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Enhanced Optomotor Efficiency by Expression of the Human Gene *Superoxide Dismutase* Primarily in *Drosophila* Motorneurons

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Abstract: Mutation of the human gene *superoxide dismutase* (*hSOD1*) triggers the fatal neurodegenerative motorneuron disorder, familial amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease). Broad expression of this gene in *Drosophila* has no effect on longevity or functional senescence. We show here that restricting expression of human *SOD1* primarily to motorneurons of *Drosophila* has significant effects on optomotor efficiency during in-flight tracking of rapidly moving visual targets. Under high-stress workloads with a recursive visual-motion stimulus cycle, young isogenic controls failed to track rapidly changing visual cues, whereas their same-aged *hSOD1*-activated progeny maintained coordinated in-flight tracking of the target by phase locking to the dynamic visual movement patterns. Several explanations are considered for the observed effects, including antioxidant intervention in motorneurons, changes in signal transduction pathways that regulate patterns of gene expression in other cell types, and expression of *hSOD1* in a small set of neurons in the central brain. That *hSOD1* overexpression improves sensorimotor coordination in young organisms may suggest possible therapeutic strategies for early-onset ALS in humans.

Keywords: behavior, *Drosophila*, motorneuron, *superoxide dismutase*

INTRODUCTION

Familial amyotrophic lateral sclerosis (FALS) or Lou Gherig's disease is a progressive motorneuron disease associated with a gain-of-function mutation of the human gene *superoxide dismutase* (*hSOD1*; Rosen et al., 1993). Broad expression of *hSOD1* in *Drosophila* has no effect on longevity or functional senescence (Kirby et al., 2008). Selective overexpression of *hSOD1* primarily in motorneurons of transgenic *Drosophila*, however, leads to increased longevity relative to parental-line isogenic controls and rescues short-lived *SOD1*-null mutants to near-normal life span (Parkes et al., 1998). Extension of longevity by *hSOD1* overexpression is considered to occur largely by antioxidant intervention that prevents cumulative DNA and cell damage caused by reactive oxygen species (Parkes et al., 1998). This process is likely to be further mediated by *hSOD1*-triggered changes in signal transduction pathways, possibly through the neuroendocrine system, that regulate patterns of gene expression in a variety of cell types other than motorneurons (Phillips et al., 2000).

The functional consequences of *hSOD1* overexpression on behavioral traits of transgenic *Drosophila* are unknown. This is an important question for two reasons. First, different life-extending genes, such as *hSOD1*, *Methuselah*, and *INDY* (I'm not dead yet) likely cause different patterns of decline or enhancement of behavioral traits (sensory, motor, learning, and memory), and therefore changes in functional abilities of one life-extended line may possibly not generalize to those of another, necessitating line-specific investigations of the effects of each gene on behavior. Second, current theory suggests that the life span of a single critical cell type, the motorneuron, may set the limit on an organism's life span, i.e., death from aging (Parkes et al., 1998; Phillips et al., 2000). Nearly all studies of life-extending genes have rightly used age at death as a practical measure of a gene's effect on life span, leaving open the question of how the expression of such genes affects either physiological state or behavioral functions early in life. Here we examine how *hSOD1* overexpression in motorneurons affects complex behaviors during early stages of life. If *hSOD1* overexpression positively affects complex behaviors in early life, then it confers an adaptive advantage (during critical reproductive periods) beyond extension of motorneuron longevity. This advantage may at least

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partially be attributable to prevention of oxidative damage, which is detected in *Drosophila* flight muscles as early as 1 day of age (Wheeler et al., 1995).

The current study investigated the effects of *hSOD1* overexpression on optomotor coordination during in-flight navigation. The optomotor response of *Drosophila* is a central feature of its corrective flight-control system for maintaining a stable trajectory in reaction to changes in optic flow patterns that signal involuntary deviations from current heading. This response has been extensively studied in *Drosophila* and has served as a rich experimental framework for the study of complex sensorimotor functions in flies (Hecht & Wald, 1933; Kalmus, 1943; Siegel, 1967; Poggio & Reichardt, 1976; Collett, 1980; Warzecha & Egelhaaf, 1996; Van Swinderen & Flores, 2006; Petrosyan et al., 2007; Mronz & Lehmann, 2008; Theobald et al., 2010; Haag et al., 2010; Duistermars et al., 2012; Wardill et al., 2012). Originally described in a number of species including fish, acquatic and hovering insects, crabs, and honey bees in the early 1900s, the optomotor response in *Drosophila* has been investigated since the early 1930s (Hecht & Wald, 1933) using a variety of experimental paradigms from locomotion in rotating striped cylinders (Götz, 1970), to flight-box experiments that allow monitoring of free flight (Miller et al., 2008), bifurcation binary-choice mazes (Van Swinderen & Flores, 2006), and in tethered simulated flight (Vogel, 1965; Lehmann & Dickinson, 1998; Petrosyan et al., 2007). *Drosophila*'s response to translational or rotational changes in the visual scene typically involves head and body orientation (e.g., using body saccades in free flight or wingbeat modulation in tethered flight) toward the direction of change in order to reduce motion blur and stabilize gaze (Collett, 1980; Mronz & Lehmann, 2008). The neural mechanisms that underlie the optomotor response in flies originate in retinula cells and the labula plate units of the optic lobe that synapse directly onto neurons that control a number of motor functions (Heisenberg & Buckner, 1977; Heisenberg et al., 1978; Krapp & Hengstenberg, 1996; Chan et al., 1998; Schnell et al., 2010), and include a horizontal system (HS) network that responds preferentially to translational and rotational visual motion (Hausen 1982a, 1982b; Krapp et al., 2001; Schnell et al., 2010). The optomotor response has also been instrumental in theoretical developments in visual-motion detection and computational vision (e.g., Hassenstein & Reichardt, 1956; Reichardt, 1961; Poggio & Reichardt, 1976; Reichardt & Poggio, 1976; Clark et al., 2011), and in genetic dissection of several mutations and their behavioral phenotypes (Kalmus, 1945; Heisenberg, 1972; Heisenberg et al., 1978; Pflugfelder 1998; Petrosyan et al., 2007; Fei et al., 2010).

We used a tethered-flight paradigm (Petrosyan et al., 2007) to measure how robustly the wingbeat frequencies of young *hSOD1*-overexpressed and control flies phase lock to rapidly moving visual targets to counterbalance the shift in perceived heading and maintain a steady flight trajectory. Our results show that under high-stress workloads with a fast iterative visual-motion cycle, parental isogenic controls fail to track the rapidly changing visual cues, whereas their same-aged *hSOD1*-activated progeny maintain coordinated in-flight tracking of the target.

MATERIALS AND METHODS

hSOD1-expressed and control lines were generated at the University of Guelph as described fully in Parkes et al. (1998). Briefly, expression of a human *SOD1* transgene in *Drosophila* motorneurons was achieved using the yeast GAL4/UAS system (Brand et al., 1993; Gustafson & Boulianne, 1996; Yeh et al., 1995). The D42-GAL4 activator used here is expressed broadly during embryogenesis, becomes restricted to motorneurons and interneurons during larval stages, and with the exception of a small number of unidentified neurons in the central brain, is restricted to motorneurons within the ventral ganglia in the adult fly. The *hSOD1* transgene consisted of a human *SOD1* cDNA coupled to a yeast UAS element within a *Drosophila* P-transformation vector. Because both life span and behavior are affected by variation in genetic background, a number of genetic measures were taken in introducing the D42-GAL4 and UAS-*hSOD1* transgenes into a uniform *Sod*+/+ genetic background, and to construct expressing and nonexpressing lines that were co-isogenic for most of the genome, with minimal differences in the genetic background between strains (see Parkes et al., 1998; Kirby et al., 2008). This allows tracing of behavioral phenotypes specifically to GAL4-activated *hSOD1* expression.

Virgin flies were sex-segregated within 4 h of eclosion and maintained in small laboratory vials containing fresh food media in an incubator at 25°C and 40% humidity on a 12/12-h dark-light cycle (VWR Scientific, model 2015; Radnor, PA, USA). They were transferred to fresh food vials every 3 to 4 days. We confirmed extension of *hSOD1*-expressed life span relative to isogenic controls by approximately 30% in virgin females (68 vs. 52 days) and 48% in virgin males (65 vs. 44 days) measured at 50% mortality levels of a population of approximately 400 flies. Kaplan-Meier survival analyses (log rank Mantel-Cox test) showed that *hSOD1*-expressed males significantly outlived control males ($\chi^2 = 119.1$, $P < 0.001$) and *hSOD1*-expressed females significantly outlived control females ($\chi^2 = 123.0$, $P < 0.001$).

Wingbeat frequency was measured in tethered flight at 5 h after onset of subjective day. The tethering process involved several steps. First, an individual fly was lightly CO_2 anesthetized and transferred to a custom-made aluminum block in a Peltier cooler (Boekel Scientific,

model 260014; Feasterville, PA, USA) on which a small opening ($2 \times 1 \times 1$ mm^3) had been drilled to allow accurate positioning of an anesthetized fly. The fly remained under cold anesthesia at 4°C. The tip of a tungsten wire (130 μm in diameter) was dipped in glass glue (Loctite, New York, NY, USA) and, under a stereo microscope (Olympus SZ40; Center Valley, PA, USA) lowered using a micropositioner (Stoelting, Wood Dale, IL, USA) onto the anesthetized fly's thorax. The glue was cured with an ultraviolet (UV) gun (Electro-Lite, model ELC-403; Bethel, CT, USA) for 20 s and the fly was removed from the Peltier cooler using the micropositioner. Flies usually recovered from cold anesthesia and began flight within 3 to 4 min. Tethered flies were moved to the experimental chamber, fed with a small piece of filter paper dipped in sucrose water, and allowed to rest and become acclimated to the experimental environment for an additional 30 min prior to data collection. The tethered fly was positioned at a pitch angle $\theta = 30°$ from the horizontal plane under a solid-state infrared (IR) laser (Lasermate Group, Pomona, CA, USA; model PLC8082AE) with an adjustable focus that cast shadows of the wingbeats onto fast-response IR photodiode sensors (Photonic Detectors, Simi Valley, CA, USA; part no. PDB-C615-2). The IR wavelength was outside the fly's range of visible spectrum (Hernfindez de Salomon & Spatz, 1983). The sensors were placed in a small plastic box covered with an IR filter (Edmund Industrial Optics, Barrington, NJ, USA; part. no. NT32769). The experiment was run in complete darkness in a steel chamber ($2 \times 2 \times 2$ m^3; IAC, Bronx, NY, USA) with either a single green light-emitting diode (LED; 555 nm) positioned directly in the fly's line of sight at a distance of 15 cm to provide a visual target for phototaxis during steady-state flight, or a fast-response liquid crystal display (LCD) for measurement of the fly's optomotor response to a moving square-wave grating (see below). The output of the photodiode sensors were sent to an amplifier and fed into an analog-to-digital converter positioned outside the chamber, and recorded at a sampling rate of 10 kHz. An individual fly's steady-state wingbeat frequency (WBF) was determined as the average of five to ten 1 s samples of flight. This sampling scheme is sufficiently representative of the average WBF, which does not vary significantly during steady-state flight (Petrosyan et al., 2007). For each 1 s sample, the wingbeat waveform was fast-Fourier transformed and the frequency corresponding to the peak of this function was determined as the WBF for that sample.

To measure an individual fly's optomotor response, we tethered it as described above and positioned it in front of a high-speed flicker-free LCD (Viewsonic VX 924; Walnut, CA, USA; 4 ms rise-decay time) at a distance of 5 cm. Vertical black and white square waves (bars) with a spatial frequency of 30° served as stimuli. In front of the LCD, a 30×30 cm^2 Schott Borofloat 3.3 mm-thick high-energy hot mirror (heat shield; Navitar Coating Labs lot no. 10-815-10; Rochester, NY, USA) was positioned to eliminate near IR as well as LCD heat (passband between 390 and 700 nm). Temperature measurement at the position of the tethered fly was equal to ambient temperature in the experimental chamber (~25°C). Each trial of a 100-trial run consisted of a 600 ms presentation of a stationary grating followed by 400 ms of visual motion toward the right at a temporal frequency of 4 s^{-1}. This motion on-off cycle rate was markedly higher than that used in prior studies (Petrosyan et al., 2007; Mamiya et al., 2011; Wardill et al., 2012) in order to impose significant sensorimotor stress on flight navigation. We initially made informal observations in which the optomotor response was examined with slower stimulus cycles (e.g., 7 s period with a 3–4 s on-off cycle; Petrosyan et al., 2007). Wingbeat waveform samples from these flies (control and *hSOD1*) will be shown in the Results section. We observed the standard response but no discernable differences across genotypes, and hence decreased the motion on-off period to 1 s (0.4–0.6 s) to increase stimulus rate (tempo) and sensorimotor demand during flight. WBF was calculated for successive 200-ms segments of the wingbeat time series, zero-padded to 1 s to allow a 1-Hz frequency resolution (Manolakis & Ingle, 2011). The longest segment of sustained flight, which for most flies was the entire 100 s experimental run, was used to calculate the modulation spectrum's peak by subjecting the WBF function (i.e., frequency modulation [FM] time series) to a fast Fourier transform (FFT). All aspects of the stimulus generation, presentation, and digital recordings were microprocessor controlled via software written in MATLAB (Mathworks, Natick, MA, USA). After each experimental run, flies were CO_2 anesthetized, removed from tether, and discarded in citrus oil.

RESULTS

WBFs of tethered flies (Figure 1A) positioned in front of a stationary point light source are shown in Figure 1B. The average steady-state WBFs were nearly identical for the *hSOD1*-expressed flies ($n = 6$) and their parental control group ($n = 7$) ($t(11) = 0.661$, $P = 0.522$). We then measured optomotor flight efficiency of the same individual flies in tracking a moving black-white square-wave grating with a stop-go period of 1 s during which the grating moved for 400 ms and was stationary for the next 600 ms. Figure 1C and D show wingbeat modulation time series for an *hSOD1* and control fly, superimposed on the stimulus timeline where red and green bars represent periods of stationary and moving visual gratings respectively. The *hSOD1*-expressed fly's wingbeat frequency phase locks to the stimulus period, whereas the control line's wingbeat time series is stochastic. Figure 1E and 1F show

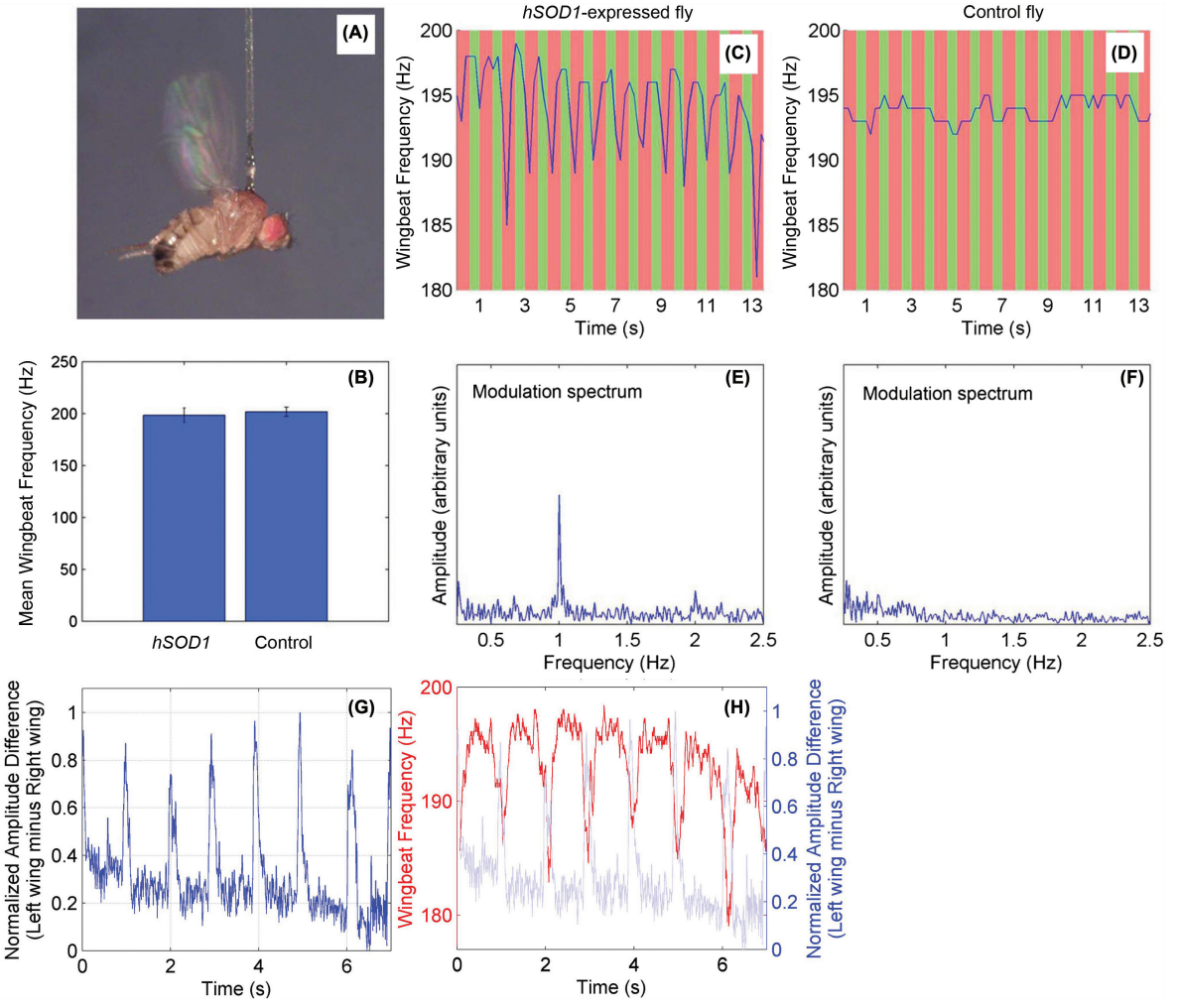

Figure 1. *Drosophila* in tethered flight phase locks to rapidly changing visual-motion cues. (**A**) *Drosophila* in tethered flight glued to a tungsten wire. (**B**) Mean wingbeat frequency in steady-state phototaxic flight. (**C**, **D**) Wingbeat modulation time series superimposed on the stimulus timeline with green and red bars respectively representing periods of stationary and moving visual stimulus. (**E**, **F**) Spectra of the waveforms shown in panels C and D, respectively. (**G**) Difference between the left- and right-wing amplitude envelopes for the fly whose data are shown in panel C. (**H**) WBF for the same fly with the data of panel G replotted in light blue for comparison.

modulation spectra of waveforms shown in the top panels (C and D). A significant peak is observed in the *hSOD1*-expressed modulation spectrum at 1 Hz, the frequency of the stop-go motion cycle, whereas no major peaks are observed in the control-line modulation spectrum.

Figure 1G shows the difference between the left- and right-wing amplitude envelopes for the fly whose data are shown in panel C, derived from the Hilbert transform of the difference between the left and right wingbeat time series. There is a significant increase in the amplitude of the left wing, relative to the right, in response to the visual grid moving toward the right. All flies that showed phase-locked WBF modulation (to fast or slow cycles) also showed this directional component of an increase in left wingbeat amplitude to generate a rightward torque in the direction of visual motion. Figure 1H shows the WBF function for this fly. To calculate WBF for the data of this panel, we measured peak-to-peak periods between every single wingbeat and estimated instantaneous WBF as the inverse of each period. Although computationally tedious, this analysis provided WBF estimates with high temporal resolution (1 wingbeat). For comparison, we also show in Figure 1H a faint tracing of the data replotted from Figure 1G time locked to changes in WBF. The two functions appear to be antiphasic, with an increase in the amplitude of the left wing coupled to a decrease in wingbeat frequency. Although the left and right wings are always synchronous and have the same WBF, a right moving visual stimulus induces an increase in the amplitude of the left wingbeat while simultaneously slowing down wingbeat frequency by 10 to 15 Hz.

Six *hSOD1* and seven control flies individually completed a 100-trial experimental run in the visual-motion

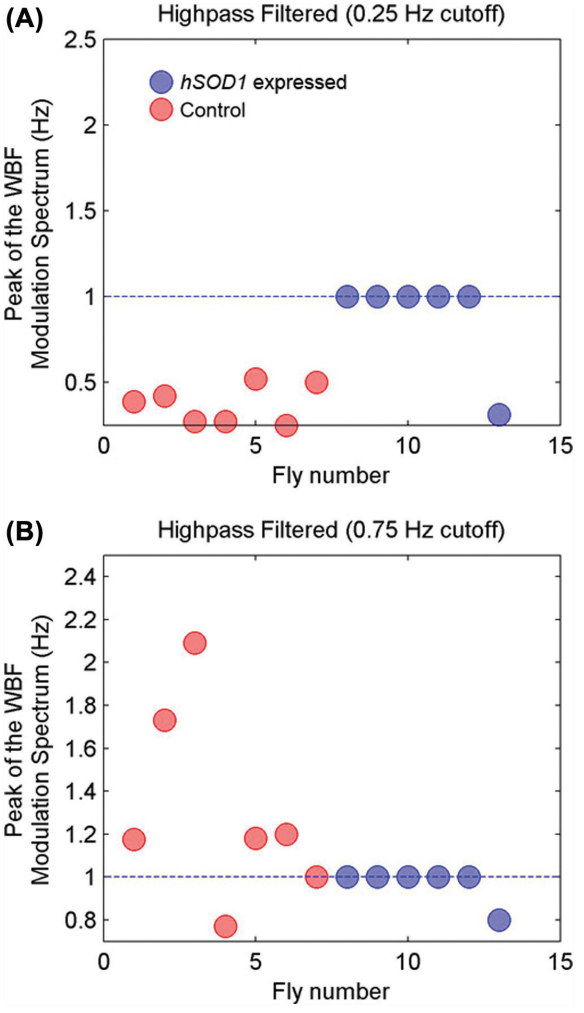

Figure 2. Spectral peaks in the wingbeat frequency modulation spectrum. (**A**) WBF modulation spectrum peaks were measured for 13 flies. Spectra were high-pass filtered at 0.25 Hz to exclude VLF components resulting from slowly drifting mean WBFs during extended flight (Figure 1F). Phase locking to the stop-go stimulus cycle of 1 s yields a spectral peak at 1 Hz. All flies were female from two age groups: 5 days (nos. 1–3, 8–10), 10 days (nos. 4–7, 11–13). (**B**) Same as A, high-pass filtered at a higher cutoff frequency of 0.75 Hz.

experiment. Figure 2A shows the results of this experiment. For five of six *hSOD1*-expressed flies, the modulation spectrum had its largest peak at 1 Hz, demonstrating robust phase locking to the visual-motion cycle. None of the seven control flies had a spectral peak at 1 Hz in their wingbeat modulation spectra, suggesting that they failed to track the rapidly changing visual cue. The modulation spectra used to calculate the peaks in Figure 2A were high-pass filtered at 0.25 Hz to exclude very-low-frequency (VLF) components in the modulation spectrum's low-pass slope (Figure 1F) that resulted from slowly drifting wingbeat frequencies during extended periods of sustained flight. Because the modulation spectrum has a low-pass slope, the largest peak in the control flies' modulation spectra occurred at low frequencies, even when high-pass filtered at 0.25 Hz (Figure 2A). To determine if limiting the spectrum's pass band to higher frequencies might reveal peaks at the stimulus repetition rate of 1 Hz, we high-pass filtered the modulation spectra at a cutoff frequency of 0.75 Hz. Figure 1B shows the values of spectral peaks for these filtered spectra for the same 13 flies. As expected peaks for *hSOD1*-expressed flies continue to occur at 1 Hz for 5 of 6 flies, but for the control group, these peaks are dispersed throughout nearly the entire range of all possible modulation frequencies, which had an upper Nyquist limit of 2.5 Hz (5 Hz sampling rate). A Levene test for homogeneity of variance confirmed that the variance of the modulation peaks for *hSOD1*-expressed flies was significantly smaller than that of the control group ($F(11) = 7.17$, $p < 0.05$). Furthermore, the likelihood that a peak occurs by chance at exactly 1 Hz, based on the spectrum's discrete sample space, is $P = \sim 0.0022$ and hence the probability that five (k) of six (n) *hSOD1*-expressed flies yield peaks at 1 Hz by chance is of course extremely small $Pr = \frac{n!}{k!(n-k)!} p^k q^{n-k} = 3.2 \times 10^{-13}$. A Mann-Whitney U test confirmed a significant genotype difference in mean spectral-peak value ($U = 4.0$, $P < 0.05$).

Figure 3 shows several interesting cases of entrainment to visual-motion. Left panels show that both control and *hSOD1* flies strongly phase lock to a slow visual-motion cycle that has a stop-go repetition period of 7 s (4 s of a stationary grid followed by 3 s of visual motion). Each panel shows data from two runs of the same fly with standard optomotor responses that are typical of all flies. Although control flies show no phase locking to rapid (1 Hz) stop-go motion cycles when the analysis window comprises the entire 100 s run (Figure 2), we wondered if they do perhaps display phase locking for brief periods, in the order of a few trials. We scanned our entire data set for potential signs of phase locking by control flies and found a very small number of cases (shown in the right panels of Figure 3) that appear to demonstrate brief and intermittent entrainment to the visual motion.

We therefore conducted a more detailed analysis of phase locking for shorter run segments by dividing each 100 s run into ten 10 s segments, and estimated the peak of the WBF modulation function for each segment. This yielded 70 estimates for control flies (7 flies), and 60 estimates for *hSOD1*-expressed flies. Histograms of these peak periodicities are shown in the upper panels of Figure 4 and the averaged modulation spectra are shown in the bottom panels. Nearly half (29 out of 60) of segments analyzed for the *hSOD1*-expressed flies showed a WBF modulation peak at 1 Hz (the visual-motion cycle), whereas only 5 out of 70 control flies showed this peak, and even that appears to simply reflect the monotonically declining value of the low-pass slope of these functions at 1 Hz (left panels).

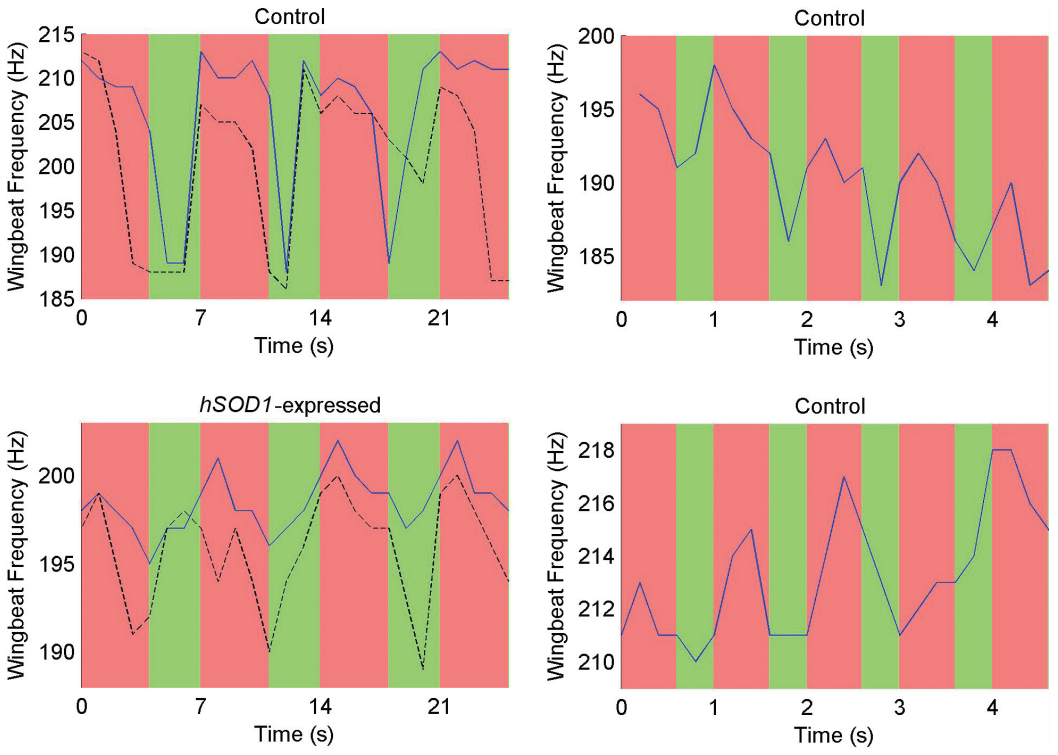

Figure 3. Four cases of entrainment to slow and fast visual motion. Left panels show that both control and *hSOD1* flies phase lock to a slow visual-motion cycle that has a stop-go repetition period of 7 s. Each panel shows data from two runs of the same fly. Right panels show two rare examples of control flies phase locking for brief periods to the fast visual-motion cycle (see text for details).

DISCUSSION

We have found that *hSOD1* overexpression primarily in motorneurons affects sensorimotor coordination in young adult flies during sustained flight. Because the *hSOD1* transgene is overexpressed primarily in the fly's motorneurons and not in brain pathways receiving input from the visual system or retina (Parkes et al., 1998), improved optomotor synchronization may possibly suggest an integrative systemwide functional transformation. Furthermore, these improvements are not a corollary of variations in overall metabolic rate as confirmed by equivalent respiration rates (Parkes et al., 1998) and the nearly identical wingbeat frequencies of *hSOD1* and control flies in steady-state flight at the age tested (Figure 1B).

Prior work has shown that overexpression of *hSOD1* in motorneurons extends life span, possibly by mitigating the adverse effects of oxidative damage (Parkes et al., 1998; Phillips et al., 2000; Kirby et al., 2008). Our results show that *hSOD1* overexpression in motorneurons additionally affects functional abilities during early stages of life, and thus may possibly provide a survival advantage by enhancing critical motor and sensory functions during reproductively active stages of life. This behavioral enhancement may reveal significant antioxidant intervention early in life, and/or possibly a novel functional role for *hSOD1* overexpression in young adults.

One should, however, be cautious in interpreting these findings. First, improved optomotor efficiency from *hSOD1* overexpression may be one of several motor system enhancements beyond the flight-control system. In a different study (Petrosyan et al., 2013), we have observed that *hSOD1* overexpression also leads to significant gerontological improvements, for example, a more vigorous locomotor response in late life (age >60 days). Furthermore, the improvement in optomotor efficiency in life-extended lines is not exclusive to *hSOD1*-expressed flies. The life-extended *methuselah* mutant, for example, also displays enhancements in optomotor abilities relative to its parental control group (Petrosyan et al., 2007). More importantly, the improved optomotor performance observed in our study may be caused either by *hSOD1*'s direct action on motorneuron function, or by a number of other complex factors. As noted earlier, although *hSOD1* is expressed primarily in motorneurons of the ventral ganglia (i.e., not all motorneurons), it is also expressed in a small number of other cell types in the central brain (Parkes et al., 1998). This allows for the possibility that *hSOD1* expression may have affected functions of other central nervous system (CNS) networks, resulting in the observed phenotypic differences. Furthermore, *hSOD1*

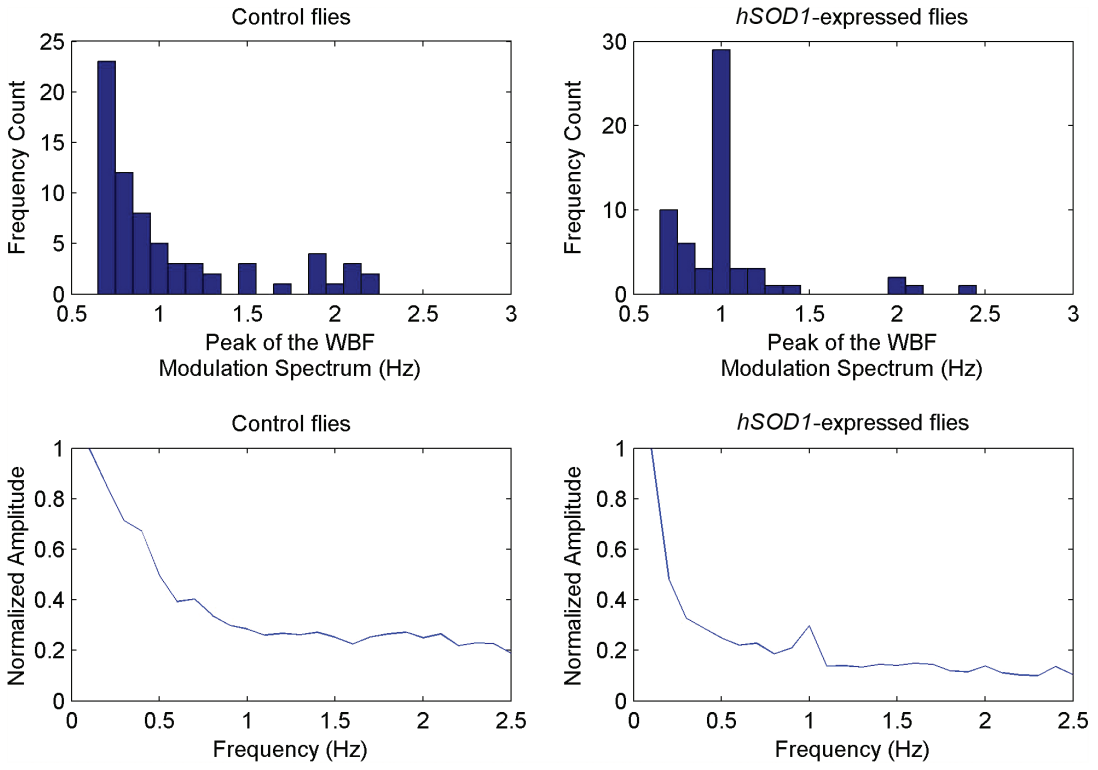

Figure 4. Histograms of WBF modulation peaks and averaged modulation spectra. *Top panels*: Data from each 100 s run was divided into ten 10 s samples and the spectrum of the WBF function was calculated from the FFT of each 10 s segment (high-pass filtered at 0.65 Hz). Histograms of these peaks for controls (70 samples) and *hSOD1* flies (60 samples) are shown in the top panels. *Bottom panels*: Unfiltered modulation spectra averaged across the 70 control and 60 *hSOD1* sample spectra.

expression may affect signal transduction pathways that regulate patterns of gene expression in cell types other than motorneurons (Phillips et al., 2000).

Our findings may also have clinical implications for treatment of ALS in humans. Although ALS tends to afflict older individuals, typically between 40 and 60 years of age, diagnostic symptoms such as dysphagia (difficulty in swallowing food or liquids) and muscle weakness particularly in upper limbs may manifest early in life and be associated with juvenile or early-onset ALS (Sabatelli et al., 2008). That *hSOD1* overexpression improves sensorimotor coordination in young organisms may suggest possible therapeutic strategies for ALS in early developmental stages of life. The determination of whether clinical interventions based on *hSOD1* overexpression may mitigate early-onset symptoms and whether such intervention may have a sustained effect if administered during early stages of life (Costantini et al., 2012) are questions that merit further investigation. An additional question for future research is whether the observed behavioral advantage of *hSOD1* overexpression is maintained throughout an organism's life span, or if the age-dependent decline of sensorimotor abilities in the *hSOD1* line converges chronologically with that of the parental control line in late life.

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