Investigations into modifications of neuromuscular physiology by axonal transport disruptions in *Drosophila* SOD1 mutants

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Abstract

Cu/Zn superoxide dismutase (SOD1) is a cytoplasmic antioxidant enzyme, which, when mutant in humans, is linked to familial cases of the motor neurodegenerative disease amyotrophic lateral sclerosis (ALS). The *Drosophila* SOD1 gene (*Sod*) shares a highly conserved sequence with the human homolog, and this study includes examinations of the established hypomorphic *n108* allele (*Sod*^{*n108*}), alongside a knock-in construct of the *G85R* allele found in human ALS patients (*Sod*^{*G85R*}). In addition to previously documented decreased adult lifespan and attenuated motor function, we show that *Sod* mutant *Drosophila* can display significant mortality during larval and pupal development. Immunostaining of neuronal membrane at neuromuscular synapses in *Sod* mutant larvae revealed presynaptic terminals of abnormal morphology, with incompletely segregated and enlarged synaptic boutons along the motor terminal branches, in which vital staining indicated mitochondrial aggregation. We demonstrate strong genetic interactions between *Sod*^{*G85R*} and the axon transport-linked *Pk* mutants *Pk*^{*Pk*} and *Pk*^{*Sple*} in larval NMJ morphology and neuromuscular transmission. Intracellular recordings of larval excitatory junction

potentials (EJPs) demonstrate enhanced EJP size in the double-mutant of *Pk*^{*Pk*} and *Sod*^{*G85R*}. To examine synaptic terminal excitability, maintained by Ca²⁺ channel action and independent of Na⁺ channel function, we used the Nav blocker TTX, along with the K_v1 blocker 4-aminopyridine (4-AP) and the commonly used broad-spectrum K⁺ channel blocker tetraethylammonium (TEA). We were able to induce prolonged “plateau-like” EJPs, which were further extended in *Pk* mutants and *Pk*;*Sod* double-mutants. These observations were corroborated with focal EJP recording from individual boutons. Altogether, this study highlights alterations in synaptic morphology and function at a developmental stage prior to neurodegeneration and death of *Sod* mutant organisms, along with a potential role of axonal transport in the maintenance of neuronal health.

Introduction

The generation of reactive oxygen species (ROS) is a ubiquitous feature of energy metabolism in living cells. ROS are a class of free radicals that readily oxidize other molecules, and the resulting damage to cellular components over time is thought to be a normal feature of aging (Harman, 1956; Lushchak, 2014). The disruption of oxidative homeostasis that occurs as a result of increased ROS load is termed ‘oxidative stress’ (Paniker et al., 1970; Sies, 1997). While ROS have been implicated as signaling molecules in healthy physiological processes ranging from cell differentiation (Thannickal & Fanburg, 2000) to synaptic plasticity (Massaad & Klann, 2011), high oxidative stress predisposes proteins to misfolding (Berlett & Stadtman, 1997), lipids to peroxidation (Altan et al. 2003), and nucleic acids to breakage and mutation (Aitken & Krausz, 2001). Excessive oxidative stress has been implicated in neurodegenerative diseases, cardiovascular diseases, and cancer (Liguori et al., 2018; Uttara et al., 2009; Dhalla et al., 2000; Sosa et al., 2013). Both

eukaryotic and prokaryotic organisms produce antioxidant enzymes to maintain oxidative homeostasis, including catalases, glutathione peroxidases, and superoxide dismutases (Matés et al., 1999).

The antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) forms homodimers that mainly localize to the cytosol, and target superoxide molecules for neutralization (McCord & Fridovich, 1969; Culotta & Daly, 2013). The reactions that SOD1 catalyzes sum to the conversion of two superoxides (O_2^-) and two protons (H^+) to molecular oxygen (O_2) and hydrogen peroxide (H_2O_2), which is targeted by the enzyme catalase for conversion into molecular oxygen and water (O_2 & H_2O) (Mondola et al., 2016; Perry et al., 2010). The gene encoding SOD1 is highly conserved across phyla, and both *Drosophila* and mice SOD1 null mutants display reduced lifespans (Phillips et al., 1989; Zhang et al., 2016). Mutations in the gene for SOD1 and decreased overall SOD1 activity have been found in some human patients with the neurodegenerative disease amyotrophic lateral sclerosis (ALS), popularly known as Lou Gehrig's disease or motor neuron disease (Rosen et al., 1993; Saccon et al., 2013). ALS is a fatal neurodegenerative disease in humans, in which the gradual death of motor neurons causes the muscles they innervate to weaken and atrophy (Rosen et al., 1993; Rowland & Schneider, 2001; Galvin et al., 2017; Hardiman et al., 2011).

Generated by EMS mutagenesis, the *n108* allele of the *Drosophila* gene for SOD1 (*Sod*) has been identified as a loss-of-function allele (Campbell et al., 1985, Phillips et al., 1989). Flies homozygous for the *n108* allele (*Sodⁿ¹⁰⁸*) display hypersensitivity to oxidative stress and severely reduced lifespans. The *Drosophila* *G85R* allele (*Sod^{G85R}*) is a knock-in construct of the missense allele of the same name found in human ALS patients (Şahin et al., 2017). The *G85R* mutation site is conserved between the human and *Drosophila* genes, so the human point mutation was introduced into the *Drosophila* gene by ends-out homologous

recombination. The G85R point mutation is in close proximity to a metal ion binding site that enables SOD1 to maintain structural stability. *Sod*^{G85R} homozygotes display locomotor defects as larvae, and near-full lethality at the pupal stage (Şahin et al., 2017).

The *Drosophila* larval NMJ (Jan & Jan, 1976; Atwood et al., 1993) is an excellent platform for the study of early motor synaptic characteristics that precede recognizable SOD1-linked neurodegeneration. Immunohistochemistry can make visible neuronal membrane and synaptic proteins at the NMJ. In particular, we used anti-horseradish peroxidase (anti-HRP: Jan & Jan, 1982) to view presynaptic terminal morphology, anti-Dlg (monoclonal antibody 4F3: Parnas et al., 2001) which also reveals subsynaptic reticulum (SSR) surround the synaptic boutons (Budnik et al., 1996), and the monoclonal antibody 22C10 (Fujita et al., 1982; Zipursky et al., 1984) to view the microtubule-associated protein Futsch that decorates mature microtubule structures (Hummel et al., 2000). We found that the presynaptic motor terminals of *Sod* mutant larvae carried enlarged synaptic boutons, which were also visible *in vivo* in our *Sod* mutant lines containing a cell membrane GFP (CD8-GFP: Lee & Luo, 2001) targeted to motor neurons (OK371-Gal4: Mahr & Aberle, 2006; C164-Gal4: Torroja et al., 1999) (Gal4-UAS system: Brand & Perrimon, 1993). Vital staining with the mitochondrial stain tetramethylrhodamine (TMRM) alongside presynaptic CD8-GFP revealed macropunctae, suggesting mitochondrial aggregation inside the enlarged boutons of *Sod* mutants. While it is known that mitochondria are important for Ca²⁺ buffering and ATP generation in the presynaptic terminal for synaptic transmission (MacAskill & Kittler, 2009), the extreme synaptic accumulation of mitochondria in *Sod* mutants may involve abnormal axonal transport. Indeed, our finding recapitulates aberrant mitochondrial distribution previously observed in human ALS patients and mouse models of ALS, with disrupted mitochondrial transport and morphology (De Vos et al., 2007; Ott et al., 2007; Smith et al., 2017; Vande Velde et al., 2011).

In *Drosophila*, the planar cell polarity gene *Prickle* (*Pk*) (Gubb & García-Bellido, 1982; Gubb et al., 1999; Gray et al., 2011; Lin & Gubb, 2009; Tree et al., 2002) is expressed in the form of the *Pk^{Pk}* (*Prickle Prickle*) and *Pk^{Sple}* (*Prickle Spiny-legs*) isoforms, and their mutants display distinctly altered microtubule-mediated transport and microtubule dynamics in larval motor axons, with related changes in neuronal excitability (Ehaideb et al., 2014; Ehaideb et al., 2016). In this study, we employ mutants of both *Pk* isoforms to demonstrate strong interactions with *Sod* in larval motor neuron synaptic morphology and neuromuscular transmission.

Synaptic transmission at the NMJ can be analyzed with the method of recording excitatory junction potentials (EJPs) with an intracellular electrode (Jan & Jan, 1976). Additionally, local loose-patch recording of synaptic currents enables characterization of the contributions of single synaptic boutons along the terminal branches to overall synaptic transmission (Renger et al., 2000; Kurdyak et al., 1994). In this study we adopt the same recording configuration with voltage recording of extracellular focal field potentials (efEJP, Xing & Wu, 2018a, b) to infer the synaptic current flow underneath the pipette, an approach first established by Fatt and Katz (1952) for analyzing miniature end-plate currents (mEPCs) and potentials (mEPPs) at the frog neuromuscular junction. We were therefore able to correlate global intracellular events and focal analysis of synaptic transmission.

Materials and Methods

Fly stocks

Flies and larvae were housed in bottles containing a cornmeal-agar medium (Frankel & Brousseau, 1968), supplemented with 3g of yeast paste prepared as a 1:1 mixture of ddH₂O and active dry yeast (Red Star Yeast Co., LLC). Flies were reared at a room temperature of 22 ± 1°C. The *Sod* mutants examined were those of alleles *n108* (Phillips et al., 1989) (gift from John Phillips, University of Guelph, Ontario, Canada) and *G85R* (Şahin et al. 2017) (gift from Barry Ganetzky, University of Wisconsin-Madison, USA). The wild-type control strain used for Figures 1, 2, 3, and 4 was Canton-S (CS). For experiments in which a motor neuron membrane tag was needed, the stock used carried a chr. II *UAS-CD8-GFP* recombined with a chr. II motor neuron GAL4 driver (Brand & Perrimon, 1993), either *C164-Gal4* (Torroja et al., 1999) or *OK371-Gal4* (Mahr and Aberle, 2006). The original recombined *OK371-Gal4 UAS-CD8-GFP* (*OK371::CD8-GFP*) was obtained from the National Centre for Biological Sciences (Bangalore, Karnataka, India). *Sod* lines used to produce the data represented in Figure 1 were balanced over a TM6 balancer with a dominant *Tubby* (*Tb*) marker (TM6, *Tb*). The *Pk^{Pk}* and *Pk^{Sple}* lines used in this study have been documented previously (Ehaideb et al., 2014). The wild-type control strain used for Figure 5 was the Oregon-R (OR) used in Ehaideb et al. (2014). All *Pk*;*Sod* double-mutant lines were generated in the Wu lab by conventional genetic crosses.

Developmental lethality studies

Housing and medium conditions for the developmental lethality study (Figure 1) were identical to those described in the main "Fly stocks" section. Flies including 20-40 females were used to seed each bottle. Counts of deaths and eclosions were performed 16-17 d. after seeding. The TM6, *Tb* balancer carried by *Sod* heterozygous wandering 3rd instar

larvae and pupae was used to distinguish them from *Sod* mutant homozygotes. Dead larvae appear as darkened corpses on the wall of the housing bottle. Dead pupae were entirely black, or contained a mixture of dark and empty patches, as confirmed by transmitted light under a dissection scope. Juvenile flies dead during eclosion were distinguished from flies in the process of eclosing by a second observation 15-30 min after the first. Juvenile fly corpses were also frequently darker than their live counterparts.

Larval preparations, vital staining, & immunohistochemistry

Wandering third instar larvae were dissected, as per the "fillet preparation method", in HL 3.1 saline (Feng et al., 2004). HL3.1 used in immunohistochemistry or vital staining experiments contained 0.1 mM Ca^{2+} . Tetramethylrhodamine (TMRM, $\text{C}_{25}\text{H}_{25}\text{ClN}_2\text{O}_7$) (Floryk & Houštěk, 1999) vital staining of larval preps for mitochondria was done at 100 nM in HL 3.1 for 20 minutes (in a light-impermeable chamber to prevent TMRM photobleaching), after which the bath was replaced with TMRM-free HL 3.1 before microscopy. Before antibody staining, larval preps were fixed in 3.7% formaldehyde in HL 3.1 for 25-30 minutes. Fixed preps were incubated with an FITC-conjugated goat antibody to HRP at 1:50 in phosphate buffer saline, at 4°C for 12-48 h. Anti-HRP (Jan & Jan, 1982) recognizes a neural carbohydrate antigen in *Drosophila* (Kurosaka et al., 1991). For double-staining with anti-HRP and anti-Dlg (monoclonal AB 4F3: Parnas et al., 2001) or anti-Futsch (monoclonal AB 22C10: Fujita et al., 1982; Zipursky et al., 1984) staining, fixed larval preps were incubated with the primary monoclonal AB at 1:100 in phosphate buffer saline, at 4°C for 12-48 h, rinsed, and incubated in phosphate buffer saline containing anti-HRP(conj. FITC) and a secondary TRITC-conjugated anti-mouse IgG antibody, both at 1:50. The 4F3 and 22C10 antibodies were obtained from the Developmental Studies Hybridoma Bank (DSHB) (University of Iowa, Iowa City, IA, USA). The FITC-conjugated anti-HRP and TRITC-conjugated anti-mouse IgG were obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Larval NMJ

images, vital and fixed, were gathered on a Leica DM IL LED inverted microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA), using the Leica Application Suite X software.

Larval NMJ morphology and mitochondrial distributions

Examination of *Sod* single-mutant NMJ morphology (Figure 2) was carried out in muscles 4 (M4) and 13 (M13), in abdominal segments A3, A4, and A5, and quantifications from the different segments were pooled. Examination of *Pk;Sod* double-mutant NMJ morphology (Figure 4) was carried out in muscle 4 (M4) in abdominal segments A2, A4, and A6, and quantifications from the different segments were pooled. Counts of boutons from M4 include only type Ib boutons. Ib and Is boutons and branches in M13 were quantified separately. Counts of specific bouton morphologies ("unsegregated" boutons and large terminal boutons) are from M4 (Ib). For branch counting, a branch was defined as a terminal process carrying at least two boutons (Zhong et al., 1992). A bouton was described as incompletely segregated ("unsegregated") if the width of the narrowest point on the neck preceding it was greater than 80% of the width of the widest point on the bouton. If multiple unsegregated boutons appeared in a row, it could be difficult count them. In these cases, counts were conservative. A terminal bouton was determined to be the "largest" if it was measurably larger than any other bouton on that branch, past the last branching point.

Photography of NMJ mitochondria was completed within 30 minutes after TMRM mitochondrial staining to mitigate effects of post-dissection physiological decline on results. Counts of putative mitochondrial aggregates in enlarged terminal boutons were obtained from larvae in which the NMJ membrane carried CD8-GFP driven by C164-Gal4 or OK371-Gal4, making bouton morphology visible in the green fluorescence channel during vital staining in the red channel.

Electrophysiology

All electrophysiological recordings were based on published protocols, and performed in HL3.1 saline (Feng et al., 2004) (Composition (mM): 70 NaCl, 5 KCl, 0.1 CaCl₂, 4 MgCl₂, 10 NaHCO₃, 5 trehalose, 115 sucrose, 5 HEPES) (pH adjusted to 7.1). Intracellular recording of excitatory junction potentials (EJPs) was adapted from Ueda and Wu (2006). Recording of extracellular focal EJPs (efEJPs) was shown in Ueda and Wu (2009) and Xing and Wu (2018a,b). For nerve-evoked action potentials, 0.1 ms stimuli were applied to the cut end of the segmental nerve through a suction pipette. To enable analysis of presynaptic terminal excitability, we also performed electrotonic stimulation (Wu et al., 1978; Ganetzky & Wu, 1982; 1983). Briefly: Tetrodotoxin (TTX) was applied to block Na⁺ channels. To achieve direct electrotonic stimulation of the terminal, a 1 ms stimulus was applied to the cut segmental nerve, which was sucked into the stimulation electrode, nearly up to the entry point into the muscle, so as to allow the stimulus to passively propagate to the terminal and depolarize the endplate membrane (Lee et al., 2014). In this manner, CaV channels were directly triggered by local depolarization, independent from invasion of axonal Na⁺ action potentials. Further, a significant portion of multiple K⁺ channel species, including *Shaker* (Kv1), were blocked by co-application of 4-aminopyridine (4-AP) and tetraethylammonium (TEA) (Jan & Jan, 1977; Singh & Wu, 1999). Electrotonic stimulation of the larval NMJ with TTX, 4-AP, and TEA has been shown in Lee et al. (2014). Such manipulations allow for the examination of terminal excitability driven by Ca²⁺ channels with reduced repolarization regulation by a range of K⁺ channels, with the exclusion of axonal potential interplay.

Statistics

Overall distributions of deaths throughout the lifecycle (Figure 1) were compared using Fisher's exact test. Counts of NMJ boutons and branches and of enlarged terminal and

unsegregated boutons (Figures 2 and 3) were examined with Kruskal-Wallis testing between all groups within muscle # and parameter (eg. M4, boutons), followed by rank-sum (Mann-Whitney U) post hoc between individual groups. EJP sizes were compared with one-way ANOVA (Figure 4B). Plateau-like EJP durations were compared by F-test (Figure 4D). Counts of NMJ mitochondrial macropunctae (Figure 6) were compared with Kruskal-Wallis testing followed by rank-sum (Mann-Whitney U) post hoc.

Results

Lethality of *Sod* mutants during different developmental stages

During fly husbandry and maintenance of fly stocks, we observed striking mortality during the development of *Sod* mutants. We therefore proceeded with a systematic study, comparing mortality rates at different developmental stages in *Sod*^{*n108*} and *Sod*^{*G85R*}. The mortality rates recorded refer to percentages of the total number ($n = 150 - 360$) of individuals counted at all stages (Figure 1C). *Drosophila* of the wild-type strain *Canton-S* exhibited no death at the wandering 3rd instar larval stage, a low rate of 3% mortality during pupal development, and no death due to unsuccessful eclosion (Figure 1C). In contrast, homozygotes from both *Sod* mutant lines studied displayed clear lethality during development, that differed significantly from WT ($p < 0.001$). *n108* and *G85R* 3rd instar larvae died at similar rates, of approx. 7% and 8%, respectively, but the phenotypes diverge at the pupal stage. In the the loss-of-function *n108* mutant, 10% of individuals died during the pupal stage, but nearly all pupae of the ALS-linked *G85R* allele died before eclosion (~91% of individuals). 1 out of 210 *G85R* individuals made it into eclosion, however it died in the process. 43% of *n108* individuals died during the eclosion process, and the remaining 40% successfully eclosed as juvenile adults. The mortality patterns of the balanced heterozygotes (*Sod*/*TM6,Tb*) did not differ significantly

from WT, as none of them displayed larval death or death at eclosion, and none had a pupal death rate above 4% (WT rate: 3%), indicating no dominant or semi-dominant developmental mortality in *Sod* mutants.

Altered larval neuromuscular junction morphology

The *Sod* mutants displayed mild alterations in NMJ outgrowth, that were readily visible in counts of boutons per NMJ (Figure 2B). Counts of Ib branches and boutons in M4 and M13 in *G85R* yielded distributions that were not significantly different from WT. While the increase in *G85R* M13 Is branches was nonsignificant, the numbers of Is boutons were significantly increased compared to WT ($p < 0.05$). NMJ outgrowth was increased in *n108* compared to WT, with more more Ib boutons in M4 ($p < 0.05$) and M13 ($p < 0.001$), more Ib branches on M13 ($p < 0.01$), and a visible but nonsignificant increase in numbers of M4 Ib branches (Figure 2B). Where *n108* NMJ complexity was significantly greater than WT, it was also significantly increased compared to *G85R*, indicating physiological differences between mutants with the loss-of-function allele and those with the ALS-linked allele.

Both *Sod* mutants displayed increases in numbers of unsegregated boutons per M4 Ib branch compared to WT (*G85R*: $p < 0.001$, *n108*: $p < 0.05$) (Figure 2C). Similarly, terminal boutons that were enlarged compared to other boutons on the same M4 Ib branch appeared more frequently in both *Sod* mutants than in WT (*G85R*: $p < 0.001$, *n108*: $p < 0.01$) (Figure 2C).

Unlike the *Sod* single-mutants, phenotypes of NMJ complexity (M4, Ib) in *Pk^{Pk}*, *Pk^{Sple}*, and their double mutants with *G85R* were most visible in changes to the numbers of branches per NMJ. Compared to WT, *Pk^{Pk};G85R* displayed significantly more boutons ($p < 0.01$), and *Pk^{Sple};G85R* displayed significantly fewer ($p < 0.01$). Both double-mutants displayed fewer branches than either corresponding *Pk* single-mutant (both $p < 0.001$), and *Pk^{Sple};G85R*

displayed a number of boutons similar to the *G85R* single-mutant, strikingly fewer than the *Pk^{Sp1e}* single-mutant. ($p < 0.001$). Both *Pk* single-mutants displayed slightly more boutons than WT (both $p < 0.05$). None of the *Pk* single-mutants or *Pk;G85R* double-mutants displayed a number of unsegregated boutons that was significantly different from WT, nor was there a clear difference between WT and the *Pk* single-mutants in the rate at which terminal boutons were enlarged. However, both *Pk;G85R* double-mutants displayed enlarged terminal boutons at rates lower than *G85R*, but still greater than WT (both $p < 0.01$).

Genetic interaction between *Sod* and *Pk* in motor neuron excitability and synaptic transmission

In light of the NMJ morphological defects manifested in *Sod* through its interaction with *Pk* mutations, we examined neuromuscular transmission for related defects. When the motor axon bundle was stimulated with 0.1 ms pulses in HL3.1 saline containing 0.2 mM Ca^{2+} , we detected reliable EJP responses in *Sod* and *Pk* mutant larvae. In *Sod* mutants (hypomorphic alleles *n108* and *x39/n108*, as well as the ALS-related *G85R* allele), EJP response upon nerve stimulation was similar to that in WT ($p > 0.05$, Figure 4B). EJP size in the *Pk* alleles of *Pk* (*Pk1* and *Pk30*, data combined in Figure 4) was comparable to WT, while EJP size was significantly decreased in the mutants of the *Sp1e* allele ($p < 0.05$, One-Way ANOVA). However, the double-mutant *Pk^{Pk1};Sod^{G85R}* showed unusually large EJPs (Figure 4B, $p < 0.05$, One-Way ANOVA). Abnormal synaptic transmission can be attributed to alterations in ion channels participating in regulation of presynaptic terminal membrane depolarization to control Ca^{2+} influx that triggers release of synaptic vesicles from presynaptic terminals.

We examined potential alterations of ionic mechanisms using a previously established protocol of direct electrotonic stimulation of the axon terminal, after silencing the axon Na^+ spikes via TTX (Ganetzky & Wu, 1982, 1983; Ueda & Wu 2009; Lee et al., 2013). We also

removed the repolarization force via K⁺ channel blockers, 4-AP and TEA. 4-AP specifically blocks rapidly inactivating A-current (K_v1, *Shaker* channels) and TEA reduces a broad range of K⁺ currents (Jan & Jan, 1977). The axon terminal was depolarized by electrotonic spread of a stimulus of increased duration (1 ms, see Methods), in HL3.1 saline containing 0.1 mM Ca₂⁺. Under this condition, we observed strikingly prolonged EJPs, with durations up to a few seconds, that reflected transmitter release triggered by sustained presynaptic Ca₂⁺ action potentials (Ca_v2, *cac*: Smith et al., 1996; Kawasaki et al., 2002; Lee et al., 2014) (Figure 4C) (Ueda & Wu, 2009). This observation is consistent with electrotonically-triggered prolonged regenerative postsynaptic potentials first described in the squid giant synapse and the frog NMJ (Katz & Miledi, 1967a,b). Our results showed that, in fact, such prolonged plateau-like EJPs were enhanced in both the *Pk^{Pk}* and *Pk^{Sp/e}* mutants despite the decreased baseline transmission in *Pk^{Sp/e}* and WT-like transmission in *Pk^{Pk}* (Figure 4B). *Sod* mutation alone did not affect the plateau EJPs, but *Pk^{Pk};Sod^{G85R}* showed prolonged plateau EJPs, suggesting a complex *Sod-Pk* mutant interaction. The previously reported dominant effect of *Pk^{Pk}* (Ehaideb et al., 2014) was also confirmed when we observed explosive efEJPs in the double mutant *Pk^{Pk/+};Sodⁿ¹⁰⁸* (Supplemental Figure 1).

Extracellular focal recordings of miniature end-plate potentials (mEPPs) generated by individual releasing sites have been described in the frog neuromuscular junction by Fatt & Katz (1952). In our study, the focal extracellular field potentials (extracellular focal EJPs or efEJPs) corresponding to the explosive events were collected to compare with the intracellularly recorded plateau-like potentials. Under the same conditions used to record plateau-like EJPs intracellularly (TTX + 4-AP + TEA, 0.1 Ca₂⁺, 1 ms stimuli), a glass electrode with an opening approx. 7-10 μm was placed on the NMJ of muscles 6 and 7 (see Methods) in an attempt to record local field potentials) generated mainly by a single bouton, with some contributions from neighboring boutons (Figure 5). In fact, we recorded prolonged

synaptic activities with waveforms of long durations consistent with our intracellular recordings, but with different complex waveforms. Since the focal recording of the potentials generated by synaptic currents, the plateau potentials observed extracellularly reflects local muscle membrane potentials that necessarily change the driving force for the local synaptic currents (the difference between the membrane potential and equilibrium potential for the glutamate receptor current, that is nearly 0 (Jan & Jan, 1976; Stewart et al., 1994; Kurdyak et al., 1994). Therefore, the efEJPs collected during the plateau-like EJPs are not strictly proportional to the amount of transmitter release. Our results suggest a synergistic effect between *sod* and *pk* alleles in controlling the excitability of presynaptic terminals, which was governed by the local expression of Ca²⁺ and K⁺ channels.

Disrupted mitochondrial distribution at the larval NMJ

Mitochondrial staining with TMRM of the larval NMJ in *Sod*^{G85R} reared at RT revealed brightly stained "macropunctae"(Figure 6A) in over half (52%) of all terminal boutons compared to just 11% in WT and 16% in *Sod*ⁿ¹⁰⁸ (both *p* < 0.001 compared to *Sod*^{G85R}) (Figure 6B). Bright spots are in contrast to the dimmer and more diffuse staining seen in most WT and *Sod*ⁿ¹⁰⁸ NMJs. In larvae expressing GFP targeted to motor neuron membrane (UAS-CD8-GFP under the control of OK371-Gal4 or C164-Gal4), an association between synaptic bouton morphology and the presence of mitochondrial aggregates became apparent in both WT and *Sod*^{G85R} (Figure 6C). Of terminal boutons that were enlarged relative to other boutons in the branch (Figure 2A), 53% in WT and 77% in *Sod*^{G85R} contained TMRM macropunctae. In contrast, only 12% of WT and 32% of *Sod*^{G85R} boutons that were not relatively enlarged were stained with macropunctae.

Discussion

Our study contrasts the loss-of-function *n108* allele of *Sod* with the ALS-linked *G85R* allele, by demonstrating clear differences in the developmental stages at which the mutants of each allele die. The contrast is further enhanced by differences between their neuromuscular synapse morphologies. We found potential mechanistic links between abnormal distribution of mitochondria in *Sod^{G85R}* and synaptic bouton enlargement, consistent with the striking interaction with *Pk*, known to affect axonal transport. Further, following pharmacological blockade of Na⁺ and K⁺ currents in our electrophysiological experiments, the remaining Ca²⁺ currents appeared to initiate more physiological actions, further enhanced in *Pk* mutants and *Pk;Sod* double-mutants.

Developmental death in *Sod* mutants

This study of *Drosophila Sod* mutant lethality extends specificity and aspects of mutational effects on the developmental stages in which deaths occur. Specifically, we distinguish between death of the fully developed juvenile fly during eclosion from the pupal casing, and death mid-pupal development, which is characterized by a pupal casing that remains closed. As a result, we established allele-dependent developmental lethality phenotypes. The *n108* lethality occurs largely during eclosion and may be due to physical weakness of the juvenile. The vast majority of *G85R* death occurs during pupal development, often prior to formation of adult morphology, as seen by a lack of red adult eyes visible through the pupal casing of late-stage pupae (Bainbridge & Bownes, 1981).

Aberrant NMJ morphology and physiology

Overgrowth of the *Sodⁿ¹⁰⁸* NMJ has been documented previously, and another oxidative stress-sensitive mutant (*spinster*) was shown to have similar overgrowth. Further, the two

genes show synergistic interactions (Milton et al., 2011). Our result also demonstrated overgrowth in *n108* lb tonic synapses, but this did not hold true for Is phasic synapses. The *G85R* larval NMJ has also been characterized (Held et al., 2019) although the bouton number did not seem to differ significantly from their control. In this study, we also found no clear difference in bouton numbers, but striking bouton morphological alterations could be demonstrated in *G85R* larvae (Figure 2A). Additional immunostaining of proteins constituting the presynaptic machinery, such as synaptotagmin and dynamin (Estes et al., 1996), or postsynaptic elements such as glutamate receptors (Harris & Littleton, 2015), could elucidate the functional relevance of the unusual bouton morphologies in *Sod* mutants.

We found similarly increased NMJ outgrowth in *Pk^{Sple}* and *Pk^{Pk}*. The increased NMJ outgrowth observed in the *Pk^{Sple}* single-mutant may be related to its previously reported increased neuronal excitability (Ehaideb et al., 2014). Other hyperexcitable mutants, such as the double-mutant of K⁺ channel mutants *Shaker* and *eag*, also exhibit significant NMJ overgrowth (Zhong et al., 1992). The similarly increased outgrowth in *Pk^{Pk}* is in spite of a lack of previously documented hyperexcitability. However, our electrophysiological studies with electrotonic stimulation under blockade of Na⁺ channels (TTX) and K⁺ channels (4-AP & TEA) revealed plateau-like potentials *Pk* and *Pk;Sod* mutants. These long potentials may be driven by the action of the Ca²⁺ channel *cacophony* (*cac*), as these waveforms are suppressed by mutation of *cac* (Lee et al., 2013) and application of Co²⁺, which blocks Ca²⁺ channels (Ueda & Wu, 2009). The extended plateau-like potentials in *Pk* and *Pk;Sod* could therefore reflect a joint effect, with extended Ca²⁺ channel currents. The reduced transport (Ehaideb et al., 2014) in *Pk^{Pk}* may affect local channel distributions, which could in turn alter ion currents. Furthermore, *Pk* has been reported to physically interact with Synapsin1 (Paemka et al., 2013), and consequences of an interaction between *Sod* and *Pk* may therefore involve Synapsin1 (Klagges et al., 1996) function, which influences synaptic

development (Vasin et al., 2014), and synaptic vesicle formation and release (Akbergenova & Bykhovskaia, 2007).

The outgrowth of $Pk^{Pk};Sod^{G85R}$ between the levels of Pk^{Pk} and Sod^{G85R} resembles an averaging effect. $Pk^{Sple};Sod^{G85R}$ exhibits an even simpler NMJ morphology than Sod^{G85R} in spite of the increased outgrowth in Pk^{Sple} , demonstrating an unexpected undergrowth phenotype associated with the genetic interaction. Additionally, the plateau-like EJPs we observed under Na⁺ and K⁺ channel blockade appear in $Pk^{Pk};Sod^{G85R}$ and $Pk^{Sple};Sod^{G85R}$. Pk apparently interacts with Sod in a dominant manner, because $Pk^{Pk/+}$ and Sod ($Pk^{Pk/+};Sod^{n108}/Sod^{n108}$) produced a strong effect on synaptic transmission, comparable to $Pk^{Pk};Sod^{G85R}$ (Supplementary Figure 1).

Mitochondrial aggregation associated with bouton morphology

The brightly TMRM-stained NMJ boutons in Sod^{G85R} could reach several-fold, within the resolution of the fluorescent microscopy, the increased signal may reflect accumulated mitochondria. These potential mitochondrial aggregates could be related to dysfunctional transport, which has previously been reported in SOD1-based models of ALS (Bilsland et al., 2010; De Vos et al., 2007). Transport disruption could also contribute to the bouton enlargement we found appeared to be linked to bright mitochondrial staining. Due to the mechanics of TMRM, which is pulled into mitochondria by membrane potential (Floryk & Houštěk, 1999), it remains to be further explored whether the brightly stained "macropunctae" we identified reflect mitochondria of higher membrane potential, increased mitochondrial volume, or a combination of the two.

Mutant SOD1 is the most commonly identifiable risk factor in human patients with familial ALS (Zarei et al., 2015). By exploiting the amenability of the *Drosophila* larval neuromuscular

preparation to the study of motor synapses at an early developmental stage, we detect clear signs of synaptic physiology and morphology alterations in *Sod* mutants. Additionally, abnormal distributions of synaptic mitochondria in *Sod*, and severe NMJ phenotypes in *Sod* double-mutants with transport-affecting *Pk* mutations, suggest dysfunctional transport may contribute to early SOD1-linked pathology. Our work therefore contributes to documentation of phenotypes and potential mechanisms that may facilitate future studies to uncover critical events leading to irreversible and terminal motor neuron degeneration in ALS.

Figure 1. Developmental lethality in *Sod* mutants.

(A) Representative images of larvae, pupae, and empty pupal casings of *Sod* mutant homozygotes (*Sod/Sod*) and balanced heterozygotes (*Sod/TM6, Tb*). *Sod* homozygotes have wild-type body morphology, and the TM6 balancer granted the heterozygotes shorter and fatter *Tubby* (*Tb*) body shape. The larvae represented are specifically of the wandering 3rd instar stage, immediately pre-pupation. Empty pupal casings are left over after successful eclosion of the juvenile fly.

(B) Images of *Sod* homozygotes dead during the wandering 3rd instar larval, pupal, or eclosion stages. Dead larvae are identifiable by their immobility and dark color and dead pupae have unevenly distributed contents and dark patches. Juvenile flies dead during eclosion were distinguished from flies in the process of eclosing by a second observation 15-30 min after the first. Juvenile fly corpses were also frequently darker than their live counterparts.

(C) Rates of lethality during development in wild-type (WT), *Sod*^{G85R} (*G85R/G85R*), *Sod*^{G85R}/*TM6, Tb* (*G85R/TM6*), *Sod*ⁿ¹⁰⁸ (*n108/n108*), and *Sod*ⁿ¹⁰⁸/*TM6, Tb* (*n108/TM6*). Dead individuals were counted at the 3rd instar larval stage (black), pupal stage (solid gray), and eclosion (gray stripes). Rates of successful eclosions (white) are from counts of empty pupal casings. *** indicates $p < 0.001$ (Fisher's exact test). *n* of individuals are indicated at the bottom of each bar.

Figure 2. Morphological defects in larval motor synapses of *Sod* mutants.

(A) Representative NMJ of larval muscles 4 (M4) and 13 (M13) from WT, *Sod*^{G85R}, and *Sod*ⁿ¹⁰⁸, immunostained with anti-HRP (neuronal membrane, green in merged image) and anti-Dlg (subs synaptic reticulum, red in merged image). Type Ib (tonic glutamatergic) branches were examined in both muscles, and type Is (phasic glutamatergic) branches were examined in M13. Is branches are distinguishable from Ib by their smaller size, and weaker anti-HRP and anti-Dlg immunostaining. Labels on the anti-HRP images indicate branch types. The much smaller type II boutons are also labeled, but were not studied. Select portions of the M4 and M13 images are magnified to show phenotypic incomplete bouton segregation (third row, green boxes) and terminal bouton enlargement (fourth row, gold boxes) in *Sod*^{G85R} and *Sod*ⁿ¹⁰⁸, compared to common WT variation. Note the "satellite" buds present on the magnified terminal bouton from *Sod*ⁿ¹⁰⁸. Scale bars in the upper left and lower left images are 10 μ m.

(B) Per NMJ, number of branches (top) and synaptic boutons (bottom) of type Ib in M4 and M13, and type Is in M13. Branches are defined here as terminal processes composed of two or more synaptic boutons, distal of any bifurcations. Counts from larval abdominal segments 3, 4, and 5 are pooled. WT counts are plotted as black circles, *Sod*^{G85R} (G85R) as maroon diamonds, *Sod*ⁿ¹⁰⁸ (n108) as red diamonds. Means are indicated by enlarged symbols, SEM by bars. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ (Kruskal-Wallis test, rank-sum post hoc). n numbers (n of NMJ/ n of larvae) are indicated above the genotype labels.

(C) Number of incompletely segregated (unsegregated) boutons per Ib branch per M4 NMJ (top), and percentage of Ib branches per M4 NMJ in which the terminal bouton was the largest after the last bifurcation. A bouton was described as incompletely segregated if the width of the narrowest point on the neck preceding it was greater than 80% of the width of the widest point on the bouton. The statistical parameters and their layout are identical to those of panel B.

Figure 3. Larval NMJ morphology in double-mutants of *Pk* and *Sod*.

(A) Representative NMJ of larval muscle 4 (M4) (Ib branches) from WT, *Sod*^{G85R}, *Pk*^{Pk}, *Pk*^{Sple}, and the double mutants *Pk*^{Pk};*Sod*^{G85R} and *Pk*^{Sple};*Sod*^{G85R}. Presynaptic neuronal membrane is immunostained with anti-HRP (green in merged image) and the microtubule-associated protein Futsch is stained with the monoclonal AB 22C10 (red in merged image). Ib branches are labeled where appropriate, but were not studied here. Select portions of the full-scale images are magnified to show characteristic bouton morphologies and cytoskeletal structures (bottom three rows). Note the greater and irregular intensities of Futsch staining in *Sod*^{G85R}, *Pk*^{Pk}, and *Pk*^{Pk};*Sod*^{G85R}, and the strongly stained "loop" structures in *Pk*^{Pk};*Sod*^{G85R}. Scale bars in the full-scale and magnified HRP images are 10 μm.

(B) Per M4 NMJ, number of type Ib branches (top) and synaptic boutons (bottom). Counts from larval abdominal segments 2, 4, and 6 are pooled. WT counts are plotted as black circles, *Pk*^{Pk} as blue inverted triangles, *Pk*^{Pk};*Sod*^{G85R} (*Pk*^{Pk};*G85R*) as purple inverted triangles, *Sod*^{G85R} (*G85R*) as maroon diamonds, *Pk*^{Sple};*Sod*^{G85R} (*Sple*;*G85R*) as purple upright triangles, (*Sple*) as blue upright triangles. Means are indicated by enlarged symbols, SEM by bars. * indicates $p < 0.05$, ** indicates $p < 0.01$, *** indicates $p < 0.001$ (Kruskal-Wallis test, rank-sum post hoc). n numbers (n of NMJ/ n of larvae) are indicated above the genotype labels.

(C) Number of incompletely segregated (unsegregated) boutons per Ib branch per M4 NMJ (top), and percentage of Ib branches per M4 NMJ in which the terminal bouton was the largest after the last bifurcation. A bouton was described as incompletely segregated if the width of the narrowest point on the neck preceding it was greater than 80% of the width of the widest point on the bouton. The statistical parameters and their layout are identical to those of panel B.

Figure 4. Whole-cell EJP recordings in *Sod* and *Pk;Sod*.

(A) Increased size and variability of excitatory junctional potentials (EJPs) from larval body wall muscles in *pk;sodG85R*. EJPs from two different muscles are shown as examples for each genotype.

(B) a, b, and c indicates that differences within the group is not significant ($p > 0.05$, One-way ANOVA). Error bars = SEM. Number of NMJs are indicated. Ejps were measured in HL3.1 saline containing 0.2 mM Ca^{2+} .

(C) Excitability in *pk* and *sod* motor axon terminals. Synaptic transmission was induced by direct activation of the motor axon terminals by electrotonic stimulation in the presence of a Na^+ channel blocker (TTX, 3 μM) and K^+ channel blockers (4-AP, 200 μM and TEA, 20 mM). Under this condition, prolonged ejps supported by continuous transmitter release was recorded (see Text). Amplitude is normalized to the peak.

(D) The duration of plateaued ejps were prolonged and more variable among preparations in *sple*, *pk*, and *pk;sodG85R*. a, b, and c indicates that differences within the group is not significant ($p > 0.05$, F-test). Error bars = SEM. Number of NMJs are indicated.

Figure 5. Focal recording of plateau-like potentials induced at neuromuscular synapses by K⁺ channel blockers.

(A) Sample focal recording (efEJP) traces from neuromuscular synapses of WT, *Sod*^{G85R}, *Pk*^{Pk}, *Pk*^{Sple}, *Pk*^{Pk};*Sod*^{G85R}, and *Pk*^{Sple};*Sod*^{G85R}, upon electrotonic stimulation after application of TTX (3 μM), 4-AP (200 μM), and TEA (20 mM). Recordings are from the NMJ of muscles 6 and 7, in larval segments A4 and A5. Each recording site contained between 1 and 4 boutons, and could contain a mixture of type Ib and type Is boutons. Note that trace amplitude is dependent on the variable seal resistance between the recording electrode and the NMJ. All artifacts have been truncated.

(B) Durations of plateau-like efEJP. At each recording site, a series of single stimulation pulses with incrementally increasing voltage was applied. The plotted plateau efEJP duration for each site is a measurement from the first plateau observed in the stimulation series. WT counts are plotted as black circles, *Pk*^{Pk} as blue inverted triangles, *Pk*^{Pk};*Sod*^{G85R} (*Pk*^{Pk};*G85R*) as purple inverted triangles, *Sod*^{G85R} (*G85R*) as maroon diamonds, *Pk*^{Sple};*Sod*^{G85R} (*Sple*;*G85R*) as purple upright triangles, *Pk*^{Sple} (*Sple*) as blue upright triangles. Means are indicated by enlarged symbols, SEM by bars. *n* numbers (*n* of recording sites/*n* of NMJ/*n* of larvae) are indicated above the genotype labels.

Figure 6. Mitochondrial macropunctae in NMJ terminals of *Sod*^{G85R} larvae are frequently present in enlarged terminal boutons.

(A) Mitochondrial live tissue staining (TMRM, first row) in muscle 4 NMJ terminal branches in wild-type (WT), *Sod*^{G85R} (G85R), and *Sod*ⁿ¹⁰⁸ (n108) larvae. The second row shows NMJ morphologies, visible with a presynaptic membrane-targeted GFP (CD8-GFP), expression of which was driven by a Gal4 construct (OK371). The third row shows overlays of the mitochondrial staining and presynaptic terminal images. The fourth row contains magnified portions of the whole-NMJ TMRM images (first row, white boxes). The larger white arrowhead indicates an *Sod*^{G85R} terminal bouton with a large, bright spot of mitochondrial staining, the smaller white arrowhead indicates bright staining in a non-terminal bouton. We describe these points of bright mitochondrial staining as "macropunctae". Scale bars in the upper left and lower left images are 10 μ m.

(B) Percentages of branches per NMJ (muscle 4, type Ib boutons) in which staining revealed mitochondrial macropunctae in the terminal bouton in WT (black circles), *Sod*^{G85R} (maroon diamonds), *Sod*ⁿ¹⁰⁸ (red diamonds). Counts from larval abdominal segments 2, 3, 4, 5, and 6 are pooled. Means are indicated by enlarged symbols, SEM by bars. *** indicates $p < 0.001$ (Kruskal-Wallis test, rank-sum post hoc). n numbers (n of NMJ/ n of larvae) are indicated above the genotype labels.

(C) Percentages of large terminal boutons (enlarged compared to other boutons on the branch) (black) and unenlarged boutons (gray) in which staining revealed mitochondrial macropunctae, in WT and *Sod*^{G85R}. Bouton morphology was visible with visible with CD8-GFP. n number of boutons per category is indicated above each genotype label.

Supplemental Figure 1. Focal recording examination of the excitability in pk^{pk} and sod^{n108} synaptic terminals.

Electrotonic stimuli (1 ms pulse duration) were applied to NMJ terminals at 0.1mM Ca^{2+} with Na^{+} channel blockers (TTX, 3 μ M) and K^{+} channel blockers (4-AP, 200 μ M and quinidine, 100 μ M). Only type Ib boutons were recorded. (A) WT and (B) sod^{n108} terminals quickly ran down after adding both 4-AP and quinidine and gave poor responses, whereas (C) pk^{pk} heterozygous (over CyO-GFP) and (D) $pk^{pk}/CyO-GFP$; sod^{n108} displayed currents of striking amplitudes. Note that $pk^{pk}/CyO-GFP$; sod^{n108} has lower stimulation voltage (in V, labeled on the side) threshold and wider response current width. All artifacts have been truncated. The circles above each trace indicate the locations of the artifacts induced by stimuli.

References

- Aitken RJ, Krausz C. (2001) Oxidative stress, DNA damage, and the Y chromosome. *Reproduction* 122: 497-506.
- Akbergenova Y, Bykhovskaia M. (2007) Synapsin maintains the reserve vesicle pool and spatial segregation of the recycling pool in *Drosophila* presynaptic boutons. *Brain Research* 1178: 52-64.
- Albrecht SC, Barata AG, Großhans J, Teleman AA, Dick TP. (2011) In vivo mapping of hydrogen peroxide and oxidize glutathione reveals chemical and regional specificity of redox homeostasis. *Cell Metabolism* 14: 819-829.
- Ali S, Jain SK, Abdulla M, Athar M. (1996) Paraquat induced DNA damage by reactive oxygen species. *Biochemistry and Molecular Biology International* 39(1): 63-67.
- Altan O, Pabuccuoglu A, Altan A, Konyalioglu S, Bayraktar H. (2003) Effect of heat stress on oxidative stress, lipid peroxidation, and some stress parameters in broilers. *British Poultry Science* 44(4): 545-550.
- Atwood HL, Govind CK, Wu CF. (1993) Differential ultrastructure of synaptic terminals on ventral longitudinal abdominal muscles in *Drosophila* larvae. *Journal of Neurobiology* 24(8):1008-1024.
- Ayala GX, Tapia R. (2003) Expression of heat shock protein 70 induced by 4-aminopyridine through glutamate-mediated excitotoxic stress in rat hippocampus in vivo. *Neuropharmacology* 45: 649-660.
- Baek MK, Kim KO, Kwon HJ, Kim YW, Woo JH, Kim DY. (2016) Age-related changes in antioxidative enzyme capacity in tongue of Fischer 344 rats. *Clinical and Experimental Otorhinolaryngology* 9(4): 352-357.
- Bainbridge SP, Bownes M. (1981) Staging the metamorphosis of *Drosophila melanogaster*. *J. Embryol. exp. Morph.* 66: 57-80.
- Bassuk AG, Wallace RH, Buhr A, ..., Slusarski DC, Berkovic SF, El-Shanti HI. (2008) A homozygous mutation in human PRICKLE1 causes an autosomal-recessive progressive myoclonus epilepsy-ataxia syndrome. *The American Journal of Human Genetics* 83: 572-581.
- Beers DR, Ho BK, Siklos L, Alexianu ME, Mosier DR, Mohamed AH, ... Appel SH. (2001) Parvalbumin overexpression alters immune-mediated increases in intracellular calcium, and delays disease onset in a transgenic model of familial amyotrophic lateral sclerosis. *Journal of Neurochemistry* 79: 499-509.

- 35 Berlett BS, Stadtman ER. (1997) Protein Oxidation in Aging, Disease, and Oxidative Stress.
36 *The Journal of Biological Chemistry* 272(33): 20313-20316.
- 37 Bilsland LG, Sahai E, Kelly G, Golding M, Greensmith L, Schiavo G. (2010) Deficits in
38 axonal transport precede ALS symptoms in vivo. *Proc Natl Acad Sci* 107(47):
39 20523-20528.
- 40 Block ML, Calderón-Garcidueñas L. (2009) Air pollution: mechanisms of neuroinflammation
41 and CNS disease. *Trends in Neurosciences* 32(9): 506-516.
- 42 Brand AH, Perrimon N. (1993) Targeted gene expression as a means of altering cell fates
43 and generating dominant phenotypes. *Development* 118:401-415.
- 44 Budnik V, Koh YH, Guan B, Hartmann B, Hough C, Woods D, Gorczyca M. (1996)
45 Regulation of synapse structure and function by the *Drosophila* tumor suppressor
46 gene *dlg*. *Neuron* 17: 627-640.
- 47 Campbell SH, Hilliker AJ, Phillips JP. (1985) Cytogenetic analysis of the cSOD microregion
48 in *Drosophila melanogaster*. *Genetics* 112: 205-215.
- 49 Chen Q, Vasquez EJ, Moghaddas S, Hoppel CL, Lesnefsky EJ. (2003) Production of
50 reactive oxygen species by mitochondria. *The Journal of Biological Chemistry*
51 278(38): 36027-36031.
- 52 Chen Y, Cohen TJ. (2019) Aggregation of the nucleic acid– binding protein TDP-43 occurs
53 via distinct routes that are coordinated with stress granule formation. *J Biol Chem*
54 294(10): 3696-3706.
- 55 Chowdhury SR, Djordjevic J, Albensi BC, Fernyhough P. (2016) Simultaneous evaluation of
56 substrate-dependent oxygen consumption rates and mitochondrial membrane
57 potential by TMRM and safranin in cortical mitochondria. *Biosci Rep* 36: e00286
- 58 Culotta & Daly. (2013) Manganese Complexes: Diverse Metabolic Routes to Oxidative
59 Stress Resistance in Prokaryotes and Yeast. *Antioxidants and Redox Signaling*
60 19(9): 933-944.
- 61 De Vos KJ, Chapman AL, Tennant ME, Manser C, Tudor EL, ... Grierson AJ. (2007)
62 Familial amyotrophic lateral sclerosis-linked SOD1 mutants perturb fast axonal
63 transport to reduce axonal mitochondria content. *Hum Mol Genet* 16(22): 2720-
64 2728.
- 65 Dhalla NS, Temsah RM, Netticadan, T. (2000) Role of oxidative stress in cardiovascular
66 diseases. *Journal of Hypertension*, 18(6): 655-673.
- 67 Ehaideb SN, Iyengar A, Ueda A, Iacobucci GJ, Cranston C, Bassuk AG, Gubb D, Axelrod
68 JD, Gunawardena S, Wu CF, Manak RJ. (2014) *prickle* modulates microtubule

69 polarity and axonal transport to ameliorate seizures in flies. *Proc. Natl. Acad. Sci.*
70 *USA* 111(30): 11187-11192.

71 Ehaideb SN, Wignall EA, Kasuya J, Evans WH, Iyengar A, Koerselman HL, Lilienthal AJ,
72 Bassuk AG, Kitamoto T, Manak RJ. (2016) Mutation of orthologous prickles genes
73 causes a similar epilepsy syndrome in flies and humans. *Annals of Clinical and*
74 *Translational Neurology* 3 (9): 695-707.

75 Estes PS, Roos J, van der Bliek A, Kelly RB, Krishnan KS, Ramaswami M. (1996) Traffic of
76 Dynamin within individual *Drosophila* synaptic boutons relative to compartment-
77 specific markers *J. Neurosci.* 1996 16(17): 5443-5456.

78 Fatt P, Katz B. (1951) An analysis of the end-plate potential recorded with an intra-cellular
79 electrode. *J. Physiol.* 115: 320-370.

80 Fatt P, Katz B. (1952) Spontaneous subthreshold activity at motor nerve endings. *J.*
81 *Physiol.* 117: 109-128.

82 Feng Y, Ueda A, Wu CF. (2004) A modified minimal hemolymph-like solution, HL3. 1, for
83 physiological recordings at the neuromuscular junctions of normal and mutant
84 *Drosophila* larvae. *Journal of Neurogenetics*, 18(2): 377-402.

85 Floryk D, Houštěk J. (1999) Tetramethyl rhodamine methyl ester (TMRM) is suitable for
86 cytofluorometric measurements of mitochondrial membrane potential in cells treated
87 with digitonin. *Bioscience Reports* 19(1): 27-34.

88 Forsberg K, Andersen PM, Marklund SL, Brannstrom T. (2011) Glial nuclear aggregates of
89 superoxide dismutase-1 are regularly present in patients with amyotrophic lateral
90 sclerosis. *Acta Neuropathol* 121: 623-634.

91 Frankel, A., & Brousseau, G. (1968). *Drosophila* medium that does not require dried yeast.
92 *Drosophila Information Service* 43, 184.

93 Fujita SC, Zipursky SL, Benzer S, Ferrús, Shotwell SL. (1982) Monoclonal antibodies
94 against the *Drosophila* nervous system. *Proc. Natl. Acad. Sci USA* 79: 7929-7933.

95 Galvin (2017) The path to specialist multidisciplinary care in amyotrophic lateral sclerosis:
96 A population- based study of consultations, interventions and costs. *PLoS ONE*
97 12(6): e0178796.

98 Ganetzky B, Wu CF. (1982) *Drosophila* mutants with opposing effects on nerve excitability:
99 Genetic and spatial interactions in repetitive firing. *Journal of Neurophysiology* 47(3):
100 501-514.

Ganetzky B, Wu CF. (1983) Neurogenetic analysis of potassium currents in *Drosophila*: Synergistic effects on neuromuscular transmission in double mutants. *Journal of Neurogenetics* 1: 17-28.

Guegan S, Prezedborski C. (2003) Programmed cell death in amyotrophic lateral sclerosis. *J Clin Invest* 111:153-161.

Giorgi C, Marchi S, Pinton P. (2018) The machineries, regulation and cellular functions of mitochondrial calcium. *Nature Reviews: Molecular Cell Biology* 19: 713-730.

Gubb D, García-Bellido A. (1982) A genetic analysis of the determination of cuticular polarity during development in *Drosophila melanogaster*. *J. Embryol. exp. Morph.* 68: 337-57.

Gubb D, Green C, Huen D, Coulson D, Johnson G, Tree D, Collier S, Roote J. (1999) The balance between isoforms of the Prickle LIM domain protein is critical for planar polarity in *Drosophila* imaginal discs. *Genes & Development* 13: 2315-2327.

Gray RS, Roszko I, Solnica-Krezel L. (2011) Planar cell polarity: coordinating morphogenetic cell behaviors with embryonic polarity. *Developmental Cell* 21: July 19, 2011.

Hardiman O, van den Berg LH, Kiernan MC. (2011) Clinical diagnosis and management of amyotrophic lateral sclerosis. *Nature Reviews: Neurology* 7: 639-649.

Harman D. (1956) Aging: A theory based on free radical and radiation chemistry. *Journal of Gerontology* 11(3): 298-300.

Harris KP, Littleton T. (2015) Transmission, development, and plasticity of synapses. *Genetics* 201: 345-375.

Heitzler P, Coulson D, Saenz-Robles M-T, Ashburner M, Roote J, Simpson P, Gubb D. (1993) Genetic and cytogenetic analysis of the 43A-E region containing the segment polarity gene *costa* and the cellular polarity genes *prickle* and *spiny-legs* in *Drosophila melanogaster*. *Genetics* 135: 105-115.

Held A, Major P, Sahin A, Reenan RA, Lipscombe D, Wharton KA. (2019) Circuit dysfunction in SOD1-ALS model first detected in sensory feedback prior to motor neuron degeneration is alleviated by BMP signaling. *Neurobiology of Disease* 39(12): 2347-2364.

Hummel T, Krukkert K, Roos J, Davis G, Klämbt C. (2000) *Drosophila* Futsch/22C10 is a MAP1B-like protein required for dendritic and axonal development. *Neuron* 26: 357-370.

- 134 Jan LY, Jan YN. (1976a) Properties of the larval neuromuscular junction in *Drosophila*
135 *melanogaster*. *J. Physiol.* 262: 189-214.
- 136 Jan LY, Jan YN. (1976b) L-glutamate as an excitatory transmitter at the *Drosophila* larval
137 neuromuscular junction. *J. Physiol.* 262: 215-236.
- 138 Jan YN, Jan LY, Dennis MJ. (1977) Two mutations of synaptic transmission in *Drosophila*.
139 *Proc. R. Soc. Lond. B.* 198: 87-108.
- 140 Jan LY, Jan YN. (1982) Antibodies to horseradish peroxidase as specific neuronal markers
141 in *Drosophila* and in grasshopper embryos. *Proc. Natl. Acad. Sci. USA* 79: 2700-
142 2704.
- 143 Kannan K, Jain SK. (2000) Oxidative stress and apoptosis. *Pathophysiology* 7(27) 153-163.
- 144 Katz B, Miledi R. (1967a) A study of synaptic transmission in the absence of nerve
145 impulses. *J. Physiol.* 192: 407-436.
- 146 Katz B, Miledi R. (1967b) Tetrodotoxin and neuromuscular transmission. *Proceedings of the*
147 *Royal Society B* 167 (1006): 8-22.
- 148 Kawasaki F, Collins SC, Ordway RW. (2002) Synaptic calcium-channel function in
149 *Drosophila*: Analysis and transformation rescue of temperature-sensitive paralytic
150 and lethal mutations of *cacophony*. 22(14): 5856-5864.
- 151 Klagges BRE, Heimbeck G, Godenschwege TA, Hofbauer A, Pflugfelder GO, Reifegerste
152 R, Reisch D, Schaupp M, Buchner S, Buchner E. (1996) Invertebrate synapsins: A
153 single gene codes for several isoforms in *Drosophila*. *J. Neurosci.* 16(10): 3154-
154 3165.
- 155 Kurdyak P, Atwood HL, Stewart BA, Wu CF. (1994) Differential physiology and morphology
156 of motor axons to ventral longitudinal muscles in larval *Drosophila*. *The Journal of*
157 *Comparative Neurology* 350: 463-472.
- 158 Kurosaka A, Yano A, Itoh N, Kuroda Y, Nakagawa T, Kawasaki T. (1991) The structure of a
159 neural specific carbohydrate epitope of horseradish peroxidase antiserum. *the*
160 *Journal of Biological Chemistry* 266(7): 4168-4172.
- 161 Landis G, Shen J, Tower J. (2012) Gene expression changes in response to again
162 compared to heat stress, oxidative stress and ionizing radiation in *Drosophila*
163 *melanogaster*. *Aging* 4(11): 768-789.
- 164 Lee T, Luo L. (2001) Mosaic analysis with a repressible cell marker (MARCM) for
165 *Drosophila* neural development. *Trends in Neurosciences* 24(5): 251-254.
- 166 Lee J, Ueda A, Wu CF (2014) Distinct roles of *Drosophila* *cacophony* and *Dmca1D* Ca²⁺
167 channels in synaptic homeostasis: Genetic interactions with *slowpoke* Ca²⁺-

activated BK channels in presynaptic excitability and postsynaptic response. *Dev. Neurobiol.* 141: 4548-4557.

Lee J, Wu CF. (2010) Orchestration of Stepwise Synaptic Growth by K⁺ and Ca²⁺ Channels in *Drosophila*. *J. Neurosci.* 30(47): 15921-15833.

Liguori I, Russo G, Curcio F, Bulli G, Aran L, Della-Morte D, Gargiulo G, ... Abete P. (2018) Oxidative stress, aging, and diseases. *Clinical interventions in Aging* 13: 757-772.

Lin CLG, Bristol LA, Jin L, Dykes-Hoberg M, Crawford T, Clawson L, Rothstein JD. (1998) Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Neuron* 20: 589-602.

Lin YY, Gubb D. (2009) Molecular dissection of *Drosophila* Prickle isoforms distinguishes their essential and overlapping roles in planar cell polarity. *Developmental Biology* 325: 386-399.

Lushchak, V. I. (2014) Free radicals, reactive oxygen species, oxidative stress and its classification. *Chem-Biol Interact* 224: 164–175.

MacAskill AF, Kittler JT. (2009) Control of mitochondrial transport and localization in neurons. *Trends in Cell Biology* 20(2): 102-112.

Mackenzie IRA, Rademakers R. (2008) Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. *Curr Opin Neurol* 21(6): 693-700.

Mahr A, Aberle H. (2006) The expression pattern of the *Drosophila* vesicular glutamate transporter: A marker protein for motoneurons and glutamatergic centers in the brain. *Gene Expression Patterns* 6: 299-309.

Massaad CA, Klann E. (2011) Reactive Oxygen Species in the Regulation of Synaptic Plasticity and Memory. *Antioxidants and Redox Signaling* 14(10): 2013-2054.

Mates, J. M., Perez-Gomez, C., de Castro, IN. (1999). Antioxidant enzymes and human diseases. *Clinical Biochemistry* 32(8): 595-603.

McCord JM, Fridovich I. (1969) Superoxide dismutase: An enzymatic function for erythrocyte (hemocuprein). *J. Biol. Chem.* 244: 6049-6055.

Mei X, Wu S, Bassuk AG, Slusarski DC. (2013) Mechanisms of *prickle1a* function in zebrafish epilepsy and retinal neurogenesis. *Disease Models & Mechanisms* 6: 679-688.

- Milton VJ, Jarrett HE, Gowers K, Chalak S, Briggs L, Robinson IM, Sweeney ST. (2011) Oxidative stress induces overgrowth of the *Drosophila* neuromuscular junction. *Proc Natl Acad Sci* 108(42): 17521-17526.
- Mondola P, Damiano S, Sasso A, Santillo M. (2016) The Cu,Zn superoxide dismutase: not only a dismutase enzyme. *Front Physiol* 7: 594.
- Morfini GA, Bosco DA, Brown H, Gatto R, Kaminska A, Song Y, Molla L, Baker L, Marangoni MN, Berth S, Tavassoli E, Bagnato C, Tiwari A, Hayward LJ, Pigino GF, Watterson DM, Huang CF, Banker G, Brown RH Jr, Brady ST. (2013) Inhibition of fast axonal transport by pathogenic SOD1 involves activation of p38 MAP kinase. *PLOS ONE* 8(6): e65235.
- Osking Z, Ayers JI, Hildebrandt R, Skrubber K, Brown H, Ryu D, ... Vitriol EA. (2019) ALS-linked SOD1 mutants enhance neurite outgrowth and branching in adult motor neurons. *iScience* 11: 294-304.
- Ott M, Gogvadze V, Orrenius S, Zhivotovsky B. (2007) Mitochondria, oxidative stress and cell death. *Apoptosis* 12: 913-922.
- Paemka L, Mahajan VB, Skeie JM, ..., Carvill GL, Manak RJ, Bassuk AG. (2013) PRICKLE1 interaction with SYNAPSIN I reveals a role in autism spectrum disorders. *PLOS ONE* 8 (12): e80737.
- Paniker NV, Srivastava SK, Beutler E. (1970) Glutathione metabolism of the red cells effect of glutathione reductase deficiency on the stimulation of hexose monophosphate shunt under oxidative stress. *Biochim. Biophys. Acta* 215: 456-460.
- Parnas D, Haghighi P, Fetter RD, Kim SW, Goodman CS. (2001) Regulation of postsynaptic structure and protein localization by the rho-type guanine nucleotide exchange factor dPix. *Neuron* 32: 415-424.
- Patel A, Lee HO, Jawerth L, Maharana S, Jahnel M, Hein MY, Stoyanov S, ... Alberti S. (2015) A liquid-to-solid phase transition of the ALS protein FUS accelerated by disease mutation. *Cell* 162, 1066-1077.
- Peng IF, Berke BA, Zhu Y, Lee WH, Chen W, Wu CF. (2007) Temperature-dependent developmental plasticity of *Drosophila* neurons: Cell-autonomous roles of membrane excitability, Ca²⁺ influx, and cAMP signaling. *The Journal of Neuroscience* 27(46): 12611-12622.
- Perry JJP, Shin DS, Getzoff ED, Tainer JA. (2010) The structural biochemistry of the superoxide dismutases. *Biochim Biophys Acta* 1804(2): 245-262.

233 Phillips JP, Campbell SD, Michaud D, Charbonneau M, Hilliker, AJ. (1989) Null mutation of
234 copper/zinc superoxide dismutase in *Drosophila* confers hypersensitivity to paraquat
235 and reduced longevity. *Proc Natl Acad Sci* 86(8): 2761-2765.

236 Renger JJ, Ueda A, Atwood HL, Govind CK, Wu CF. (2000) Role of cAMP cascade in
237 synaptic stability and plasticity: Ultrastructural and physiological analyses of
238 individual synaptic boutons in *Drosophila* memory mutants. *J. Neurosci.* 20(11):
239 3980-3992.

240 Rosen DR, Siddique T, Patterson D, Figlewicz DA, Sapp P, Hentati A, ... Brown RH Jr.
241 (1993) Mutations in Cu/Zn superoxide dismutase gene are associated with familial
242 amyotrophic lateral sclerosis. *Nature* 362: 59-62.

243 Rowland LP, Shneider NA. (2001). Amyotrophic lateral sclerosis. *The New England Journal*
244 *of Medicine* 344(22): 1688-1700.

245 Ruan, H., & Wu, C. F. (2008). Social interaction-mediated lifespan extension of *Drosophila*
246 Cu/Zn superoxide dismutase mutants. *Proc Natl Acad Sci* 105(21): 7506-7510.

247 Saccon RA, Bunton-Stasyshyn RKA, Fisher EMC, Fratta P. (2013) Is SOD1 loss of function
248 involved in amyotrophic lateral sclerosis. *Brain* 136: 2342-2358.

249 Şahin A, Held A, Bredvik K, Major P, Achilli TM, Kerson, AG, ... Reenan, R. (2017). Human
250 SOD1 ALS mutations in a *Drosophila* knock-in model cause severe phenotypes and
251 reveal dosage-sensitive gain- and loss-of-function components. *Genetics* 205(2):
252 707-723.

253 Salińska E, Danysz W, Łazarewicz JW. (2005) The role of excitotoxicity in
254 neurodegeneration. *Folia Neuropathol* 43(4): 322-339.

255 Sies H. (1997) Oxidative Stress: Oxidants and Antioxidants (Physiological Society
256 Symposium: Impaired endothelial and smooth muscle cell function in oxidative
257 stress). *Experimental Physiology* 82: 291-295.

258 Singh S, Wu CF. (1999) Ionic currents in larval muscles of *Drosophila*. *International Review*
259 *of Neurobiology* 43: 191-220.

260 Smith EF, Shaw PJ, De Vos KJ. (2017) The role of mitochondria in amyotrophic lateral
261 sclerosis. *Neurosci Lett* 710: 132933.

262 Smith LA, Wang XJ, Peixoto AA, Neumann EK, Hall LM, Hall JC. (1996) A *Drosophila*
263 calcium channel $\alpha 1$ subunit gene maps to a genetic locus associated with
264 behavioral and visual defects. *J. Neurosci.* 16(24): 7868-7879.

- Sørensen JG, Nielsen MM, Kruhøffer M, Justesen J, Loeschcke V. (2005) Full genome gene expression analysis of the heat stress response in *Drosophila melanogaster*. *Cell Stress Chaperones*. 10(4): 312-328.
- Sosa V, Moliné T, Somoza R, Paciucci R, Kondoh H, Lleonart ME. (2013) Oxidative stress and cancer: An overview. *Ageing Research Reviews* 12: 376-390.
- Stewart BA, Atwood HL, Renger JJ, Wang J, Wu CF. (1994) Improved stability of *Drosophila* larval neuromuscular preparations in haemolymph-like physiological solutions. *J Comp Physiol A* 175: 179-191.
- Tang H, Tian E, Liu C, Wang Q, Deng H. (2013) Oxidative stress induces monocyte necrosis with enrichment of cell-bound albumin and overexpression of endoplasmic reticulum and mitochondrial chaperones. *PLoS ONE* 8(3): e59610.
- Tao H, Manak RJ, Sowers L, ..., Wemmie J, Ueno N, Bassuk AG. (2011) Mutations in Prickle orthologs cause seizures in flies, mice, and humans. *The American Journal of Human Genetics* 88: 138-149.
- Thannickal VJ, Fanburg BL. (2000) Reactive oxygen species in cell signaling. *Am J Physiol Lung Cell Mol Physiol* 279: L1005-L1028.
- Torroja L, Packard M, Gorczyca M, White K, Budnik V. (1999) The *Drosophila* β -amyloid precursor protein homolog promotes synapse differentiation at the neuromuscular junction. *The Journal of Neuroscience* 19(18): 7793-7803.
- Tree DRP, Shulman JM, Rousset R, Scott MP, Gubb D, Axelrod JD. (2002) Prickle mediates feedback amplification to generate asymmetric planar cell polarity signaling. *Cell* 109: 371-381.
- Trotti D, Rolfs A, Danbolt NC, Brown RH Jr, Hediger MA. (1999) SOD1 mutants linked to amyotrophic lateral sclerosis selectively inactivate a glial glutamate transporter. *Nature Neuroscience* 2(5): 427-433.
- Tsang CK, Liu Y, Thomas J, Zhang Y, Zheng XFS. (2015) Superoxide dismutase 1 acts as a nuclear transcription factor to regulate oxidative stress resistance. *Nat Commun* 5:3446.
- Ueda A, Wu CF. (2006) Distinct frequency-dependent regulation of nerve terminal excitability and synaptic transmission by I_A and I_K potassium channels revealed by *Drosophila Shaker* and *Shab* mutations. *The Journal of Neuroscience* 26(23): 6238-6248.

297 Ueda A, Wu CF. (2008) Effects of *Hyperkinetic*, a beta-subunit of *Shaker* voltage-
 298 dependent K⁺ channels, on the oxidation state of presynaptic nerve terminals. *J*
 299 *Neurogenet* 22(2): 1-13.

300 Ueda A, Wu CF. (2009) Role of *rut* adenylyl cyclase in the ensemble regulation of
 301 presynaptic terminal excitability: Reduced synaptic strength and precision in a
 302 *Drosophila* memory mutant. *J Neurogenet* 23: 185-199.

303 Uttara B, Singh AV, Zamboni P, Mahajan RT. (2009) Oxidative stress and
 304 neurodegenerative diseases: a review of upstream and downstream antioxidant
 305 therapeutic options. *Current Neuropharmacology* 7: 65-74.

306 Van Raamsdonk JM, Hekimi S. (2012) Superoxide dismutase is dispensable for normal
 307 animal lifespan. *Proc Natl Acad Sci* 109(15): 5785-5790.

308 Vande Velde C, McDonald KK, Boukhedimi Y, ... Cleveland DW. (2011) Misfolded SOD1
 309 Associated with Motor Neuron Mitochondria Alters Mitochondrial Shape and
 310 Distribution Prior to Clinical Onset. *PLoS ONE* 6(7): e22031.

311 Vasin A, Zueva L, Torrez C, Volfson A, Littleton JT, Bykhovskaia M. (2014) Synapsin
 312 regulates activity-dependent outgrowth of synaptic boutons at the *Drosophila*
 313 neuromuscular junction. *J. Neurosci.* 34(32): 10554-10563.

314 Wang J, Farr GW, Zeiss CJ, Rodriguez-Gil DJ, Wilson JH, ... Horwich AL. (2008)
 315 Progressive aggregation despite chaperone associations of a mutant SOD1-YFP in
 316 transgenic mice that develop ALS. *Proc Natl Acad Sci* 106(5): 1392-1897.

317 Wang J, Farr GW, Hall DH, Li F, Furtak K, Dreier L, ... Horwich AL. (2009) An ALS-Linked
 318 Mutant SOD1 Produces a Locomotor Defect Associated with Aggregation and
 319 Synaptic Dysfunction When Expressed in Neurons of *Caenorhabditis elegans*. *PLoS*
 320 *Genetics* 5(1): e1000350.

321 Wang JW, Humphreys JM, Phillips JP, Hilliker AJ, Wu CF. (2000) A novel leg-shaking
 322 *Drosophila* mutant defective in a voltage-gated K⁺ current and hypersensitive to
 323 reactive oxygen species. *Journal of Neuroscience* 20(16): 5958-5964.

324 Wu CF, Ganetzky B, Jan LY, Jan YN, Benzer S. (1978) A *Drosophila* mutant with a
 325 temperature-sensitive block in nerve conduction. *Proc Natl Acad Sci USA* 75(8):
 326 4047-4051.

327 Xia R, Liu Y, Yang L, Gal J, Zhu H, Jia J. (2012) Motor neuron apoptosis and
 328 neuromuscular junction perturbation are prominent features in a *Drosophila* model of
 329 Fus-mediated ALS. *Molecular Neurodegeneration* 7:10.

- 330 Xing X, Wu CF. (2018a) Unraveling synaptic GCaMP signals: Differential excitability and
331 clearance mechanisms underlying distinct Ca^{2+} dynamics in tonic and phasic
332 excitatory, and aminergic modulatory motor terminals in *Drosophila*. *eNeuro* 5(1):
333 e0362-17.2018 1-28
- 334 Xing X, Wu CF. (2018b) Inter-relationships among physical dimensions, distal–proximal
335 rank orders, and basal GCaMP fluorescence levels in Ca^{2+} imaging of functionally
336 distinct synaptic boutons at *Drosophila* neuromuscular junctions. *J Neurogenet*
337 32(3): 195-208.
- 338 Zarei S, Carr K, Reiley L, Diaz K, Guerra O, Fernandez Altamirano P, ... China A. (2015) A
339 comprehensive review of amyotrophic lateral sclerosis. *Surgical Neurology*
340 *International* 6:171.
- 341 Zhang Y, Liu Y, Walsh M, Bokov A, Ikeno Y, Jang YC, Perez VI, ... Richardson A. (2016)
342 Liver specific expression of Cu/ZnSOD extends the lifespan of *Sod1* null mice. *Mech*
343 *Ageing Dev* 154:1-8.
- 344 Zhong Y, Budnik V, Wu CF. (1992) Synaptic plasticity in *Drosophila* memory and
345 hyperexcitable mutants: role of cAMP cascade. *J Neurosci* 12(2): 644-651.
- 346 Zhong Y, Wu CF. (1991) Altered synaptic plasticity in *Drosophila* memory mutants with a
347 defective cyclic AMP cascade. *Science* 251(4990): 198-201.
- 348 Zipursky SL, Venkatesh TR, Teplow DB, Benzer S. (1984) Neuronal development in the
349 *Drosophila* retina: Monoclonal antibodies as molecular probes. *Cell* 36: 15-26.