In contrast, Drosophila that express α-synuclein in a pan-neural distribution (elav→GAL4 driver) show a marked, age-dependent loss of dorsomedial dopaminergic neurons (Fig. 1d). In young adult flies expressing wild-type and mutant forms of α-synuclein, the dorsomedial cluster consists of the normal complement of four or five neurons. In 30–60-day-old α-synuclein transgenic flies, however, the cluster is either absent or consists of a single tyrosine-hydroxylase-positive cell (Fig. 1d). These dopaminergic neurons disappear in flies expressing wild-type, A30P or A53T α-synuclein.

To confirm that dopaminergic neurons degenerate in α-synuclein transgenic animals, we used a driver line containing the promotor for the DOPA decarboxylase gene (Ddc→GAL4), another marker of dopaminergic neurons. The Ddc line drives reporter-gene expression specifically in dopaminergic cells, allowing us to identify them. Transgenic flies expressing A30P α-synuclein under the control of the Ddc driver line show robust immunostaining for α-synuclein at one day of age. However, after 30 days, there is no α-synuclein immunostaining associated with cell bodies, consistent with degeneration of dopaminergic neurons. To ensure that the Ddc driver line remains active at 30 days, the marker protein β-galactosidase was expressed instead of α-synuclein. β-Galactosidase immunoreactivity is present at 30 days when the Ddc line is used to drive β-galactosidase expression. We also confirmed that dorsomedial cluster neurons are depleted at 30 days by tyrosine hydroxylase immunostaining brains from flies expressing wild-type, A30P or A53T α-synuclein under the control of the Ddc→GAL4 driver.

As a final test for degeneration of dopaminergic neurons, both β-galactosidase and A30P α-synuclein were expressed at the same time, in the same dopaminergic neurons, using the Ddc driver line. In one-day-old transgenic flies, dopaminergic neurons are readily identified by β-galactosidase immunostaining. In contrast, at 10 days of age, β-galactosidase expression is undetectable (Fig. 1f, compare with 10-day-old controls in Fig. 1e).

Not all dopaminergic neurons degenerate in α-synuclein transgenic flies. Substantial numbers of tyrosine-hydroxylase-positive cells remain in aged flies that express α-synuclein in a pan-neural distribution (elav→GAL4 driver) or specifically in dopaminergic neurons (Ddc→GAL4 driver). Preserved neurons may reflect variation in the amount of GAL4 activator protein available to drive α-synuclein expression in particular cells. Alternatively, certain subsets of Drosophila dopaminergic neurons may be particularly sensitive to α-synuclein toxicity. In patients with Parkinson's disease, preferential degeneration of specific dopaminergic neurons occurs even within the same neuronal nucleus.

Neurodegeneration induced by α-synuclein shows specificity for dopaminergic neurons. Pan-neural expression of α-synuclein in the brain produces no demonstrable loss of volume in the outer cellular cortex or in the central neuropil area (Fig. 1b). The excess vacuolization characteristic of other, more generalized, neurodegenerative mutations in Drosophila is not present. In addition, no excess of degenerating neurons is detected by toluidine blue staining or ultrastructural examination. Thus, most neurons are preserved in flies expressing α-synuclein. Of course, such anatomical investigations do not exclude degeneration of a minor neuronal subpopulation.

To address the possibility that subsets of non-dopaminergic neurons are vulnerable to α-synuclein toxicity, we examined a second major amine transmitter in Drosophila, serotonin. Serotonergic neurons can degenerate in patients with Parkinson's disease. Whole-mount preparations of adult brains stained with an antibody against serotonin revealed no differences between 30-day-old flies expressing A30P α-synuclein in a pan-neural pattern and controls.

**Figure 1**  Histological and immunocytochemical analysis of α-synuclein transgenic flies. a,b, Frontal sections of 60-day-old control fly (a, elav→GAL4/+ and 60-day-old A30P α-synuclein transgenic fly (b, UAS→A30P α-synuclein/elav→GAL4) stained with haematoxylin and eosin. Overall brain volume, including the outer cellular cortex layer containing neuronal and glial cell bodies (arrows) and central neuropil areas, and overall architecture are preserved. c, 30-day-old control fly (elav→GAL4/) shows immunostaining for tyrosine hydroxylase in 4–5 cells in the dorsomedial cluster. d, 30-day-old α-synuclein-expressing fly (elav+/; UAS→wild-type α-synuclein/elav→GAL4) shows no cell-body-associated immunostaining in the same area. e, 10-day-old control fly expressing α-galactosidase in both cell cortex and neuronal processes (arrows) of dopaminergic neurons (UAS→lacZ/Ddc→GAL4). f, 10-day-old fly carrying in addition an α-synuclein transgene (UAS→A30P α-synuclein+/; UAS→lacZ/Ddc→GAL4) shows no α-galactosidase expression in the outer cellular cortex or central neuropil. g, Immunostaining of α-synuclein inclusions in the brain from a 30-day-old transgenic fly (UAS→A30P α-synuclein/elav→GAL4) in the area of the subesophageal ganglia with an antibody against α-synuclein. h, Human cortical Lewy body (arrow) from the cingulate cortex of a patient with diffuse Lewy body disease, stained with an antibody against ubiquitin (same scale as g). i, Immunostaining of three α-synuclein inclusions in the brain of a young adult fly (1 day post-eclosion; Ddc→GAL4/UAS→wild-type α-synuclein) showing irregularity of selected inclusions (arrow) and diffuse immunoreactivity in a larger neuron (arrowhead). j, Neuropathic pathology consisting of α-synuclein immunoreactive thread-like structures and grain-like structures. 60-day-old fly (UAS→A30P α-synuclein/elav→GAL4). Compare with abnormal neurite in the cingulate cortex of a Lewy body disease patient (h, arrowhead). k, Immunofluorescence staining of eye imaginal disc from wandering third instar larva (UAS→A53T α-synuclein+/; gmr→GAL4/) with antibody against α-synuclein showing no inclusions. l, Diffuse cytoplasmic α-synuclein immunoreactivity in the adult gut from a 30-day-old fly (UAS→A53T α-synuclein+/; e29c→GAL4/). Scale bars: a,b,e,f, 30 μm; c, d, g, h, 10 μm; i, j, 5 μm.
The anatomical arrangement of serotonergic cells has been described in detail in *Drosophila* [1]. We can identify all the major serotonergic cell groups in experimental animals and controls. However, we cannot exclude an effect of α-synuclein expression on a small subgroup of serotonergic cells.

Expression of human α-synuclein in flies thus replicates three key features of the pathology of Parkinson’s disease: adult onset, involvement restricted to the nervous system and anatomical specificity within the nervous system.

The most specific and diagnostic feature of Parkinson’s disease is an α-synuclein-rich cytoplasmic filamentous aggregate called the Lewy body. Lewy bodies in the cerebral cortex are best visualised by immunostaining [16-18]. When we immunostain brains from aged flies expressing normal and mutant α-synuclein in a pan-neuronal pattern with antibodies against human α-synuclein, we observe a distinct punctate pattern of staining suggestive of aggregate formation (Fig. 1g). The fly α-synuclein inclusions strongly resemble cortical Lewy bodies from patients with diffuse Lewy body disease, a disorder closely related to Parkinson’s disease (Fig. 1h). Most neuronal cytoplasmic inclusions are single, round and regular, as described in detail in [16-17]. We never saw similar inclusions in brains of flies expressing wild-type, A30P or A53T α-synuclein transgenic flies.

Electron microscopy reveals cytoplasmic inclusions in brains from flies expressing α-synuclein (pan-neuronal *elav*-GAL4 driver) that have a relatively homogenous core and are edged by radiating filaments projecting into a surrounding halo (Fig. 2a). The filaments are 7–10 nm in diameter and are sometimes associated with cellular organelles (Fig. 2b, arrow). Granular material is admixed with loosely packed filaments in less compact inclusions (Fig. 2b, arrowhead), as in cortical Lewy bodies [16-18]. The overall morphology of the inclusions, the filamentous and granular nature of the aggregates and the size and disposition of the component filaments are all reminiscent of human Lewy bodies [16-18]. We never saw similar inclusions in aged control flies. Immunoelectron microscopy reveals Hrp labelling concentrated over the inclusions (Fig. 2c). Faint radiating filaments are visible peripherally in the unstained preparation (Fig. 2c, arrow). No immunoreactivity was present in identically treated specimens from control flies of the same age.

The time of appearance of α-synuclein inclusions in the fly brain depends on the level of α-synuclein expression. The inclusions appear at 20–30 days of age when α-synuclein expression is pan-neural. In younger flies, the immunostaining pattern is diffuse and cytoplasmic (Fig. 1i, arrowhead). Immunoreactivity is also diffuse and cytoplasmic in developing tissues (Fig. 1k, eye imaginal disc) and non-neuronal tissues (Fig. 1l, adult gut) when the GAL4 drivers induce expression in these tissues. Inclusion formation thus parallels α-synuclein toxicity in both its restriction to the nervous system and its timing. Inclusions are present in 2–10% of neurons throughout the central body complex at 30 days of age (pan-neural *elav*-GAL4 driver), and are not restricted to dopaminergic neurons.

Lewy bodies in classic Parkinson’s disease and in diffuse Lewy body disease are also present in a variety of nuclei and neuronal subtypes, not all of which show obvious degeneration.

Given the neuronal degeneration and widespread inclusion formation present in α-synuclein transgenic flies, we searched for behavioural manifestations of nervous system dysfunction. Locomotor behaviour is grossly preserved in young flies. However, as transgenic flies with pan-neuronal expression of α-synuclein age, they develop locomotor dysfunction (Fig. 3). Normal *Drosophila* display a strong negative geotactic response. When tapped to the bottom of a vial they rapidly climb to the top of the vial, and most flies remain there. As they get older, normal flies no longer climb to the top of the vial, but instead make short abortive climbs and fall back to the bottom of the vial. The loss of the climbing response has been used to monitor aging-related changes in *Drosophila* [19,20]. Flies transgenic for α-synuclein initially climb as well as control flies. However, over time they decline in performance more rapidly than controls (Fig. 3). The progressive, accelerated decline in climbing ability in α-synuclein transgenic flies demonstrates a functional deficit produced by α-synuclein expression in the nervous system. The time course of locomotor dysfunction parallels the degeneration of dopaminergic neurons and the appearance of α-synuclein inclusions.

*Drosophila* expressing A30P α-synuclein lose their climbing ability earlier than flies expressing wild-type or A53T α-synuclein. This small but statistically significant effect may reflect either enhanced biological toxicity of the A30P mutant protein or small variations in the amount of α-synuclein produced in the transgenic lines. We examined α-synuclein expression in multiple independent transgenic lines for each α-synuclein variant by western blotting, by immunofluorescence on eye imaginal discs and by immunohistochemical staining on sections of adult *Drosophila*, and we have...
Table 1 Effects of expressing normal and mutant α-synuclein in Drosophila

<table>
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<th>Transgene</th>
<th>Nervous system</th>
<th>Eye</th>
<th>Presumptive mesoderm</th>
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<th>Imaginal disc, adult tissues</th>
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<td>Retinal degeneration</td>
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<td>A30P α-synuclein</td>
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<td>Viable</td>
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<td>Crumpled wing lethality</td>
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SCA1-82, spinocerebellar ataxia type 1 with an expanded repeat length encoding 82 glutamines. GAL4 enhancer lines with the following patterns were used: elav-GAL4; all neurons of the developing and mature central and peripheral nervous system; gmr-GAL4, developing and mature retina, including photoreceptors and pigment cells; Ddc-GAL4, presumptive mesoderm and muscle; and e29c-GAL4, widespread embryonic, eye and wing imaginal disc; dpp-GAL4, imaginal disc anterior–posterior border; e29c-GAL4, widespread imaginal disc and adult imaginal disc derivatives. UAS-SCA1-82/+; dpp-GAL4/+ transheterozygotes showed crumpled wings; animals of the genotype UAS-SCA1-82/+; e29c-GAL4/+ died as larvae.

Figure 4 Retinal degeneration in α-synuclein transgenic flies. a, One-day-old control fly. b, Normal retina in one-day-old α-synuclein transgenic fly. c, Well preserved retina in 30-day-old control fly. d, 30-day-old α-synuclein-expressing fly showing retinal degeneration with vacuolization and architectural distortion. Scale bar, 15 μm. Genotypes include control: gmr–GAL4/++; transgenic: UAS–wild-type α-synuclein/gmr–GAL4 flies.

Compared transgenic lines with similar levels of α-synuclein as determined by all three assays. However, small differences in α-synuclein protein levels may have escaped our detection.

Degenerative changes are not restricted to the brain. Retinal degeneration occurs when α-synuclein is expressed specifically in the eye (gmr–GAL4 driver). Expression of wild-type or mutant α-synuclein during development of the eye produces no effect. However, continued expression of α-synuclein in the adult eye produces retinal degeneration that is detectable by ten days, and marked at 30 days in transgenic flies expressing wild-type (Fig. 4), A30P or A53T α-synuclein. Retinal degeneration can be readily monitored under the dissecting microscope in live flies by examining an optical effect termed the retinal pseudopupil21. The pseudopupil is quite sensitive to disruptions in the normal architecture of the retina, and becomes abnormal as the retinas of the α-synuclein transgenic flies degenerate. The presence of an easily assayed, nervous-system-specific, degenerative phenotype will facilitate generation of second site modifiers and other pharmacological and transgenic manipulations designed to modify neurodegeneration.

Retinal degeneration in α-synuclein transgenic flies shows that in flies, as in people, α-synuclein-related degenerative changes show relative rather than absolute specificity for dopaminergic neurons. In fact, the Drosophila retina may be resistant to α-synuclein toxicity because more transgenic α-synuclein is produced in the eye (gmr–GAL4) than the brain (elav–GAL4).

The modest difference between the toxicity of wild-type and A30P mutant α-synuclein in the locomotor assay, and the similar effects of all three α-synuclein variants on dopaminergic neuronal loss and retinal degeneration, are consistent with the fact that most patients with Parkinson’s disease have the wild-type form of the protein, which does become incorporated into Lewy bodies.

We cloned normal and mutant α-synuclein and SCA1 cDNAs into the GAL4-responsive PUAST expression vector, and created transgenic strains by embryo injection. At least four independent transgenic lines were derived for wild-type α-synuclein and each α-synuclein mutant, and all were tested for protein expression. Western blots with two monoclonal antibodies (1B599 and clone 42, see below) revealed an appropriately sized band (M, 19 kDa) in heads of transheterozygous flies of the following six genotypes: (1) elav–GAL4/++; UAS–wild-type α-synuclein/++; (2) UAS–A30P α-synuclein/elav–GAL4/++; (3) UAS–A53T α-synuclein/elav–GAL4/++; (4) UAS–wild-type α-synuclein/gmr–GAL4/++; (5) UAS–A30P α-synuclein/gmr–GAL4/++; (6) UAS–A53T α-synuclein/gmr–GAL4/+. Wild-type and mutant lines expressed 30% or less α-synuclein per mg total brain protein compared with rat and human controls. For wild-type α-synuclein and each mutant, we analysed 2–5 independent transgenic lines with varying α-synuclein expression in the assays described.

The GAL4 driver lines used included: 24B–GAL4 (ref. 6), 32B–GAL4 (ref. 6), dpp–GAL4, Ddc–GAL4 (ref. 28), e29c–GAL4, elav–GAL4 (ref. 29) and gmr–GAL4 (ref. 30). Flies expressing GAL4 from the Ddc, elav or gmr promoter, but without a UAS–α-synuclein target, were used as controls, and had the following genotypes: Ddc–GAL4/++; elav–GAL4/++; and gmr–GAL4/+. The wild-type chromosome from the control heterozygotes was derived from the w background strain used for embryo injections. The GAL4-responsive lacZ reporter construct was UAS–lacZ42/++; (ref. 6).

We used a Ddc–GAL4 line to express α-synuclein and β-galactosidase in dopaminergic cells. At the time points reported, in flies of the genotype Ddc–GAL4/UAS–lacZ42/++; all cells clearly positive for β-galactosidase also expressed tyrosine hydroxylase by double-label immunohistochemistry. Only a subset of tyrosine-hydroxylase-positive cells expressed β-galactosidase at our level of detection. We thus restrict our conclusions to a subset of dopaminergic cells in experiments with the Ddc–GAL4 line.

Methods

Transgenic Drosophila

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Sectioning, immunostaining and electron microscopy

Adult flies were fixed in formalin at 1, 10, 30 and 60 days, and embedded in paraffin. Whole-mount preparations fixed in formalin were also used. Immunostaining on paraffin sections was performed using an avidin-biotin-peroxidase complex (ABC) method. Antibodies, sources and dilutions included; anti-tyrosine hydroxylase, Chemicon, 1:500; LB509, Zymed Laboratories, 1:500; clone 42, Transduction Laboratories, 1:200; anti-serotonin, Sigma, 1:500; anti-β-galactosidase, Promega, 1:500; anti-ubiquitin polyclonal serum, Chemicon, 1:1,000. No endogenous immunoreactivity was revealed in tissue sections from nontransgenic control Drosophila stained with the anti-α-synuclein antibodies LB509 and clone 42.

To assess brain morphology, serial 4-μm sections including the entire brain were prepared from formalin-fixed, paraffin-embedded heads from flies of the following three
genotypes: (1) elav→GAL4/+; UAS→wild-type α-synuclein/+; (2) UAS→A30P α-synuclein/elav→GAL4; and (3) UAS→A53T α-synuclein/elav→GAL4. Sections were stained with haematoxylin and eosin. Time points monitored were 1, 10, 30 and 60 days. In addition, serial 1-μm sections of glutaraldehyde-fixed heads from flies of the same genotypes prepared at 1, 30 and 60 days were stained with toluidine blue to highlight degenerating cells. No evidence of excess neurodegeneration was detected using either technique.

To evaluate dopaminergic cells of the dorsomalateral cluster by tyrosine hydroxylase immunostaining, serial 4-μm sections were cut to include the entire brain. Immunocytochemical staining at the cell body level was performed by whole mount immunocytochemistry. Control flies were of the genotype elav→GAL4/+. Experimental flies were of the following genotypes: (1) elav→GAL4/+; UAS→wild-type α-synuclein/+; (2) UAS→A30P α-synuclein/elav→GAL4; and (3) UAS→A53T α-synuclein/elav→GAL4. Sections were stained with haematoxylin and eosin. Time points monitored were 1, 10, 30 and 60 days. In addition, serial 1-μm sections of glutaraldehyde-fixed heads from flies of the same genotypes prepared at 1, 30 and 60 days were stained with toluidine blue to highlight degenerating cells. No evidence of excess neurodegeneration was detected using either technique.

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