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 Ca^{2+} channel action and independent of Na^{+} channel function, we used the Na_V 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),

53 and nucleic acids to breakage and mutation (Aitken & Krausz, 2001). Excessive oxidative
54 stress has been implicated in neurodegenerative diseases, cardiovascular diseases, and
55 cancer (Liguori et al., 2018; Uttara et al., 2009; Dhalla et al., 2000; Sosa et al., 2013). Both
56 eukaryotic and prokaryotic organisms produce antioxidant enzymes to maintain oxidative
57 homeostasis, including catalases, glutathione peroxidases, and superoxide dismutases
58 (Matés et al., 1999).
59
60 The antioxidant enzyme Cu/Zn superoxide dismutase (SOD1) forms homodimers that mainly
61 localize to the cytosol, and target superoxide molecules for neutralization (McCord &
62 Fridovich, 1969; Culotta & Daly, 2013). The reactions that SOD1 catalyzes sum to the
63 conversion of two superoxides (O_2^-) and two protons (H^+) to molecular oxygen (O_2) and
64 hydrogen peroxide (H_2O_2), which is targeted by the enzyme catalase for conversion into
65 molecular oxygen and water (O_2 & H_2O) (Mondola et al., 2016; Perry et al., 2010). The gene
66 encoding SOD1 is highly conserved across phyla, and both *Drosophila* and mice SOD1 null
67 mutants display reduced lifespans (Phillips et al., 1989; Zhang et al., 2016). Mutations in the
68 gene for SOD1 and decreased overall SOD1 activity have been found in some human
69 patients with the neurodegenerative disease amyotrophic lateral sclerosis (ALS), popularly
70 known as Lou Gehrig's disease or motor neuron disease (Rosen et al., 1993; Saccon et al.,
71 2013). ALS is a fatal neurodegenerative disease in humans, in which the gradual death of
72 motor neurons causes the muscles they innervate to weaken and atrophy (Rosen et al.,
73 1993; Rowland & Schneider, 2001; Galvin et al., 2017; Hardiman et al, 2011).
74
75 Generated by EMS mutagenesis, the *n108* allele of the *Drosophila* gene for SOD1 (*Sod*) has
76 been identified as a loss-of-function allele (Campbell et al., 1985, Phillips et al., 1989). Flies
77 homozygous for the *n108* allele (Sod^{n108}) display hypersensitivity to oxidative stress and
78 severely reduced lifespans. The *Drosophila G85R* allele (Sod^{G85R}) is a knock-in construct of

79 the missense allele of the same name found in human ALS patients (Şahin et al., 2017). The
80 *G85R* mutation site is conserved between the human and *Drosophila* genes, so the human
81 point mutation was introduced into the *Drosophila* gene by ends-out homologous
82 recombination. The G85R point mutation is in close proximity to a metal ion binding site that
83 enables SOD1 to maintain structural stability. Sod^{G85R} homozygotes display locomotor
84 defects as larvae, and near-full lethality at the pupal stage (Şahin et al., 2017).
85
86 The *Drosophila* larval NMJ (Jan & Jan, 1976; Atwood et al., 1993) is an excellent platform for
87 the study of early motor synaptic characteristics that precede recognizable SOD1-linked
88 neurodegeneration. Immunohistochemistry can make visible neuronal membrane and
89 synaptic proteins at the NMJ. In particular, we used anti-horseradish peroxidase (anti-HRP:
90 Jan & Jan, 1982) to view presynaptic terminal morphology, anti-Dlg (monoclonal antibody
91 4F3: Parnas et al., 2001) which also reveals subsynaptic reticulum (SSR) surround the
92 synaptic boutons (Budnik et al., 1996), and the monoclonal antibody 22C10 (Fujita et al.,
93 1982; Zipursky et al., 1984) to view the microtubule-associated protein Futsch that decorates
94 mature microtubule structures (Hummel et al., 2000). We found that the presynaptic motor
95 terminals of *Sod* mutant larvae carried enlarged synaptic boutons, which were also visible *in*
96 *vivo* in our *Sod* mutant lines containing a cell membrane GFP (CD8-GFP: Lee & Luo, 2001)
97 targeted to motor neurons (OK371-Gal4: Mahr & Aberle, 2006; C164-Gal4: Torroja et al.,
98 1999) (Gal4-UAS system: Brand & Perrimon, 1993). Vital staining with the mitochondrial
99 stain tetramethylrhodamine (TMRM) alongside presynaptic CD8-GFP revealed
100 macropunctae, suggesting mitochondrial aggregation inside the enlarged boutons of *Sod*
101 mutants. While it is known that mitochondria are important for Ca^{2+} buffering and ATP
102 generation in the presynaptic terminal for synaptic transmission (MacAskill & Kittler, 2009),
103 the extreme synaptic accumulation of mitochondria in *Sod* mutants may involve abnormal
104 axonal transport. Indeed, our finding recapitulates aberrant mitochondrial distribution

105 previously observed in human ALS patients and mouse models of ALS, with disrupted
106 mitochondrial transport and morphology (De Vos et al., 2007; Ott et al., 2007; Smith et al.,
107 2017; Vande Velde et al., 2011).
108
109 In *Drosophila*, the planar cell polarity gene *Prickle* (*Pk*) (Gubb & García-Bellido, 1982; Gubb
110 et al., 1999; Gray et al., 2011; Lin & Gubb, 2009; Tree et al., 2002) is expressed in the form
111 of the Pk^{Pk} (*Prickle Prickle*) and Pk^{Sple} (*Prickle Spiny-legs*) isoforms, and their mutants display
112 distinctly altered microtubule-mediated transport and microtubule dynamics in larval motor
113 axons, with related changes in neuronal excitability (Ehaideb et al., 2014; Ehaideb et al.,
114 2016). In this study, we employ mutants of both *Pk* isoforms to demonstrate strong
115 interactions with *Sod* in larval motor neuron synaptic morphology and neuromuscular
116 transmission.
117
118 Synaptic transmission at the NMJ can be analyzed with the method of recording excitatory
119 junction potentials (EJPs) with an intracellular electrode (Jan & Jan, 1976). Additionally, local
120 loose-patch recording of synaptic currents enables characterization of the contributions of
121 single synaptic boutons along the terminal branches to overall synaptic transmission (Renger
122 et al., 2000; Kurdyak et al., 1994) In this study we adopt the same recording configuration
123 with voltage recording of extracellular focal field potentials (efEJP, Xing & Wu, 2018a, b) to
124 infer the synaptic current flow underneath the pipette, an approach first established by Fatt
125 and Katz (1952) for analyzing miniature end-plate currents (mEPCs) and potentials (mEPPs)
126 at the frog neuromuscular junction. We were therefore able to correlate global intracellular
127 events and focal analysis of synaptic transmission.
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134 **Materials and Methods**

135

136 Fly stocks

137 Flies and larvae were housed in bottles containing a cornmeal-agar medium (Frankel &
138 Brousseau, 1968), supplemented with 3g of yeast paste prepared as a 1:1 mixture of ddH_2O
139 and active dry yeast (Red Star Yeast Co., LLC). Flies were reared at a room temperature of
140 22 ± 1°C. The *Sod* mutants examined were those of alleles *n108* (Phillips et al., 1989) (gift
141 from John Phillips, University of Guelph, Ontario, Canada) and *G85R* (Şahin et al. 2017) (gift
142 from Barry Ganetzky, University of Wisconsin-Madison, USA). The wild-type control strain
143 used for Figures 1, 2, 3, and 4 was Canton-S (CS). For experiments in which a motor neuron
144 membrane tag was needed, the stock used carried a chr. II *UAS-CD8-GFP* recombined with
145 a chr. II motor neuron GAL4 driver (Brand & Perrimon, 1993), either *C164-Gal4* (Torroja et
146 al., 1999) or *OK371-Gal4* (Mahr and Aberle, 2006). The original recombined *OK371-Gal4*
147 *UAS-CD8-GFP* (*OK371::CD8-GFP*) was obtained from the National Centre for Biological
148 Sciences (Bangalore, Karnataka, India). *Sod* lines used to produce the data represented in
149 Figure 1 were balanced over a TM6 balancer with a dominant *Tubby* (*Tb*) marker (TM6, *Tb*).
150 The Pk^{Pk} and Pk^{Sple} lines used in this study have been documented previously (Ehaideb et
151 al., 2014). The wild-type control strain used for Figure 5 was the Oregon-R (OR) used in
152 Ehaideb et al. (2014). All *Pk;Sod* double-mutant lines were generated in the Wu lab by
153 conventional genetic crosses.

154

155 Developmental lethality studies

156 Housing and medium conditions for the developmental lethality study (Figure 1) were
157 identical to those described in the main "Fly stocks" section. Flies including 20-40 females
158 were used to seed each bottle. Counts of deaths and eclosions were performed 16-17 d.
159 after seeding. The TM6, *Tb* balancer carried by *Sod* heterozygous wandering 3rd instar
160 larvae and pupae was used to distinguish them from *Sod* mutant homozygotes. Dead larvae
161 appear as darkened corpses on the wall of the housing bottle. Dead pupae were entirely
162 black, or contained a mixture of dark and empty patches, as confirmed by transmitted light
163 under a dissection scope. Juvenile flies dead during eclosion were distinguished from flies in
164 the process of eclosing by a second observation 15-30 min after the first. Juvenile fly corpses
165 were also frequently darker than their live counterparts.
166
167 Larval preparations, vital staining, & immunohistochemistry
168 Wandering third instar larvae were dissected, as per the "fillet preparation method", in HL 3.1
169 saline (Feng et al., 2004). HL3.1 used in immunohistochemistry or vital staining experiments
170 contained 0.1 mM Ca^{2+}. Tetramethylrhodamine (TMRM, $C_{25}H_{25}ClN_2O_7$) (Floryk & Houštěk,
171 1999) vital staining of larval preps for mitochondria was done at 100 nM in HL 3.1 for 20
172 minutes (in a light-impermeable chamber to prevent TMRM photobleaching), after which the
173 bath was replaced with TMRM-free HL 3.1 before microscopy. Before antibody staining,
174 larval preps were fixed in 3.7% formaldehyde in HL 3.1 for 25-30 minutes. Fixed preps were
175 incubated with an FITC-conjugated goat antibody to HRP at 1:50 in phosphate buffer saline,
176 at 4°C for 12-48 h. Anti-HRP (Jan & Jan, 1982) recognizes a neural carbohydrate antigen in
177 *Drosophila* (Kurosaka et al., 1991). For double-staining with anti-HRP and anti-Dlg
178 (monoclonal AB 4F3: Parnas et al., 2001) or anti-Futsch (monoclonal AB 22C10: Fujita et al.,
179 1982; Zipursky et al., 1984) staining, fixed larval preps were incubated with the primary
180 monoclonal AB at 1:100 in phosphate buffer saline, at 4°C for 12-48 h, rinsed, and incubated
181 in phosphate buffer saline containing anti-HRP(conj. FITC) and a secondary TRITC-

182 conjugated anti-mouse IgG antibody, both at 1:50. The 4F3 and 22C10 antibodies were
183 obtained from the Developmental Studies Hybridoma Bank (DSHB) (University of Iowa, Iowa
184 City, IA, USA). The FITC-conjugated anti-HRP and TRITC-conjugated anti-mouse IgG were
185 obtained from Jackson ImmunoResearch Laboratories (West Grove, PA, USA). Larval NMJ
186 images, vital and fixed, were gathered on a Leica DM IL LED inverted microscope (Leica
187 Microsystems Inc., Buffalo Grove, IL, USA), using the Leica Application Suite X software.
188

189 Larval NMJ morphology and mitochondrial distributions

190 Examination of *Sod* single-mutant NMJ morphology (Figure 2) was carried out in muscles 4
191 (M4) and 13 (M13), in abdominal segments A3, A4, and A5, and quantifications from the
192 different segments were pooled. Examination of *Pk;Sod* double-mutant NMJ morphology
193 (Figure 4) was carried out in muscle 4 (M4) in abdominal segments A2, A4, and A6, and
194 quantifications from the different segments were pooled. Counts of boutons from M4 include
195 only type Ib boutons. Ib and Is boutons and branches in M13 were quantified separately.
196 Counts of specific bouton morphologies ("unsegregated" boutons and large terminal boutons)
197 are from M4 (Ib). For branch counting, a branch was defined as a terminal process carrying
198 at least two boutons (Zhong et al., 1992). A bouton was described as incompletely
199 segregated ("unsegregated") if the width of the narrowest point on the neck preceding it was
200 greater than 80% of the width of the widest point on the bouton. If multiple unsegregated
201 boutons appeared in a row, it could be difficult count them. In these cases, counts were
202 conservative. A terminal bouton was determined to be the "largest" if it was measurably
203 larger than any other bouton on that branch, past the last branching point.
204

205 Photography of NMJ mitochondria was completed within 30 minutes after TMRM
206 mitochondrial staining to mitigate effects of post-dissection physiological decline on results.
207 Counts of putative mitochondrial aggregates in enlarged terminal boutons were obtained

208 from larvae in which the NMJ membrane carried CD8-GFP driven by C164-Gal4 or OK371-
209 Gal4, making bouton morphology visible in the green fluorescence channel during vital
210 staining in the red channel.
211
212 Electrophysiology
213 All electrophysiological recordings were based on published protocols, and performed in
214 HL3.1 saline (Feng et al., 2004) (Composition (mM): 70 NaCl, 5 KCl, 0.1 $CaCl_2$, 4 $MgCl_2$, 10
215 $NaHCO_3$, 5 trehalose, 115 sucrose, 5 HEPES) (pH adjusted to 7.1). Intracellular recording of
216 excitatory junction potentials (EJPs) was adapted from Ueda and Wu (2006). Recording of
217 extracellular focal EJPs (efEJPs) was shown in Ueda and Wu (2009) and Xing and Wu
218 (2018a,b). For nerve-evoked action potentials, 0.1 ms stimuli were applied to the cut end of
219 the segmental nerve through a suction pipette. To enable analysis of presynaptic terminal
220 excitability, we also performed electrotonic stimulation (Wu et al., 1978; Ganetzky & Wu,
221 1982; 1983). Briefly: Tetrodotoxin (TTX) was applied to block Na+ channels. To achieve
222 direct electrotonic stimulation of the terminal, a 1 ms stimulus was applied to the cut
223 segmental nerve, which was sucked into the stimulation electrode, nearly up to the entry
224 point into the muscle, so as to allow the stimulus to passively propagate to the terminal and
225 depolarize the endplate membrane (Lee et al., 2014). In this manner, CaV channels
226 were directly triggered by local depolarization, independent from invasion of axonal Na+
227 action potentials. Further, a significant portion of multiple K+ channel species, including
228 *Shaker* (Kv1), were blocked by co-application of 4-aminopyridine (4-AP) and
229 tetraethylammonium (TEA) (Jan & Jan, 1977; Singh & Wu, 1999). Electrotonic stimulation of
230 the larval NMJ with TTX, 4-AP, and TEA has been shown in Lee et al. (2014). Such
231 manipulations allow for the examination of terminal excitability driven by Ca2+ channels with
232 reduced repolarization regulation by a range of K+ channels, with the exclusion of axonal
233 potential interplay.

234

235 Statistics

236 Overall distributions of deaths throughout the lifecycle (Figure 1) were compared using
237 Fisher's exact test. Counts of NMJ boutons and branches and of enlarged terminal and
238 unsegregated boutons (Figures 2 and 3) were examined with Kruskal-Wallis testing between
239 all groups within muscle # and parameter (eg. M4, boutons), followed by rank-sum (Mann-
240 Whitney U) post hoc between individual groups. EJP sizes were compared with one-way
241 ANOVA (Figure 4B). Plateau-like EJP durations were compared by F-test (Figure 4D).
242 Counts of NMJ mitochondrial macropunctae (Figure 6) were compared with Kruskal-Wallis
243 testing followed by rank-sum (Mann-Whitney U) post hoc.

244

245

246 **Results**

247

248 Lethality of *Sod* mutants during different developmental stages

249 During fly husbandry and maintenance of fly stocks, we observed striking mortality during the
250 development of Sod mutants. We therefore proceeded with a systematic study, comparing
251 mortality rates at different developmental stages in Sod^{n108} and Sod^{G85R}. The mortality rates
252 recorded refer to percentages of the total number (n = 150 - 360) of individuals counted at all
253 stages (Figure 1C). *Drosophila* of the wild-type strain *Canton-S* exhibited no death at the
254 wandering 3rd instar larval stage, a low rate of 3% mortality during pupal development, and
255 no death due to unsuccessful eclosion (Figure 1C). In contrast, homozygotes from both *Sod*
256 mutant lines studied displayed clear lethality during development, that differed significantly
257 from WT ($p < 0.001$). *n108* and *G85R* 3rd instar larvae died at similar rates, of approx. 7%
258 and 8%, respectively, but the phenotypes diverge at the pupal stage. In the the loss-of-
259 function *n108* mutant, 10% of individuals died during the pupal stage, but nearly all pupae of

260 the ALS-linked *G85R* allele died before eclosion (~91% of individuals). 1 out of 210 *G85R*
261 individuals made it into eclosion, however it died in the process. 43% of *n108* individuals died
262 during the eclosion process, and the remaining 40% successfully eclosed as juvenile adults.
263 The mortality patterns of the balanced heterozygotes (*Sod/TM6,Tb*) did not differ significantly
264 from WT, as none of them displayed larval death or death at eclosion, and none had a pupal
265 death rate above 4% (WT rate: 3%), indicating no dominant or semi-dominant developmental
266 mortality in *Sod* mutants.
267
268 <u>Altered larval neuromuscular junction morphology</u>
269 The *Sod* mutants displayed mild alterations in NMJ outgrowth, that were readily visible in
270 counts of boutons per NMJ (Figure 2B). Counts of Ib branches and boutons in M4 and M13
271 in *G85R* yielded distributions that were not significantly different from WT. While the increase
272 in *G85R* M13 Is branches was nonsignificant, the numbers of Is boutons were significantly
273 increased compared to WT ($p < 0.05$). NMJ outgrowth was increased in *n108* compared to
274 WT, with more more Ib boutons in M4 ($p < 0.05$) and M13 ($p < 0.001$), more Ib branches on
275 M13 ($p < 0.01$), and a visible but nonsignificant increase in numbers of M4 Ib branches
276 (Figure 2B). Where *n108* NMJ complexity was significantly greater than WT, it was also
277 significantly increased compared to *G85R*, indicating physiological differences between
278 mutants with the loss-of-function allele and those with the ALS-linked allele.
279 Both *Sod* mutants displayed increases in numbers of unsegregated boutons per M4 Ib
280 branch compared to WT (*G85R*: $p < 0.001$, *n108*: $p < 0.05$) (Figure 2C). Similarly, terminal
281 boutons that were enlarged compared to other boutons on the same M4 Ib branch appeared
282 more frequently in both *Sod* mutants than in WT (*G85R*: $p < 0.001$, *n108*: $p < 0.01$) (Figure
283 2C).
284

285 Unlike the *Sod* single-mutants, phenotypes of NMJ complexity (M4, Ib) in Pk^{Pk}, Pk^{Sple}, and
286 their double mutants with *G85R* were most visible in changes to the numbers of branches
287 per NMJ. Compared to WT, Pk^{Pk};*G85R* displayed significantly more boutons ($p < 0.01$), and
288 Pk^{Sple};*G85R* displayed significantly fewer ($p < 0.01$). Both double-mutants displayed fewer
289 branches than either corresponding *Pk* single-mutant (both $p < 0.001$), and Pk^{Sple};*G85R*
290 displayed a number of boutons similar to the *G85R* single-mutant, strikingly fewer than the
291 Pk^{Sple} single-mutant. ($p < 0.001$). Both *Pk* single-mutants displayed slightly more boutons
292 than WT (both $p < 0.05$). None of the *Pk* single-mutants or *Pk*;*G85R* double-mutants
293 displayed a number of unsegregated boutons that was significantly different from WT, nor
294 was there a clear difference between WT and the *Pk* single-mutants in the rate at which
295 terminal boutons were enlarged. However, both *Pk*;*G85R* double-mutants displayed enlarged
296 terminal boutons at rates lower than *G85R*, but still greater than WT (both $p < 0.01$).
297
298 <u>Genetic interaction between *Sod* and *Pk* in motor neuron excitability and synaptic</u>
299 <u>transmission</u>
300 In light of the NMJ morphological defects manifested in *Sod* through its interaction with *Pk*
301 mutations, we examined neuromuscular transmission for related defects. When the motor
302 axon bundle was stimulated with 0.1 ms pulses in HL3.1 saline containing 0.2 mM Ca2+, we
303 detected reliable EJP responses in *Sod* and *Pk* mutant larvae. In *Sod* mutants (hypomorphic
304 alleles *n108* and *x39*/*n108*, as well as the ALS-related *G85R* allele), EJP response upon
305 nerve stimulation was similar to that in WT ($p > 0.05$, Figure 4B). EJP size in the *Pk* alleles of
306 *Pk* (*Pk1* and *Pk30*, data combined in Figure 4) was comparable to WT, while EJP size was
307 significantly decreased in the mutants of the *Sple* allele ($p<0.05$, One-Way ANOVA).
308 However, the double-mutant Pk^{Pk1};Sod^{G85R} showed unusually large EJPs (Figure 4B, $p <$
309 0.05, One-Way ANOVA). Abnormal synaptic transmission can be attributed to alterations in

310 ion channels participating in regulation of presynaptic terminal membrane depolarization to
311 control Ca^{2+} influx that triggers release of synaptic vesicles from presynaptic terminals.
312
313 We examined potential alterations of ionic mechanisms using a previously established
314 protocol of direct electrotonic stimulation of the axon terminal, after silencing the axon Na+
315 spikes via TTX (Ganetzky & Wu, 1982, 1983; Ueda & Wu 2009; Lee et al., 2013). We also
316 removed the repolarization force via K+ channel blockers, 4-AP and TEA. 4-AP specifically
317 blocks rapidly inactivating A-current (K_v1, *Shaker* channels) and TEA reduces a broad range
318 of K+ currents (Jan & Jan, 1977). The axon terminal was depolarized by electrotonic spread
319 of a stimulus of increased duration (1 ms, see Methods), in HL3.1 saline containing 0.1 mM
320 Ca2+. Under this condition, we observed strikingly prolonged EJPs, with durations up to a
321 few seconds, that reflected transmitter release triggered by sustained presynaptic Ca2+
322 action potentials (Ca_V2, *cac*: Smith et al., 1996; Kawasaki et al., 2002; Lee et al., 2014)
323 (Figure 4C) (Ueda & Wu, 2009). This observation is consistent with electrotonically-triggered
324 prolonged regenerative postsynaptic potentials first described in the squid giant synapse and
325 the frog NMJ (Katz & Miledi, 1967a,b). Our results showed that, in fact, such prolonged
326 plateau-like EJPs were enhanced in both the Pk^{Pk} and Pk^{Sple} mutants despite the decreased
327 baseline transmission in Pk^{Sple} and WT-like transmission in Pk^{Pk} (Figure 4B). Sod mutation
328 alone did not affect the plateau EJPs, but Pk^{Pk};Sod^{G85R} showed prolonged plateau EJPs,
329 suggesting a complex *Sod-Pk* mutant interaction. The previously reported dominant effect of
330 Pk^{Pk} (Ehaideb et al., 2014) was also confirmed when we observed explosive efEJPs in the
331 double mutant Pk^{Pk}/+;Sod^{n108} (Supplemental Figure 1).
332
333 Extracellular focal recordings of miniature end-plate potentials (mEPPs) generated by
334 individual releasing sites have been described in the frog neuromuscular junction by Fatt &
335 Katz (1952). In our study, the focal extracellular field potentials (extracellular focal EJPs or

336 efEJPs) corresponding to the explosive events were collected to compare with the
337 intracellularly recorded plateau-like potentials. Under the same conditions used to record
338 plateau-like EJPs intracellularly (TTX + 4-AP + TEA, 0.1 Ca2+, 1 ms stimuli), a glass
339 electrode with an opening approx. 7-10 μm was placed on the NMJ of muscles 6 and 7 (see
340 Methods) in an attempt to record local field potentials) generated mainly by a single bouton,
341 with some contributions from neighboring boutons (Figure 5). In fact, we recorded prolonged
342 synaptic activities with waveforms of long durations consistent with our intracellular
343 recordings, but with different complex waveforms. Since the focal recording of the potentials
344 generated by synaptic currents, the plateau potentials observed extracellularly reflects local
345 muscle membrane potentials that necessarily change the driving force for the local synaptic
346 currents (the difference between the membrane potential and equilibrium potential for the
347 glutamate receptor current, that is nearly 0 (Jan & Jan, 1976; Stewart et al., 1994; Kurdyak et
348 al., 1994). Therefore, the efEJPs collected during the plateau-like EJPs are not strictly
349 proportional to the amount of transmitter release. Our results suggest a synergistic effect
350 between sod and pk alleles in controlling the excitability of presynaptic terminals, which was
351 governed by the local expression of Ca2+ and K+ channels.
352
353
354 <u>Disrupted mitochondrial distribution at the larval NMJ</u>
355 Mitochondrial staining with TMRM of the larval NMJ in Sod^{G85R} reared at RT revealed brightly
356 stained "macropunctae"(Figure 6A) in over half (52%) of all terminal boutons compared to
357 just 11% in WT and 16% in Sod^{n108} (both $p < 0.001$ compared to Sod^{G85R}) (Figure 6B). Bright
358 spots are in contrast to the dimmer and more diffuse staining seen in most WT and Sod^{n108}
359 NMJs. In larvae expressing GFP targeted to motor neuron membrane (UAS-CD8-GFP under
360 the control of OK371-Gal4 or C164-Gal4), an association between synaptic bouton
361 morphology and the presence of mitochondrial aggregates became apparent in both WT and

362 *Sod*^{G85R} (Figure 6C). Of terminal boutons that were enlarged relative to other boutons in the
363 branch (Figure 2A), 53% in WT and 77% in *Sod*^{G85R} contained TMRM macropunctae. In
364 contrast, only 12% of WT and 32% of *Sod*^{G85R} boutons that were not relatively enlarged were
365 stained with macropunctae.
366
367
368 **Discussion**
369
370 Our study contrasts the loss-of-function *n108* allele of *Sod* with the ALS-linked *G85R* allele,
371 by demonstrating clear differences in the developmental stages at which the mutants of each
372 allele die. The contrast is further enhanced by differences between their neuromuscular
373 synapse morphologies. We found potential mechanistic links between abnormal distribution
374 of mitochondria in *Sod*^{G85R} and synaptic bouton enlargement, consistent with the striking
375 interaction with *Pk*, known to affect axonal transport. Further, following pharmacological
376 blockade of Na+ and K+ currents in our electrophysiological experiments, the remaining
377 Ca2+ currents appeared to initiate more physiological actions, further enhanced in *Pk*
378 mutants and *Pk*;*Sod* double-mutants.
379
380 <u>Developmental death in *Sod* mutants</u>
381 This study of *Drosophila Sod* mutant lethality extends specificity and aspects of mutational
382 effects on the developmental stages in which deaths occur. Specifically, we distinguish
383 between death of the fully developed juvenile fly during eclosion from the pupal casing, and
384 death mid-pupal development, which is characterized by a pupal casing that remains closed.
385 As a result, we established allele-dependent developmental lethality phenotypes. The *n108*
386 lethality occurs largely during eclosion and may be due to physical weakness of the juvenile.
387 The vast majority of *G85R* death occurs during pupal development, often prior to formation of

388 adult morphology, as seen by a lack of red adult eyes visible through the pupal casing of
389 late-stage pupae (Bainbridge & Bownes, 1981).
390
391 Aberrant NMJ morphology and physiology
392 Overgrowth of the *Sod*^{n108} NMJ has been documented previously, and another oxidative
393 stress-sensitive mutant (*spinster*) was shown to have similar overgrowth. Further, the two
394 genes show synergistic interactions (Milton et al., 2011). Our result also demonstrated
395 overgrowth in *n108* Ib tonic synapses, but this did not hold true for Is phasic synapses. The
396 *G85R* larval NMJ has also been characterized (Held et al., 2019) although the bouton
397 number did not seem to differ significantly from their control. In this study, we also found no
398 clear difference in bouton numbers, but striking bouton morphological alterations could be
399 demonstrated in *G85R* larvae (Figure 2A). Additional immunostaining of proteins constituting
400 the presynaptic machinery, such as synaptotagmin and dynamin (Estes et al., 1996), or
401 postsynaptic elements such as glutamate receptors (Harris & Littleton, 2015), could elucidate
402 the functional relevance of the unusual bouton morphologies in *Sod* mutants.
403
404 We found similarly increased NMJ outgrowth in *Pk*^{Sple} and *Pk*^{Pk}. The increased NMJ
405 outgrowth observed in the *Pk*^{Sple} single-mutant may be related to its previously reported
406 increased neuronal excitability (Ehaideb et al., 2014). Other hyperexcitable mutants, such as
407 the double-mutant of K^+ channel mutants *Shaker* and *eag*, also exhibit significant NMJ
408 overgrowth (Zhong et al., 1992). The similarly increased outgrowth in *Pk*^{Pk} is in spite of a lack
409 of previously documented hyperexcitability. However, our electrophysiological studies with
410 electrotonic stimulation under blockade of Na+ channels (TTX) and K+ channels (4-AP &
411 TEA) revealed plateau-like potentials *Pk* and *Pk*;*Sod* mutants. These long potentials may be
412 driven by the action of the Ca2+ channel *cacophony* (*cac*), as these waveforms are
413 suppressed by mutation of *cac* (Lee et al., 2013) and application of Co2+, which blocks Ca2+

414 channels (Ueda & Wu, 2009). The extended plateau-like potentials in *Pk* and *Pk*;*Sod* could
415 therefore reflect a joint effect, with extended Ca2+ channel currents. The reduced transport
416 (Ehaideb et al., 2014) in Pk^{Pk} may affect local channel distributions, which could in turn alter
417 ion currents. Furthermore, Pk has been reported to physically interact with Synapsin1
418 (Paemka et al., 2013), and consequences of an interaction between *Sod* and *Pk* may
419 therefore involve Synapsin1 (Klagges et al., 1996) function, which influences synaptic
420 development (Vasin et al., 2014), and synaptic vesicle formation and release (Akbergenova
421 & Bykhovskaia, 2007).
422
423 The outgrowth of $Pk^{Pk};Sod^{G85R}$ between the levels of Pk^{Pk} and Sod^{G85R} resembles an
424 averaging effect. $Pk^{Sple};Sod^{G85R}$ exhibits an even simpler NMJ morphology than Sod^{G85R} in
425 spite of the increased outgrowth in Pk^{Sple}, demonstrating an unexpected undergrowth
426 phenotype associated with the genetic interaction. Additionally, the plateau-like EJPs we
427 observed under Na+ and K+ channel blockade appear in $Pk^{Pk};Sod^{G85R}$ and $Pk^{Sple};Sod^{G85R}$. *Pk*
428 apparently interacts with *Sod* in a dominant manner, because Pk^{Pk}/+ and *Sod*
429 (Pk^{Pk}/+;Sod^{n108}/Sod^{n108}) produced a strong effect on synaptic transmission, comparable to
430 $Pk^{Pk};Sod^{G85R}$ (Supplementary Figure 1).
431
432 <u>Mitochondrial aggregation associated with bouton morphology</u>
433 The brightly TMRM-stained NMJ boutons in Sod^{G85R} could reach several-fold, within the
434 resolution of the fluorescent microscopy, the increased signal may reflect accumulated
435 mitochondria. These potential mitochondrial aggregates could be related to dysfunctional
436 transport, which has previously been reported in SOD1-based models of ALS (Bilsland et al.,
437 2010; De Vos et al., 2007). Transport disruption could also contribute to the bouton
438 enlargement we found appeared to be linked to bright mitochondrial staining. Due to the
439 mechanics of TMRM, which is pulled into mitochondria by membrane potential (Floryk &

440 Houštěk, 1999), it remains to be further explored whether the brightly stained
441 "macropunctae" we identified reflect mitochondria of higher membrane potential, increased
442 mitochondrial volume, or a combination of the two.
443
444 Mutant SOD1 is the most commonly identifiable risk factor in human patients with familial
445 ALS (Zarei et al., 2015). By exploiting the amenability of the *Drosophila* larval neuromuscular
446 preparation to the study of motor synapses at an early developmental stage, we detect clear
447 signs of synaptic physiology and morphology alterations in *Sod* mutants. Additionally,
448 abnormal distributions of synaptic mitochondria in *Sod,* and severe NMJ phenotypes in *Sod*
449 double-mutants with transport-affecting *Pk* mutations, suggest dysfunctional transport may
450 contribute to early SOD1-linked pathology. Our work therefore contributes to documentation
451 of phenotypes and potential mechanisms that may facilitate future studies to uncover critical
452 events leading to irreversible and terminal motor neuron degeneration in ALS.

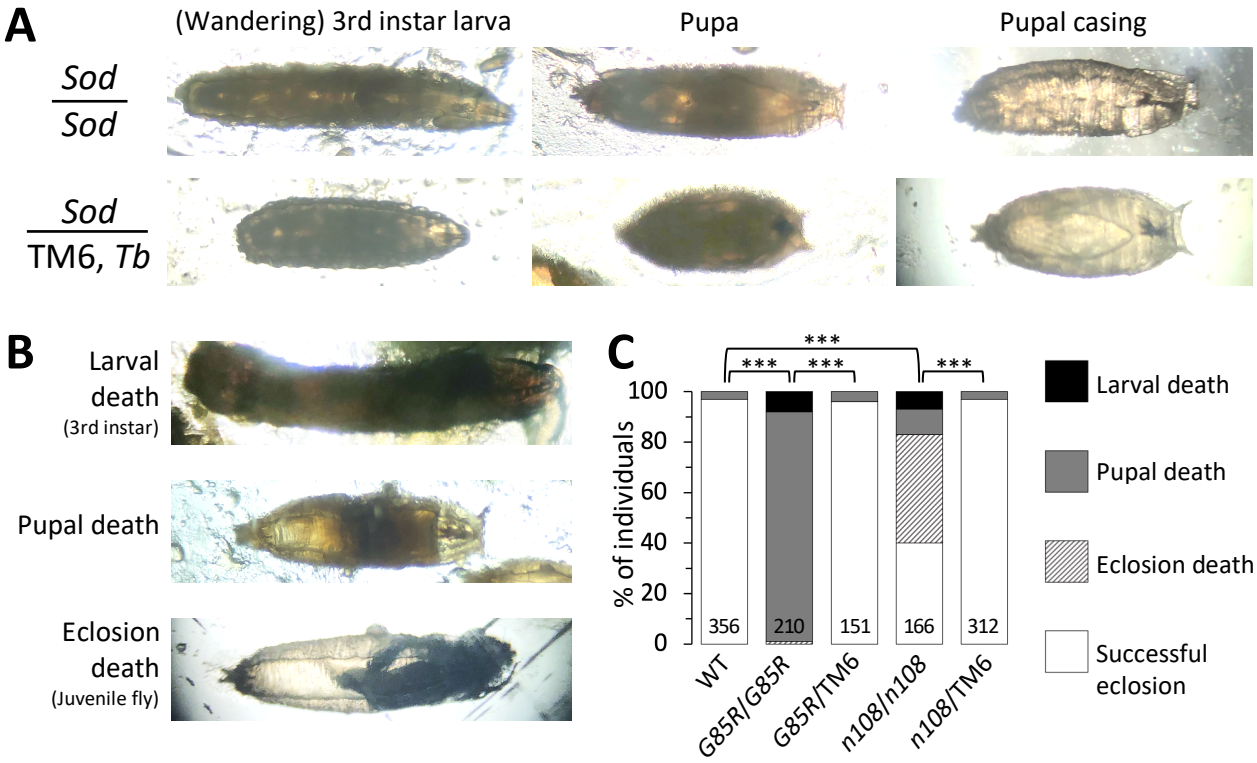
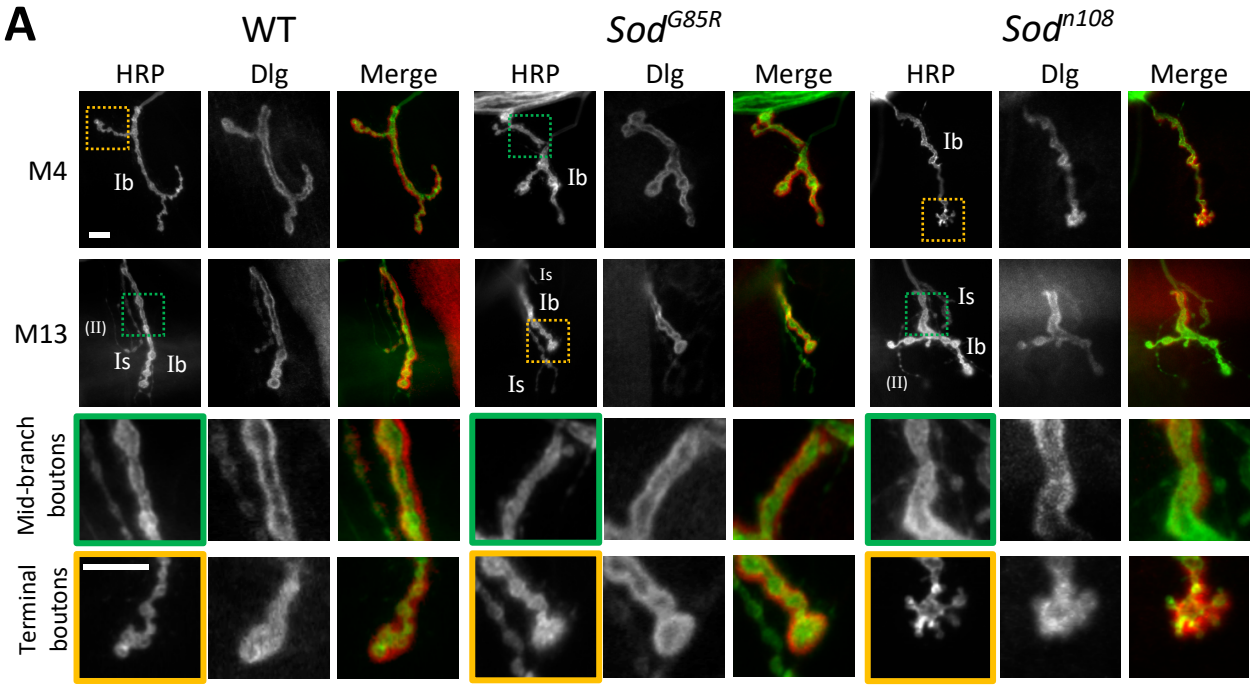

A
(Wandering) 3rd instar larva
Pupa
Pupal casing
Sod / Sod
Sod / TM6, Tb
B
Larval death (3rd instar)
Pupal death
Eclosion death (Juvenile fly)
C
% of individuals
100
80
60
40
20
0
356
210
151
166
312
WT
G85R/G85R
G85R/TM6
n108/n108
n108/TM6
***
Larval death
Pupal death
Eclosion death
Successful eclosion

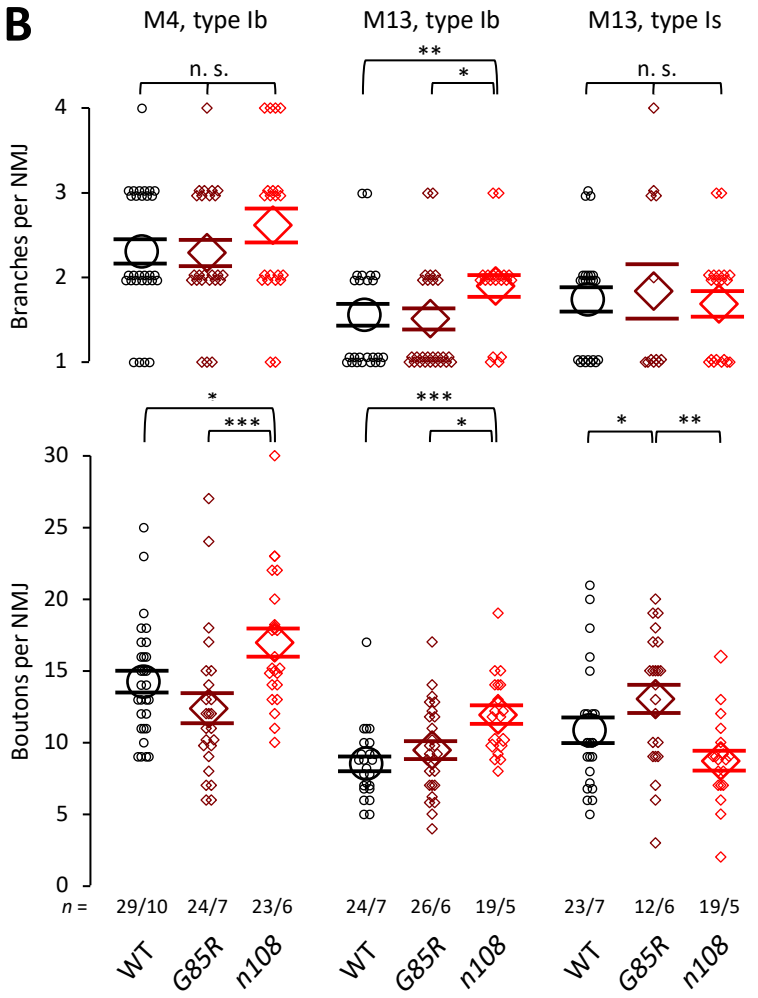
Figure 1. Developmental lethality in *Sod* mutants.

(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.


A
WT
Sod^G85R
Sod^n108
HRP
Dlg
Merge
M4
M13
Ib
Is
(II)
Mid-branch boutons
Terminal boutons


B
M4, type Ib
M13, type Ib
M13, type Is
n. s.
Branches per NMJ
Boutons per NMJ
n =
29/10
24/7
23/6
24/7
26/6
19/5
23/7
12/6
19/5
WT
G85R
n108

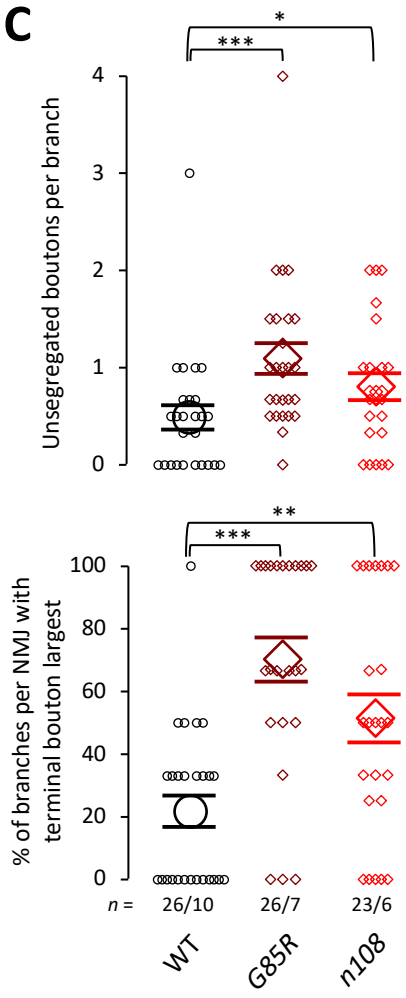

C
Unsegregated boutons per branch
% of branches per NMJ with terminal bouton largest
n =
26/10
26/7
23/6
WT
G85R
n108

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 (subsynaptic 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 μm.

(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.

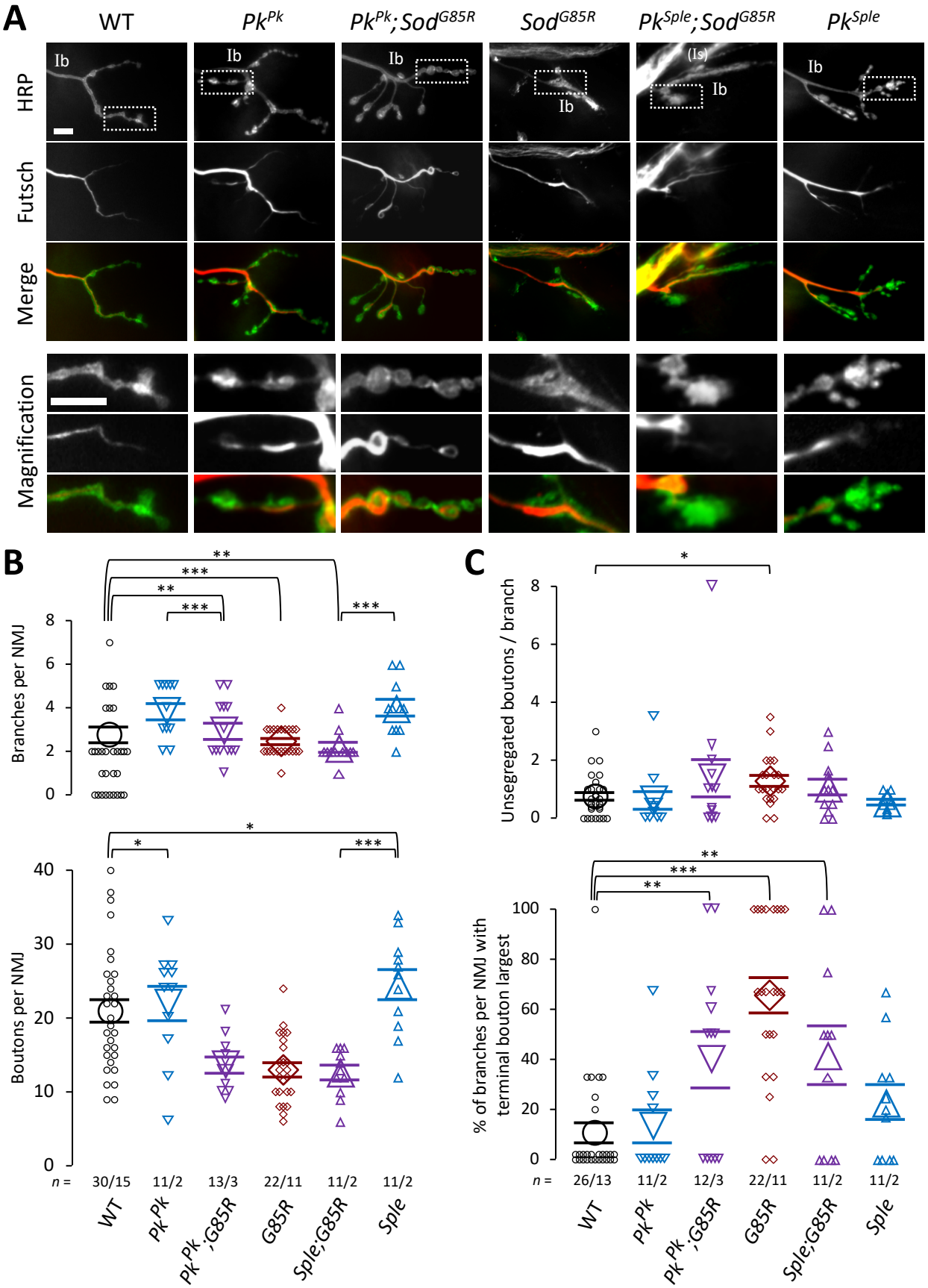

A
WT
Pk^Pk
Pk^Pk;Sod^G85R
Sod^G85R
Pk^Sple;Sod^G85R
Pk^Sple
HRP
Futsch
Merge
Magnification
Ib
(Is)
B
Branches per NMJ
Boutons per NMJ
C
Unsegregated boutons / branch
% of branches per NMJ with terminal bouton largest
n = 30/15 11/2 13/3 22/11 11/2 11/2
n = 26/13 11/2 12/3 22/11 11/2 11/2
WT
Pk^Pk
Pk^Pk;G85R
G85R
Sple;G85R
Sple

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). Is 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.

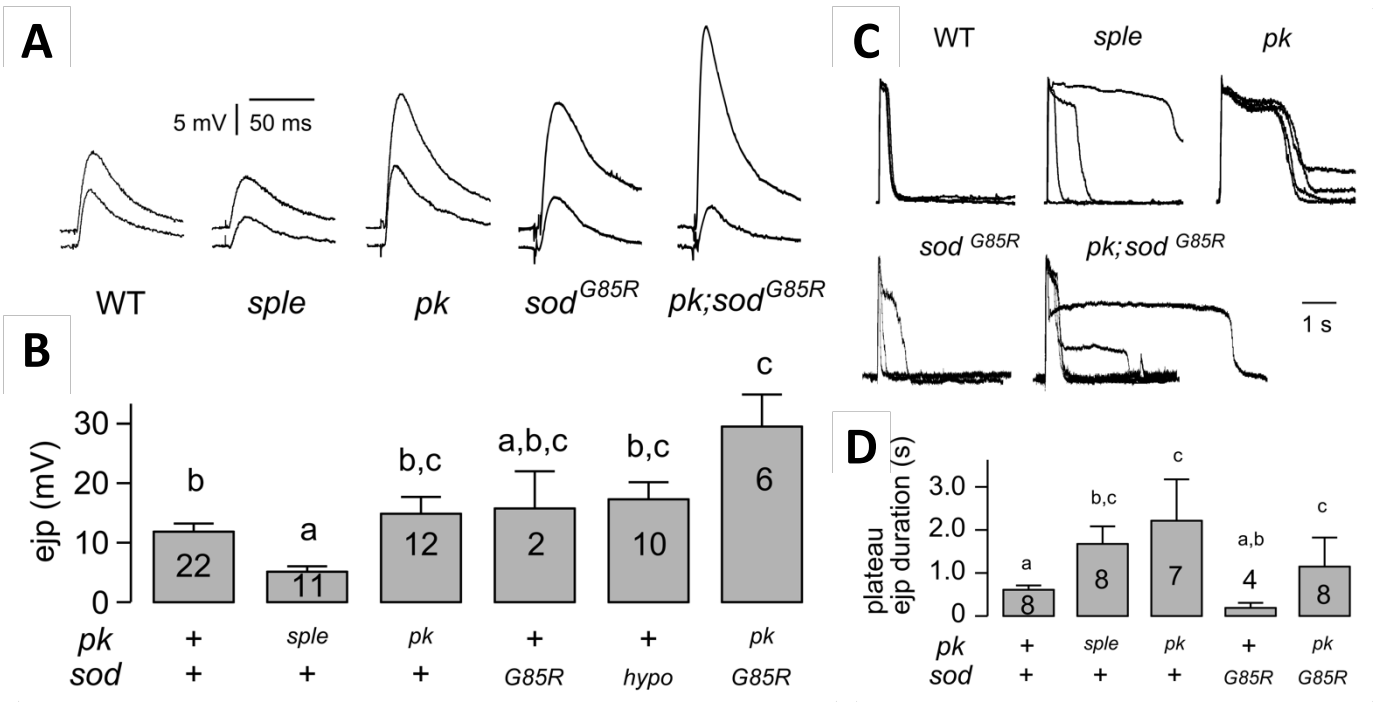

A
5 mV
50 ms
WT
sple
pk
sod G85R
pk;sod G85R
B
ejp (mV)
30
20
10
0
b
a
b,c
a,b,c
b,c
c
22
11
12
2
10
6
pk + sple pk + + pk
sod + + + G85R hypo G85R
C
WT
sple
pk
sod G85R
pk;sod G85R
1 s
D
plateau ejp duration (s)
3.0
2.0
1.0
0
a
b,c
c
a,b
c
8
8
7
4
8
pk + sple pk + pk
sod + + + G85R G85R

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 Ca2+.

(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 perparations 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.

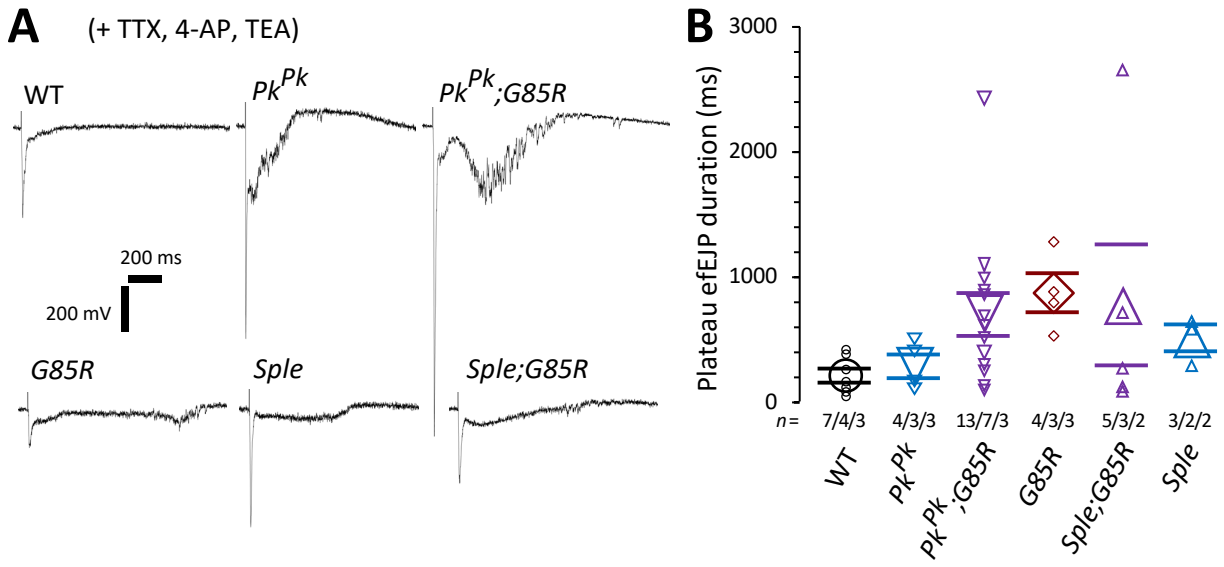

A
(+ TTX, 4-AP, TEA)
WT
Pk^Pk
Pk^Pk;G85R
200 ms
200 mV
G85R
Sple
Sple;G85R
B
Plateau efEJP duration (ms)
3000
2000
1000
0
n =
7/4/3
4/3/3
13/7/3
4/3/3
5/3/2
3/2/2
WT
Pk^Pk
Pk^Pk;G85R
G85R
Sple;G85R
Sple

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.

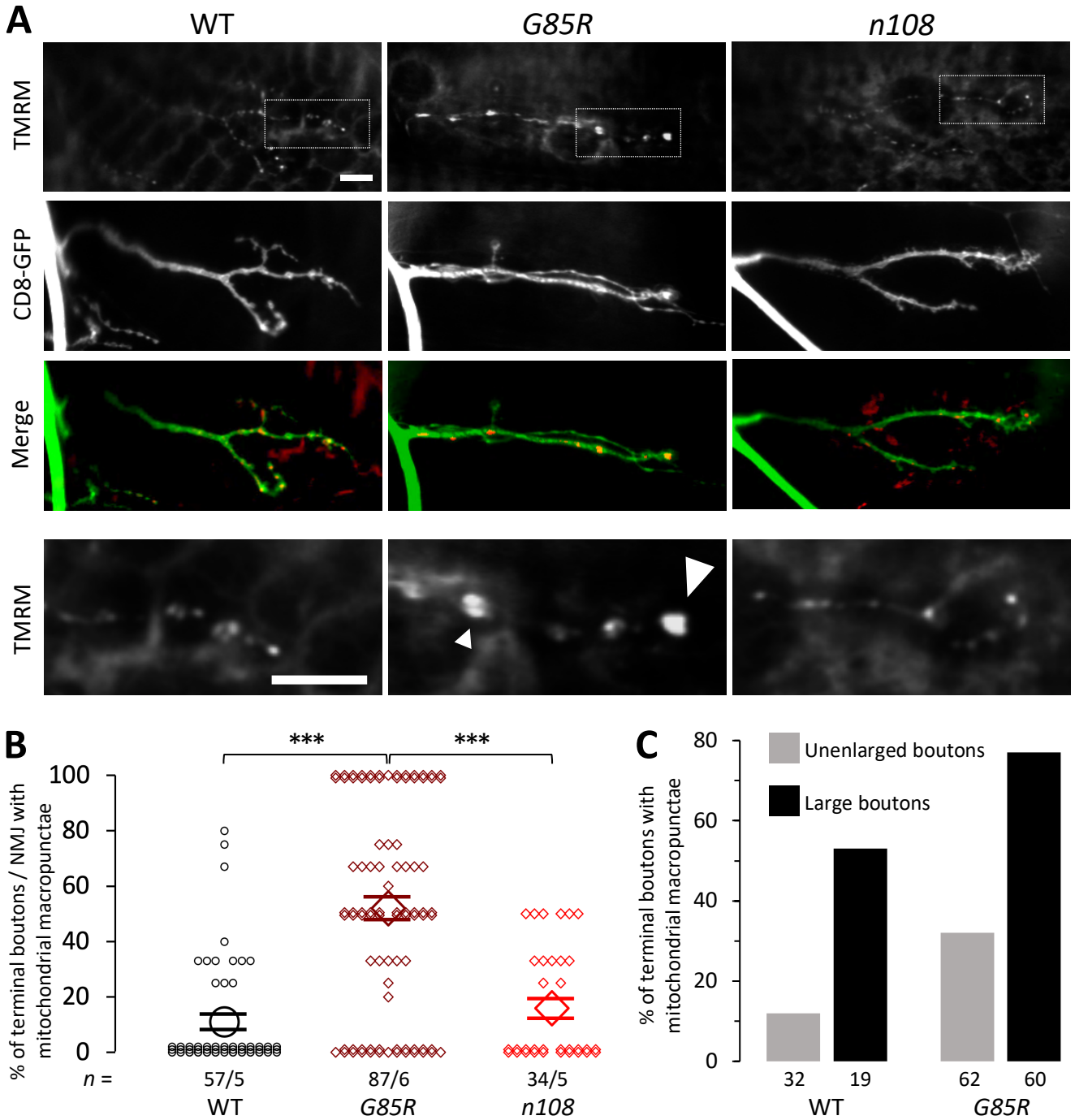
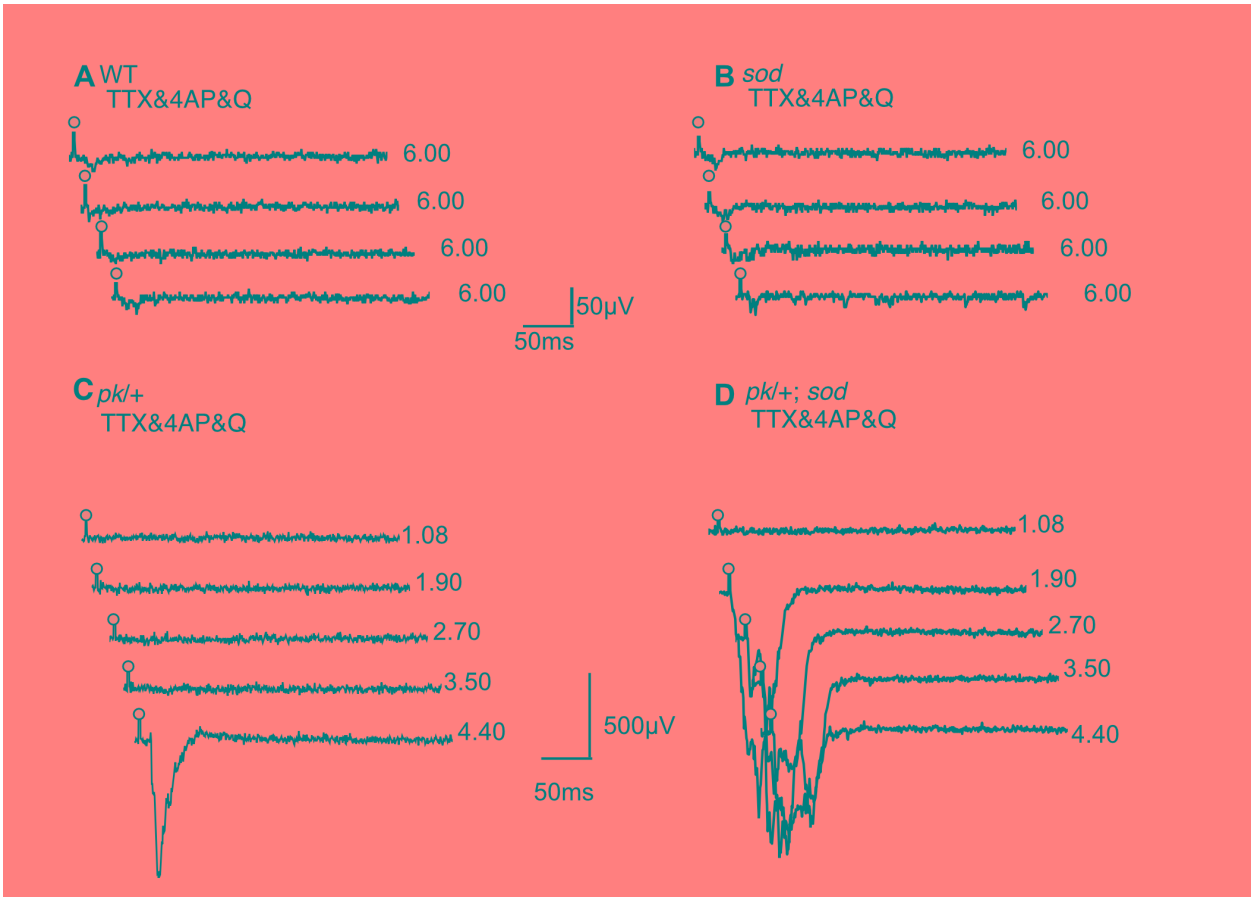

A
WT
G85R
n108
TMRM
CD8-GFP
Merge
TMRM
B
***
***
% of terminal boutons / NMJ with mitochondrial macropunctae
100
80
60
40
20
0
n =
57/5
87/6
34/5
WT
G85R
n108
C
% of terminal boutons with mitochondrial macropunctae
80
60
40
20
0
Unenlarged boutons
Large boutons
32
19
62
60
WT
G85R

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 μm.

(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.


A WT
TTX&4AP&Q
6.00
6.00
6.00
6.00
50μV
50ms
B sod
TTX&4AP&Q
6.00
6.00
6.00
6.00
C pkl+
TTX&4AP&Q
1.08
1.90
2.70
3.50
4.40
500μV
50ms
D pkl+; sod
TTX&4AP&Q
1.08
1.90
2.70
3.50
4.40

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 Ca2+ with Na^+ channel blockers (TTX, 3 μM) and K^+ channel blockers (4-AP, 200 μM and quinidine, 100 μM). Only type Ib boutons were recorded. (A) WT and (B) *sodn108* 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; *sodn108* displayed currents of striking amplitudes. Note that *pk*^{pk}/CyO-GFP; *sodn108* 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.

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