

1 **Title:** Synaptic homeostasis at the Drosophila neuromuscular junction is a reversible signaling  
2 process that is sensitive to high temperature

3

4 **Abbreviated Title:** Reversibility and limits of synaptic homeostasis

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## 23 **ABSTRACT**

24 Homeostasis is a vital mode of biological self-regulation. The hallmarks of homeostasis  
25 for any biological system are a baseline set point of physiological activity, detection of unac-  
26 ceptable deviations from the set point, and effective corrective measures to counteract devia-  
27 tions. Homeostatic synaptic plasticity (HSP) is a form of neuroplasticity in which neurons and  
28 circuits resist environmental perturbations in order to maintain appropriate levels of activity. One  
29 assumption is that if an environmental perturbation triggers homeostatic corrective changes in  
30 neuronal properties, those corrective measures should be reversed upon removal of the pertur-  
31 bation. We test the reversibility and limits of HSP at a well-studied model synapse, the *Dro-*  
32 *sophila melanogaster* neuromuscular junction (NMJ). At the *Drosophila* NMJ, impairment of glu-  
33 tamate receptors causes a decrease in quantal size, which is offset by a corrective, homeostatic  
34 increase in the number of vesicles released per evoked presynaptic stimulus, or quantal con-  
35 tent. This process has been termed presynaptic homeostatic potentiation (PHP). Taking ad-  
36 vantage of a GAL4/GAL80<sup>TS</sup>/UAS expression system, we triggered PHP by expressing a domi-  
37 nant-negative glutamate receptor subunit at the NMJ. We then reversed PHP by halting expres-  
38 sion of the dominant-negative receptor. Our data show that PHP is fully reversible over a time  
39 course of 48-72 hours after the dominant-negative glutamate receptor stops being genetically  
40 expressed. Additionally, we found that the PHP response triggered by the dominant-negative  
41 subunit was ablated at high temperatures. Our data show that the long-term maintenance of  
42 PHP at the *Drosophila* NMJ is a reversible regulatory process that is sensitive to temperature.

43

## 44 **SIGNIFICANCE STATEMENT**

45 Biological homeostatic systems must upregulate or downregulate cellular parameters in  
46 order to maintain appropriate set points of physiological activity. Homeostasis is a well-  
47 documented mode of regulation in metazoan nervous systems. True homeostatic control should  
48 be a reversible process – but due to technical difficulties of presenting and removing functional

49 challenges to living synapses, the reversibility of homeostatic forms of synapse regulation has  
50 not been rigorously examined *in vivo* over extended periods of developmental time. Here we  
51 formally demonstrate that homeostatic regulation of *Drosophila melanogaster* neuromuscular  
52 synapse function is reversible and temperature-labile. This is significant because developing  
53 methods to study how homeostatic regulatory systems are turned on and off could lead to fun-  
54 damental new insights about control of synaptic output.

55

## 56 INTRODUCTION

57 Homeostasis is a strong form of biological regulation. It permits individual cells or entire  
58 systems of cells to maintain core physiological properties that are compatible with life. In the  
59 nervous system, decades of study have shown that while synapses and circuits are inherently  
60 plastic, they also possess robust homeostatic regulatory systems in order to maintain physiolog-  
61 ical stability. Homeostatic plasticity in the nervous system is a non-Hebbian strategy to counter-  
62 act challenges to neuronal function that may threaten to disrupt essential neuronal and circuit  
63 activities (Turrigiano, 2017). Depending upon the synaptic preparation examined and the envi-  
64 ronmental challenge presented to the synapse, homeostatic responses may be executed via  
65 compensatory adjustments to presynaptic neurotransmitter release (Cull-Candy et al., 1980;  
66 Davis and Müller, 2015; Frank et al., 2006; Murthy et al., 2001; Petersen et al., 1997;  
67 Thiagarajan et al., 2005), postsynaptic neurotransmitter receptor composition (O'Brien et al.,  
68 1998; Rongo and Kaplan, 1999; Turrigiano, 2008; Turrigiano et al., 1998), neuronal excitability  
69 (Bergquist et al., 2010; Marder and Bucher, 2007; Marder and Goaillard, 2006; Marder and  
70 Prinz, 2002; Parrish et al., 2014) – or even developmentally, via changes in synaptic contact  
71 formation and maintenance (Burrone et al., 2002; Davis and Goodman, 1998; Wefelmeyer et  
72 al., 2016).

73 Bi-directionality has been documented in several homeostatic systems, perhaps most  
74 prominently in the case of synaptic scaling of neurotransmitter receptors. For vertebrate neu-

75 ronal culture preparations – such as cortical neurons or spinal neurons – global silencing of  
76 network firing can induce increases in excitatory properties, such as increased AMPA-type glu-  
77 tamate receptor accumulation; by contrast, global enhancement of activity can induce the oppo-  
78 site type of response (O'Brien et al., 1998; Turrigiano, 2008; Turrigiano et al., 1998; Wierenga et  
79 al., 2005).

80 Bi-directionality is also a key feature underlying homeostatic alterations of neurotransmit-  
81 ter release at peripheral synapses like the neuromuscular junction (NMJ). At the *Drosophila*  
82 *melanogaster* and mammalian NMJs, impairing neurotransmitter receptor function  
83 postsynaptically results in diminished sensitivity to single vesicles of transmitter.  
84 Electrophysiologically, this manifests as decreased quantal size. NMJs respond to this chal-  
85 lenge by enhancing neurotransmitter vesicle release (Cull-Candy et al., 1980; Davis et al., 1998;  
86 Frank et al., 2009; Petersen et al., 1997; Plomp et al., 1995; Plomp et al., 1992). By contrast,  
87 perturbations that enhance quantal size – for example, overexpression of the vesicular neuro-  
88 transmitter transporter – can result in decreased quantal content (Daniels et al., 2004; Gaviño et  
89 al., 2015).

90 Synapses and circuits possess myriad solutions to assume appropriate functional out-  
91 puts in the face of perturbations (Marder and Goaillard, 2006; Marder and Prinz, 2002). There-  
92 fore, a corollary to bi-directional regulation is that homeostatic forms of regulation should also be  
93 reversible. There are experimental difficulties of presenting and removing a synaptic challenge  
94 in the context of a living synapse, so homeostatic reversibility has not been rigorously studied in  
95 an *in vivo* system or over extended periods of developmental time. However, understanding  
96 how homeostatic regulatory systems are reversibly turned on and off could have profound impli-  
97 cations for elucidating fundamental properties of circuit regulation.

98 Here we focus on the aforementioned *Drosophila melanogaster* NMJ as a living synapse  
99 to test homeostatic reversibility. At the *Drosophila* NMJ, a canonical way to challenge synapse  
100 function is through glutamate receptor impairment (Frank, 2014), either genetically (Petersen et

101 al., 1997) or pharmacologically (Frank et al., 2006). Impairments of muscle glutamate receptor  
102 function decrease quantal size. Decreased quantal size spurs muscle-to-nerve signaling that  
103 ultimately results in a homeostatic increase in presynaptic vesicle release – a process that has  
104 been termed presynaptic homeostatic potentiation (PHP). The most widely used experimental  
105 homeostatic challenges to *Drosophila* NMJ function are not easily reversed – including pharma-  
106 cological inhibition with PhTox, due to an irreversible impairment of glutamate receptor function  
107 (Frank et al., 2006).

108 For this study, we engineered a way to challenge NMJ function *in vivo* for significant pe-  
109 riods of time, verify the effectiveness of the challenge at a defined time developmental time  
110 point, remove the challenge, and then assess the homeostatic capacity of the NMJ at a later  
111 developmental time point. To do this, we utilized the TARGET (temporal and regional gene ex-  
112 pression targeting) GAL4/GAL80<sup>TS</sup>/UAS expression system (McGuire et al., 2003). By using this  
113 expression system to temporally control the expression of a dominant-negative GluRIIA receptor  
114 subunit (DiAntonio et al., 1999), we found that homeostatic potentiation of neurotransmitter re-  
115 lease is fully reversible. In the course of conducting our studies, we also uncovered a high tem-  
116 perature limitation of homeostatic potentiation at the NMJ.

117

## 118 MATERIALS AND METHODS

119

### 120 *Drosophila* Stocks and Husbandry

121 Fruit fly stocks were either obtained from the Bloomington *Drosophila* Stock Center  
122 (BDSC, Bloomington, Indiana) or from the labs that generated them. *w*<sup>1118</sup> was used as a wild-  
123 type (WT) control (Hazelrigg et al., 1984). The *GluRIIA*<sup>SP16</sup> deletion was used as a genetic loss-  
124 of-function (Petersen et al., 1997). Transgenes included the UAS-driven dominant-negative glu-  
125 tamate receptor subunit, *UAS-GluRIIA*<sup>M614R</sup> (DiAntonio et al., 1999) and a ubiquitous  
126 *Tubulin*<sub>Promoter</sub>-*Gal80*<sup>TS</sup> (*Tub*<sub>P</sub>-*Gal80*<sup>TS</sup>) (McGuire et al., 2003). Muscle-specific GAL4 drivers in-

127 cluded *MHC-Gal4* (Schuster et al., 1996a, b) and *BG57-Gal4* (also known as *C57-Gal4*) (Budnik  
128 et al., 1996). For reversibility experiments, the full genotypes for the crosses were  $w^{1118}; CyO-$   
129  $GFP/UAS-GluRIIA^{M614R}; TM6b(Tb)/Tub_P-Gal80^{TS} \times w^{1118}; ; TM6b(Tb)/MHC-Gal4$  or  $BG57-Gal4$ .  
130 Non-tubby, non-GFP larvae were selected for recording. In control recordings, we found no dis-  
131 cernable differences between male and female third-instar electrophysiology, but for reversibility  
132 experiments (Figs. 3-5), single sexes of larvae were chosen to eliminate sex as a possible con-  
133 founding variable.

134 Fruit flies were raised on cornmeal, molasses, and yeast medium (see BDSC website for  
135 standard recipe) in temperature-controlled conditions. For most experiments, animals were  
136 reared at the temperatures noted (including temperature shifts) until they reached the wandering  
137 third instar larval stage, at which point they were chosen for electrophysiological recording. For  
138 experiments in Figs. 2 and 3, mated animals were placed at either 25°C or 29°C and allowed to  
139 lay eggs for 6-8 hours. Stage-matched and size-matched early third instar larvae (approximately  
140 48-54 hours after the egg laying period) were subjected to electrophysiological recording (Fig. 2)  
141 or temperature swaps (Fig. 3), as indicated.

142

### 143 **Electrophysiology and Analysis**

144 For Figs. 1 and 3-6, wandering third instar larvae were used for electrophysiological re-  
145 cordings. For Fig. 2, early third instar larvae were used. In both cases, sharp electrode electro-  
146 physiological recordings were taken from muscle 6 of abdominal segments 2 and 3. Briefly, lar-  
147 vae were dissected in a modified HL3 saline comprised of: NaCl (70 mM), KCl (5 mM), MgCl<sub>2</sub>  
148 (10 mM), NaHCO<sub>3</sub> (10 mM), sucrose (115 mM = 3.9%), trehalose (4.2 mM = 0.16%), HEPES  
149 (5.0 mM = 0.12%), and CaCl<sub>2</sub> (0.5 mM). Electrophysiological data were collected using an  
150 Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) in bridge mode, digitized using a  
151 Digidata 1440A data acquisition system (Molecular Devices), and recorded with pCLAMP 10  
152 acquisition software (Molecular Devices). A Master-8 pulse stimulator (A.M.P. Instruments, Je-

153    rusalem, Israel) and an ISO-Flex isolation unit (A.M.P. Instruments) were utilized to deliver 1 ms  
154    suprathreshold stimuli to the appropriate segmental nerve. The average spontaneous miniature  
155    excitatory postsynaptic potential (mEPSP) amplitude per NMJ was quantified by hand, approxi-  
156    mately 100-200 individual spontaneous release events per NMJ (MiniAnalysis, Synaptosoft, Fort  
157    Lee, NJ). In the case that the mEPSP frequency was extremely low (usually for expression of  
158    the dominant-negative glutamate receptor subunit), several minutes of spontaneous recording  
159    were done and all events measured. Measurements from all NMJs of a given condition were  
160    then averaged. For evoked neurotransmission, 30 excitatory postsynaptic potentials (EPSPs)  
161    were averaged to find a value for each NMJ. These were then averaged to calculate a value for  
162    each condition. Quantal content (QC) was calculated by the ratio of average EPSP and average  
163    mEPSP amplitudes for each individual NMJ. An average quantal content was then calculated  
164    for each condition.

165

## 166    **Statistical Analyses**

167            Statistical analyses were conducted using GraphPad Prism Software. Statistical signifi-  
168    cance was assessed either by Student's T-Test when one experimental data set was being di-  
169    rectly compared to a control data set, or one-way ANOVA with Tukey's post-hoc test when mul-  
170    tiple data sets were being compared. To assess potential correlations between incubation tem-  
171    perature recovery times and electrophysiological parameters (Fig. 4), Pearson correlation coef-  
172    ficients were calculated ( $r$ ) and reported on the graphs, and two-tailed statistical analyses per-  
173    formed to check correlation significance.

174            Specific  $p$  value ranges are noted in the Fig. legends and shown in graphs as follows: \*  $p$   
175    < 0.05, \*\*  $p$  < 0.01, and \*\*\*  $p$  < 0.001. For some  $p$  values that potentially trend toward statistical  
176    significance ( $0.05 < p < 0.1$ ), specific values are given. Most values reported in Table 1 or plot-  
177    ted on bar graphs are mean  $\pm$  SEM.

178

179 **RESULTS**

180

181 **Homeostatic Potentiation Utilizing a Dominant-Negative Glutamate Receptor Subunit**

182 A prior study described a transgene encoding a dominant-negative GluRIIA subunit,  
183 *UAS-GluRIIA<sup>M614R</sup>* (herein termed *UAS-GluRIIA<sup>M/R</sup>* or “dominant-negative”) (DiAntonio et al.,  
184 1999). The GluRIIA M614R amino-acid substitution resides in the ion conduction pore of the  
185 GluRIIA subunit, and it cripples channel function (DiAntonio et al., 1999). Transgenic expression  
186 of *UAS-GluRIIA<sup>M/R</sup>* in muscles renders a strong homeostatic challenge (markedly diminished  
187 quantal size) and an equally strong compensatory response (increase in quantal content). The  
188 prior report demonstrated that evoked amplitudes remain normal as a result of this compensa-  
189 tion (DiAntonio et al., 1999).

190 We acquired this dominant-negative transgenic line to test homeostatic reversibility.  
191 First, we replicated the published experiments – this time raising fruit fly larvae at temperatures  
192 compatible with the TARGET system that we planned to use to test reversibility (McGuire et al.,  
193 2003). We tested 25°C (a common culturing temperature) and 29°C (a temperature at which the  
194 GAL80<sup>TS</sup> protein ceases to inhibit GAL4). We drove *UAS-GluRIIA<sup>M/R</sup>* expression with *MHC-Gal4*,  
195 which turns on in first-instar larval muscles (Schuster et al., 1996a, b). We recorded from NMJs  
196 of control and dominant-negative wandering third instar larvae.

197 For animals reared at 25°C, wild-type (WT) electrophysiology was robust (Figs. 1A, C, D,  
198 Table 1 – see Table 1 for all raw electrophysiological data). By contrast, muscle-specific *UAS-*  
199 *GluRIIA<sup>M/R</sup>* expression at 25°C caused a large diminishment of quantal size compared to WT,  
200 evident from a small average miniature excitatory postsynaptic potential (mEPSP) amplitude  
201 (Figs. 1A, C, Table 1). Despite this decrease in quantal size, there was no diminishment in the  
202 average evoked excitatory postsynaptic potential (EPSP) amplitude for dominant-negative  
203 NMJs (Figs. 1A, C, D, Table 1) because of an offsetting homeostatic increase in quantal content  
204 (QC) (Figs. 1A, C, Table 1). In sum, recordings at 25°C agreed with the prior finding for the



205 dominant-negative transgene (DiAntonio et al., 1999): perfect presynaptic homeostatic potentia-  
206 tion (PHP). We did note one previously unreported phenotype at 25°C: starkly diminished  
207 quantal frequency (Fig. 1C). This diminished mEPSP frequency phenotype mirrored numerous  
208 cases in which there was greatly diminished glutamate receptor subunit expression or function  
209 (Brusich et al., 2015; Daniels et al., 2006; Featherstone et al., 2005; Kim et al., 2012; Kim et al.,  
210 2015; Ramos et al., 2015).

211 For animals raised at 29°C, we garnered similar results as 25°C, noting a few differ-  
212 ences. First, at 29°C, WT NMJs had slightly smaller mEPSP quantal amplitude and frequency  
213 than 25°C WT NMJs (Figs. 1A-D, Table 1). This finding was consistent with a prior report meas-  
214 uring NMJ physiology at elevated temperatures (Ueda and Wu, 2015). Nevertheless, WT  
215 evoked amplitudes and QC at 29°C were robust, similar to the values garnered at 25°C (Figs.  
216 1A-D, Table 1). *UAS-GluRIIA<sup>M/R</sup>*-expressing NMJs from 29°C showed a profound decrease in  
217 mEPSP size, a strong compensatory increase in QC, and markedly reduced quantal frequency  
218 compared to WT controls (Fig. 1B). However, unlike at 25°C, homeostatic compensation was  
219 not perfect at 29°C for dominant-negative NMJs. Even though QC was significantly increased in  
220 dominant-negative NMJs compared to WT, it did not increase enough to bring average *MHC-*  
221 *Gal4 >> UAS-GluRIIA<sup>M/R</sup>* EPSP amplitudes at 29°C fully back to control levels (Figs. 1B, C, D).

222

### 223 **Early Third Instar Larvae Express Homeostatic Plasticity**

224 Our initial experiments showed that it is possible to observe compensatory increases in  
225 quantal content for *UAS-GluRIIA<sup>M/R</sup>*-expressing animals raised throughout life, at either 25°C or  
226 29°C. We sought to test homeostatic reversibility using the TARGET system. For this study,  
227 TARGET augments GAL4/UAS expression adding a ubiquitously expressed *Tubulin<sub>P</sub>-Gal80<sup>TS</sup>*  
228 GAL4 inhibitor transgene (McGuire et al., 2003) to the *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* genetic  
229 background. The temperature sensitivity of the GAL80<sup>TS</sup> protein permits tight control over when  
230 GAL4-responsive transgenes are expressed (McGuire et al., 2003). We predicted that the dom-

231 inant-negative transgene should be repressed by GAL80<sup>TS</sup> at low, permissive temperatures; and  
232 conversely, *UAS-GluRIIA<sup>M/R</sup>* should be actively expressed when GAL80<sup>TS</sup> is inactive (~29°C or  
233 higher) (McGuire et al., 2003). In order to study the reversibility of PHP, animals could be reared  
234 at high temperature and then swapped to a lower temperature at an appropriate developmental  
235 time point.

236 We needed to identify a suitable developmental stage for temperature swaps. An ideal  
237 swap point would be late enough to detect PHP, but early enough to allow recovery time after  
238 cessation of the dominant-negative *UAS-GluRIIA<sup>M/R</sup>* expression. We crossed *UAS-GluRIIA<sup>M/R</sup>*;  
239 *Tub<sub>P</sub>-Gal80<sup>TS</sup>* x *MHC-Gal4* stocks. Mated animals were transferred to 25°C or 29°C for egg lay-  
240 ing and subsequent larval development. We selected early third instar progeny for electrophysi-  
241 ological recording. At temperature ranges of 25-29°C, early third instar larvae emerge roughly  
242 48-60 hours after an egg-laying period. We staged small animals unambiguously by examining  
243 their posterior spiracles for an orange-colored tip.

244 For animals raised entirely at 25°C, early third instar NMJ mEPSP size was large – sig-  
245 nificantly larger than one would observe for third instar larvae (Figs 2A, B, Table 1). This was  
246 expected because for developing NMJs, small muscles have a significantly greater input re-  
247 sistance and enhanced quantal size (Davis and Bezprozvanny, 2001; Lnenicka and Mellon,  
248 1983a, b) (Table 1). Early third instar larval NMJs showed normal evoked amplitudes, consistent  
249 with a stable level of evoked muscle excitation throughout development (Figs 2A, B, Table 1).

250 We predicted genotypically equivalent early third instars raised entirely at 29°C would  
251 express the dominant-negative transgene. As expected, NMJs from these animals showed  
252 sharply reduced mEPSP amplitude and frequency compared to their stage- and size-matched  
253 counterparts raised and 25°C (Figs. 2A, B, Table 1). There was a robust increase in quantal  
254 content at 29°C, resulting in EPSP amplitudes that were nearly normal, but not quite at the  
255 same level as at 25°C. As with the earlier experiments, presynaptic homeostatic potentiation

256 (PHP) for early third instar NMJs raised exclusively at 29°C was present, but not perfect (Figs.  
257 2A, B, Table 1).

258

### 259 **Imperfect Homeostatic Potentiation is Reversible**

260 At 29°C, PHP was not perfect, but QC increases versus controls were robust, making it  
261 possible to test reversibility. We generated additional *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* larvae with  
262 the *TubP-GAL80<sup>TS</sup>* transgene. This time we chose 21°C as a permissive GAL80<sup>TS</sup> shift tempera-  
263 ture to examine because 21°C permitted multiple electrophysiological time point measurements  
264 over a long recovery window (Fig. 3A).

265 Control (no PHP) animals raised entirely at 21°C took ~120 hours after the egg laying  
266 period to reach the wandering third instar stage, while control (PHP) animals raised entirely at  
267 29°C took ~96 hours to reach the same stage (Fig. 3A). A 1-day recovery condition was utilized  
268 – exposing animals to the *UAS-GluRIIA<sup>M/R</sup>* challenge for ~72 hours (29°C) and allowing them to  
269 recover at 21°C for 1 day prior to recording. We also tested 2- and 3-day recovery conditions,  
270 rearing larvae at 29°C for approximately ~49-50 hours and then swapping them to 21°C until  
271 they reached wandering third instar stage. Due to the length of the egg lays and small variations  
272 in developmental time, some animals reached wandering third instar stage after about 2 days at  
273 21°C, while others took closer to 3 days (Fig. 3A).

274 Control *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* larvae with ubiquitously expressed *TubP-Gal80<sup>TS</sup>*  
275 and raised at 21°C showed physiology indistinguishable from WT control animals reared at  
276 25°C. This was true for all electrophysiological parameters (Compare Figs. 1, 3, Table 1). By  
277 contrast, genetically identical control animals raised at 29°C throughout life showed electrophys-  
278 iological phenotypes similar to dominant-negative *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* animals raised  
279 at 29°C (Compare Figs. 1, 3, Table 1). Compared to counterparts raised at 21°C, animals raised  
280 at 29°C showed reduced mEPSP frequency, reduced mEPSP size, slightly below-normal EPSP  
281 amplitudes, and increased QC, indicating robust PHP (Fig. 3, Table 1).

282 Recovery conditions showed physiological signatures that corresponded with how much  
283 time was spent at 21°C. Compared to the 29°C condition, the 1-day 21°C recovery condition  
284 showed no significant changes in physiological properties and no reversal of PHP (Figs 3B-D).  
285 By contrast, the 2-day recovery condition showed intermediate phenotypes. Compared to con-  
286 stant exposure to 21°C, the 2-day recovery condition still had diminished mEPSP amplitude and  
287 frequency – but not nearly as diminished as constant exposure to 29°C (Figs. 3B-D). Interest-  
288 ingly, the 2-day recovery EPSP amplitudes revealed restored levels of excitation, due to an in-  
289 crease in QC (Figs. 3C, D). The 3-day recovery condition showed electrophysiology that was  
290 not significantly different from the constant 21°C condition (Figs 3B-D), indicating a full reversal  
291 of PHP.

292 We further analyzed the aggregate data from the reversibility experiment. We wished to  
293 test for hallmarks of PHP and reversal related to recovery time. Prior studies of homeostatic  
294 plasticity at the *Drosophila* NMJ have shown that by plotting hundreds individual recording val-  
295 ues, quantal content inversely scales with quantal size across genotypes – and as a result,  
296 evoked excitation levels remain stable (Davis and Müller, 2015; Frank et al., 2006; Gaviño et al.,  
297 2015). For our temperature swap experiments – this time conducting a comparison within a sin-  
298 gle genotype – this also proved to be the case (Fig. 4A).

299 Next, we plotted mEPSP and QC values versus the number of animals spent at the re-  
300 covery temperature, with a specific recovery time value for each NMJ, time locked to the egg  
301 laying period and the recording time after recovery/development at 21°C. Both plots showed  
302 hallmarks of PHP reversal: mEPSP amplitudes significantly positively correlated with time at  
303 21°C (Fig. 4B), and QC values significantly inversely correlated with time at 21°C (Fig. 4C).  
304 Consistent with the observation that PHP was present – but not perfect – at 29°C (Figs. 1-3),  
305 individual data points from NMJs of animals raised entirely at 29°C showed a wide variability of  
306 QC values (Fig. 4C).

307

308 **Perfect Homeostatic Potentiation is Also Reversible**

309 Homeostatic potentiation was robust, yet imperfect, at the 29°C condition. We hoped to  
310 test a condition that was both compatible with perfect PHP and the reversibility assay. We at-  
311 tempted a new temperature swap, changing a few parameters. For one change, we lowered the  
312 restrictive Gal80<sup>TS</sup> temperature from 29°C to 28.5°C. Other studies using the TARGET system  
313 in *Drosophila melanogaster* have reported that 28.5°C is somewhat effective at impairing  
314 Gal80<sup>TS</sup> function (Corrigan et al., 2014; Redhai et al., 2016; Staley and Irvine, 2010). Second,  
315 there was a formal possibility that imperfect compensation at 29°C reflected a GAL4 driver-  
316 specific phenomenon, rather than a temperature-specific phenomenon. Therefore, we replaced  
317 the *MHC-Gal4* muscle driver with the *BG57-Gal4* muscle driver (Budnik et al., 1996). Finally, we  
318 returned to 25°C as the permissive condition.

319 We generated new sets of larvae for this swap experiment – *BG57-Gal4* >> *UAS-*  
320 *GluRIIA<sup>M/R</sup>* larvae with the *TubP-Gal80<sup>TS</sup>* transgene. As expected, animals raised at 25°C  
321 throughout life (GAL80<sup>TS</sup> on) developed NMJs with electrophysiological properties similar to  
322 other control conditions already reported (Figs. 5A, C, Table 1). Also, as expected, animals  
323 raised at 28.5°C throughout life (GAL80<sup>TS</sup> impaired) had significantly diminished NMJ mEPSP  
324 amplitudes and mEPSP frequency (Figs. 5A, B, Table 1). NMJs from those 28.5°C animals also  
325 showed completely normal NMJ EPSP amplitudes because of a perfect, offsetting homeostatic  
326 increase in QC (Figs. 5A, C, Table 1). Of note, the 28.5°C NMJs had markedly diminished  
327 mEPSP frequency, an indication of successful expression of the dominant-negative *GluRIIA<sup>M/R</sup>*  
328 transgene (Fig. 1). Animals raised at 28.5°C until early third instar and then swapped to 25°C for  
329 the final two days of larval development showed NMJ electrophysiology indistinguishable from  
330 that of animals raised at 25°C throughout life, indicating a complete reversal of PHP (Fig. 5).

331

332 **Homeostatic potentiation induced by *GluRIIA<sup>M/R</sup>* is impaired at high temperatures**

333 *GluRIIA<sup>M/R</sup>* transgene-induced homeostatic potentiation was robust, yet imperfect at the  
334 29°C condition. We wondered if PHP might specifically be impaired at the NMJ when flies are  
335 raised at high temperatures. To test this idea further, we drove the dominant-negative transgene  
336 in the muscle, setting up crosses to generate both *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* and *BG57-  
337 Gal4 >> UAS-GluRIIA<sup>M/R</sup>* animals, as well as driver-specific controls. For the driver controls,  
338 quantal size was somewhat diminished at 30°C (Figs. 6A-D, Table 1). This was consistent with  
339 the idea that quantal size is generally diminished at very high temperatures (Ueda and Wu,  
340 2015) – though it could also be the case that high levels of muscle-driven GAL4 protein at high  
341 temperatures contributes to this phenotype. Evoked EPSP amplitudes were still robust for driver  
342 controls (Figs. 6A-D, Table 1). For the dominant-negative NMJs, there was a significant reduc-  
343 tion in mEPSP size – beyond what was measured for the driver controls. Moreover, evoked am-  
344 plitudes were weak, diminished significantly versus driver controls because of no significant in-  
345 crease in QC – in fact, there was a significant decrease in QC (Figs. 6A-D, Table 1). These data  
346 indicated that signaling processes that maintain PHP in response to the dominant-negative  
347 transgene may break down at high temperatures.

348 To extend this line of inquiry, we examined a second homeostatic challenge to NMJ  
349 function. We raised WT and *GluRIIA<sup>SP16</sup>* deletion flies (Petersen et al., 1997) at 30°C. WT NMJs  
350 showed relatively normal physiology at 30°C (Figs., 6E, F, Table 1). NMJs from *GluRIIA<sup>SP16</sup>* de-  
351 letion animals raised at 30°C showed significant PHP, with reduced mEPSP amplitudes and ro-  
352 bust increase in QC compared to WT (Figs. 6A, B, Table 1). We noted that EPSP amplitudes  
353 were somewhat reduced compared to WT (Table 1). Collectively, our data show that normal  
354 baseline neurotransmission is not disrupted at 30°C. Moreover, PHP is possible and robust (yet  
355 imperfect) at 30°C for *GluRIIA<sup>SP16</sup>* mutants, but it is abolished in the case of the dominant-  
356 negative *UAS-GluRIIA<sup>M/R</sup>* transgene.

357

358 **DISCUSSION**

359 We present evidence that presynaptic homeostatic potentiation (PHP) at the *Drosophila*  
360 *melanogaster* neuromuscular synapse is a reversible process. In doing so, we confirm prior find-  
361 ings showing that there is a tight inverse relationship between quantal amplitude and quantal  
362 content at the NMJ (Fig. 4). We complement those findings by conducting temperature shift ex-  
363 periments. We find that PHP is measurable at an early stage of larval development (Fig. 2) and  
364 can be erased over a matter of days (Figs. 3, 5). Interestingly, PHP fails or falls short of perfect  
365 compensation at high temperatures (Figs. 1, 2, 3, 6).

366 For the *Drosophila* NMJ, homeostatic potentiation is a robust and sensitive process. One  
367 assumption supported by all available data is that the larval NMJ is capable of modulating its  
368 vesicle release at any time point during development – in accordance with the presence or ab-  
369 sence of a homeostatic challenge to synapse function. Rapid, acute induction of homeostatic  
370 signaling has previously been demonstrated at the *Drosophila* NMJ by application of  
371 Philanthotoxin-433 (PhTox) to impair the glutamate receptors (Frank et al., 2006), but the re-  
372 versibility of this particular modulation (or any other modulation at the *Drosophila* NMJ) had not  
373 been studied. More generally, it is not clear what happens in metazoan nervous systems when  
374 harsh perturbations that induce homeostatic signaling are introduced for long periods of devel-  
375 opmental time and then later removed.

376

### 377 **Why is reversibility slow after dominant-negative GluRIIA<sup>M/R</sup> removal?**

378 There was a robust expression of presynaptic homeostatic potentiation (PHP) for NMJs  
379 of *MHC-Gal4 >> UAS-GluRIIA<sup>M/R</sup>* larvae with the *Tub $\rho$ -Gal80<sup>TS</sup>* transgene raised at 29°C for 48  
380 hours post egg-laying (Fig. 2). Once the expression of the dominant-negative *UAS-GluRIIA<sup>M/R</sup>*  
381 transgene was halted, this expression of PHP was erased over a slow 48- to 72-hour period  
382 (Fig. 3). 24 hours of halted dominant-negative expression provided no relief (Fig. 3).

383 If PHP is a readily reversible homeostatic process, why is there a days-long time lag in  
384 order to reverse it? The answer is likely a constraint of the dominant-negative GluRIIA<sup>M/R</sup> exper-

385 imental perturbation, rather than a reflection of the NMJ's capacity to respond quickly to the  
386 changed environment. In a prior study, researchers expressed functional, tagged GluRIIA trans-  
387 genic subunits at the NMJ and performed fluorescence recovery after photobleaching (FRAP)  
388 experiments (Rasse et al., 2005). Those experiments demonstrated that receptor turnover rates  
389 at the *Drosophila* NMJ are extremely slow: it appears that once postsynaptic densities (PSDs)  
390 reach a critical size, GluRIIA subunits are stably incorporated (Rasse et al., 2005). For our  
391 study, this likely means that the temperature downshift represented an opportunity for the NMJ  
392 to incorporate endogenous wild-type GluRIIA into a significant number of new PSDs while it  
393 continued to grow (Rasse et al., 2005; Schmid et al., 2008; Schmid et al., 2006). Given sufficient  
394 growth, the endogenously expressed GluRIIA would gradually overcome the previously incorpo-  
395 rated dominant-negative GluRIIA<sup>M/R</sup> subunits. As a result, this gradually restored neurotransmis-  
396 sion to normal levels over a time course of 24-48 hours (Fig. 3), during which time many of the  
397 dominant-negative GluRIIA<sup>M/R</sup> subunits that had previously been stably incorporated at the NMJ  
398 likely remained at the synapse.

399

#### 400 **Reversibility of rapid and sustained forms of homeostatic plasticity**

401 The majority of recent studies about synaptic homeostasis at the *Drosophila* NMJ have  
402 emphasized that presynaptic adjustments to neurotransmitter release properties must occur  
403 within minutes of drug-induced (PhTox) postsynaptic receptor inhibition in order to induce a rap-  
404 id and offsetting response to PhTox challenge. Important presynaptic parameters uncovered  
405 through these studies include regulation of presynaptic Ca<sup>2+</sup> influx (Frank et al., 2006; Frank et  
406 al., 2009; Müller and Davis, 2012; Wang et al., 2014; Wang et al., 2016a; Younger et al., 2013);  
407 regulation of the size of the readily releasable pool (RRP) of presynaptic vesicles (Harris et al.,  
408 2015; Müller et al., 2015; Müller et al., 2012; Wang et al., 2016a; Weyhersmüller et al., 2011);  
409 control of SNARE-mediated fusion events (Dickman and Davis, 2009; Dickman et al., 2012;  
410 Müller et al., 2011); control of neuronal excitability (Bergquist et al., 2010; Parrish et al., 2014;



411 Younger et al., 2013); and recently, ER calcium-sensing downstream of presynaptic calcium in-  
412 flux (Genç et al., 2017). For almost all of the cases in which a mutation or an experimental con-  
413 dition blocks the short-term induction of homeostatic signaling, the same perturbation has also  
414 proven to block its long-term maintenance. Interestingly, the converse is not true. Additional  
415 studies have amassed evidence that the long-term consolidation of homeostatic signaling at the  
416 NMJ can be genetically uncoupled from its induction, and select molecules seem to be dedicat-  
417 ed to a maintenance program that involves protein translation and signaling processes in both  
418 the neuron and the muscle (Brusich et al., 2015; Frank et al., 2009; Kauwe et al., 2016; Marie et  
419 al., 2010; Penney et al., 2012; Spring et al., 2016). These long-term processes seem to take six  
420 hours or more to take full effect (Kauwe et al., 2016).

421 As more molecular detail about HSP is elucidated, it will be interesting to test if the rapid  
422 induction and sustained consolidation of PHP can be reversed by similar or separate mecha-  
423 nisms – and what the time courses of those reversal mechanisms are. At the mouse NMJ, re-  
424 versibility was recently demonstrated pharmacologically. D-Tubocurarine was applied to at a  
425 sub-blocking concentration in order to impair postsynaptic acetylcholine receptors. Within se-  
426 conds of drug application, QC increased – and then within seconds of drug washout, it de-  
427 creased again (Wang et al., 2016b). Follow-up experiments suggested that those rapid, dynam-  
428 ic changes in PHP dynamics at the mouse NMJ were mediated by a calcium-dependent in-  
429 crease in the size of the readily-releasable pool (RRP) of presynaptic vesicles (Wang et al.,  
430 2016b). Since there seem to be several similarities between the mouse NMJ and the *Drosophila*  
431 NMJ (Davis and Müller, 2015; Frank, 2014), it is possible that PHP at the insect NMJ can also  
432 be rapidly reversed.

433 Finally, it is instructive to examine mammalian synaptic preparations to study how ho-  
434 meostatic forms of synaptic plasticity are turned on and off. Groundbreaking work on cultured  
435 excitatory vertebrate synapses showed that in response to activity deprivation (or promotion),  
436 synapses employ scaling mechanisms by adding (or subtracting) AMPA-type glutamate recep-

437 tors in order to counteract the perturbation (O'Brien et al., 1998; Turrigiano et al., 1998). Bi-  
438 directional scaling suggested that reversible mechanisms likely dictate homeostatic scaling pro-  
439 cesses. Complementary studies testing scaling reversibility have borne out this prediction  
440 (Rutherford et al., 1997; Swanwick et al., 2006; Wang et al., 2011). Additionally, evidence for  
441 reversible forms of homeostatic scaling have also been found in rodent sensory systems, such  
442 as auditory synapses after hearing deprivation (and restoration to reverse) (Whiting et al., 2009)  
443 and in the visual cortex after light deprivation (and restoration to reverse) (Goel and Lee, 2007).  
444 Collectively, the vertebrate and invertebrate studies support the notion that reversible fine-tuning  
445 is an efficient strategy used to stabilize activities in metazoan nervous systems. One advantage  
446 offered by the *Drosophila* system is a toolkit to uncover possible reversibility factors.

447

#### 448 **Homeostatic signaling can crash at extreme temperatures**

449 Are there environmental limitations for homeostatic potentiation at the *Drosophila* NMJ?  
450 Our data suggest that high temperatures represent a potential limitation on the system. It is not  
451 clear what the molecular or anatomical basis of this limit is. We do know that it is not an issue of  
452 NMJ excitation at high temperatures. This is because evoked neurotransmission for WT (or  
453 driver control) NMJs remains remarkably robust over a range of temperatures, including 30°C  
454 (Table 1). Nor does it seem to be an elimination of PHP in general because PHP was still pre-  
455 sent in the case of *GlurIIA*<sup>SP16</sup> animals raised at 30°C (Fig. 6, Table 1). Rather, the limitation  
456 seems to be on homeostatic signaling that supports PHP at high temperatures in the face of the  
457 dominant-negative transgene expression.

458 Temperature effects on neurophysiology are well documented. Recent work in crusta-  
459 ceans demonstrates that robust and reliable circuits like the neurons driving the rhythmicity  
460 stomatogastric nervous system can “crash” under extreme temperature challenges (Marder et  
461 al., 2015; Rinberg et al., 2013; Tang et al., 2012). For the *Drosophila* NMJ, prior studies of larval  
462 development documented a significant enhancement of synaptic arborization when larvae were

463 raised at high temperatures (Sigrist et al., 2003; Zhong and Wu, 2004). Additional studies have  
464 shown that NMJ growth plasticity can be additionally affected by mutations that affect neuronal  
465 excitability (Budnik et al., 1990; Lee and Wu, 2010; Zhong et al., 1992). Given the backdrop of  
466 these data, it is not unreasonable to hypothesize that the tolerable limits of synaptic activity  
467 challenge could be different at different temperatures.

468 For our experiments, 29-30°C represents a potential “crash” point for homeostatic poten-  
469 tiation at the *Drosophila* NMJ. We must note, however, that our data suggest that the coping  
470 capacity of the NMJ is dependent on genotype. WT NMJs cope at all temperatures. By contrast,  
471 for dominant-negative *GluRIIA*-expressing NMJs, 29°C is a point at which PHP becomes imper-  
472 fect (Figs. 1-2), and 30°C is a point at which it crashes (Fig. 6). For *GluRIIA*<sup>SP16</sup> subunit deletion  
473 NMJs, there is robust, but imperfect PHP at 30°C (Fig. 6) – not unlike the compensation seen  
474 for the dominant-negatives at 29°C. Why do these differences persist? The answer could relate  
475 to the well-documented temperature-induced alterations in NMJ growth – or alternatively, a lim-  
476 ited availability of synaptic factors that are needed in order to cope with a double challenge of  
477 high temperature and particular impairment glutamate receptor function. Future molecular and  
478 physiological work will be needed to unravel those possibilities in the contexts of different genet-  
479 ic backgrounds and culturing conditions.

480

## 481 **ACKNOWLEDGEMENTS**

482 We thank members of the Tootle, Lin, Wallrath, and Geyer labs for helpful discussions.  
483 Funding supporting this work includes a Whitehall Foundation Grant (2014-08-03), an NSF  
484 Grant (1557792), and an NIH/NINDS Grant (R01NS085164) to C.A.F. C.J.Y. was supported by  
485 a post-comprehensive pre-doctoral summer fellowship by the Graduate College at the Universi-  
486 ty of Iowa (UI) and a Ballard and Seashore Dissertation fellowship via the Neuroscience Gradu-  
487 ate Program at the UI.

488

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693 **FIGURE AND TABLE LEGENDS**

694

695 **Figure 1. Postsynaptic expression of *GluRIIA<sup>M/R</sup>* causes a decrease in mEPSP size and a**  
696 **homeostatic increase in quantal content. (A)** Electrophysiological profiles comparing *w<sup>1118</sup>*  
697 (WT) control NMJs to NMJs with postsynaptic expression of *UAS-GluRIIA<sup>M/R</sup>* (*w; UAS-*  
698 *GluRIIA<sup>M/R</sup>/+; MHC-Gal4/+*). Animals were reared at 25°C. mEPSP amplitude is markedly de-  
699 creased compared to WT (\*\**p* < 0.001). Quantal content (QC) is significantly increased for the  
700 dominant-negative NMJs (\*\**p* < 0.001), showing a homeostatic response that maintains EPSP  
701 amplitude at control levels. **(B)** The same genotypes were raised at 29°C. mEPSP amplitude is  
702 significantly decreased in the dominant-negative (\*\**p* < 0.001). EPSP size is also decreased in  
703 the dominant-negative compared to WT (\*\**p* < 0.001), with QC significantly increased (\*\**p* <  
704 0.001) but not enough to completely offset the decrease in quantal size. **(C)** Raw value compar-  
705 isons of the recordings normalized in (A) and (B). mEPSP amplitude, mEPSP frequency, EPSP  
706 amplitude and QC were compared for these four conditions: WT 25°C, WT 29°C, *w;*  
707 *GluRIIA<sup>M/R</sup>/+; MHC-GAL4/+* 25°C, and *w; GluRIIA<sup>M/R</sup>/+; MHC-GAL4/+* 29°C. In addition to the  
708 observations above, WT at 29°C had a decreased mEPSP amplitude (\* *p* < 0.05) and mEPSP  
709 frequency (\*\**p* < 0.001) compared to WT at 25°C. There was no difference in quantal content  
710 between the two WT conditions (*p* = 0.32). At 25°C, the dominant-negative showed a decrease  
711 in mEPSP frequency compared to WT at the same temperature (\*\**p* < 0.001). The dominant-  
712 negative at 29°C also showed a significant decrease in mEPSP frequency compared to WT at  
713 29°C (\*\**p* < 0.001). **(D)** Electrophysiological traces. Scale bars for EPSPs (and mEPSPs) are 5  
714 mV (1 mV) and 50 ms (1000 ms). All statistical comparisons done by one-way ANOVA with  
715 Tukey's post-hoc, collectively comparing the four total conditions.

716

717 **Figure 2. Animals expressing *GluRIIA<sup>M/R</sup>* show homeostatic compensation early in devel-**  
718 **opment. (A)** *w; GluRIIA<sup>M/R</sup>/+; MHC-GAL4/GAL80<sup>TS</sup>* animals were reared at 25°C or 29°C.

719 Compared to animals reared at 25°C, the animals reared at 29°C had a decrease in mEPSP  
720 amplitude (\*\* $p < 0.001$ , Student's T-test) and frequency ( $p < 0.001$ , see Table 1). EPSP ampli-  
721 tude was decreased (\*\*  $p < 0.01$ ), but there was a significant increase in QC (\*\* $p < 0.001$ ) **(B)**  
722 Representative electrophysiological traces. Scale bars for EPSPs (and mEPSPs) are 5 mV (1  
723 mV) and 50 ms (1000 ms).

724

725 **Figure 3. Presynaptic homeostatic potentiation is reversible. (A)** Diagram of a temperature  
726 swap paradigm. Mated animals were allowed to lay eggs for 6-8 hours. One set of animals was  
727 reared entirely at 21° from egg lay to electrophysiological recording. A second condition was  
728 raised entirely at 29°C. To test for reversibility of homeostatic potentiation, animals were reared  
729 initially at 29°C and then swapped to 21°C. Animals were allowed to recover for either 1, 2, or 3  
730 days before recording. **(B)** Expression of the dominant-negative transgene throughout life  
731 (29°C) causes a dramatic decrease in mEPSP frequency (\*\* $p < 0.001$ , one-way ANOVA with  
732 Tukey's post-hoc). Frequency remains low after 1 day of recovery at 21°C, compared 21°C rear-  
733 ing controls. By 2 days recovery, the frequency is significantly increased compared to 29°C (\*\*  
734  $p < 0.001$ ), but still significantly decreased compared to 21°C controls (\*\*  $p < 0.01$ ). By 3 days  
735 recovery, mEPSP frequency is no different from animals raised entirely at 21°C. **(C)** Normalized  
736 electrophysiological data for mEPSP amplitude, EPSP amplitude, and QC for the NMJs of ani-  
737 mals raised as described in (A). Longer recovery periods yield electrophysiology that more  
738 closely approximates the control 21°C rearing condition (\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$   
739 compared to 21°C control by one-way ANOVA with Tukey's post-hoc). **(D)** Representative elec-  
740 trophysiological traces for 21°C and 29°C. Scale bars refer to 5 mV and 50 ms for EPSPs and 1  
741 mV and 1000 ms for mEPSPs. **(E)** Traces for recovery conditions. Scale bars for EPSPs (and  
742 mEPSPs) are 5 mV (1 mV) and 50 ms (1000 ms) for both (D) and (E).

743

744 **Figure 4. Strong correlations between recovery time and classical homeostatic parameters** (A) There is a significant inverse correlation between mEPSP amplitude and QC for the  
745 swap experiments described in Figure 3 (Pearson's  $r = -0.68$ , \*\*\*  $p < 0.001$ ,  $n = 59$ ). (B) There is  
746 a significant positive correlation between the number of hours an animal has spent at 21°C and  
747 mEPSP amplitude. ( $r = 0.81$ ,  $p < 0.001$ ,  $n = 59$ ). Animals reared for “0 hours” at 21°C are the  
748 29°C condition, with animals in the 21°C condition being reared at that temperature for around  
749 120 hours before recording. (C) A significant inverse correlation between hours at 21°C and  
750 quantal content was found ( $r = -0.58$ , \*\*\*  $p < 0.001$ ,  $n = 59$ ). Animals with the highest quantal  
751 content were either at 21°C for 0 hours (29°C condition) or 24 hours (1-day recovery).  
752

753  
754 **Figure 5. Perfect homeostatic compensation is also reversible** (A) Dominant-negative ani-  
755 mals raised at 28.5°C show a significant decrease in mEPSP amplitude (\*  $p < 0.05$ , compared  
756 to 25°C condition; one-way ANOVA with Tukey's post-hoc), accompanied by a perfectly offset-  
757 ting increase in QC (\*\*  $p < 0.01$ ). Reversibility of homeostatic potentiation was demonstrated by  
758 initially rearing *w; GluRIIA<sup>M/R</sup>/+*; *BG57-GAL4/GAL80<sup>TS</sup>* animals at 28.5°C for 2 days and swap-  
759 ping them to 25°C for 2 days. mEPSP amplitude, EPSP amplitude, and quantal content returned  
760 to control (25°C throughout life) levels. EPSP amplitudes were virtually identical across all of the  
761 conditions. (B) Dominant-negative animals raised at 28.5°C show a significant decrease in  
762 mEPSP frequency (\* $p < 0.05$ , one-way ANOVA with Tukey's post-hoc). By contrast, the mEPSP  
763 frequency in animals after two days of recovery was not significantly different from that of the  
764 animals reared at 25°C. (C) Representative electrophysiological traces. Scale bars for EPSPs  
765 (and mEPSPs) are 5 mV (1 mV) and 50 ms (1000 ms).

766  
767 **Figure 6. Presynaptic homeostatic potentiation can be impaired at high temperatures** (A)  
768 *MHC-Gal4* was used to drive expression of the dominant-negative *GluRIIA<sup>M/R</sup>* subunit. Domi-  
769 nant-negative animals were compared at 30°C to driver controls, *MHC-GAL4* crossed to WT.

770 mEPSP amplitude was significantly decreased in the dominant-negative animals ( $*** p < 0.001$ ,  
771 Student's T-test). A dramatic decrease in EPSP amplitude also occurred ( $*** p < 0.001$ ) because  
772 of a significant decrease in QC in the dominant-negative animals ( $*** p < 0.001$ ). **(B)** Repre-  
773 sentative electrophysiological traces. Scale bars for EPSPs (and mEPSPs) are 5 mV (1 mV)  
774 and 50 ms (1000 ms). **(C)** *BG57-Gal4* was used to drive expression of the dominant-negative  
775 *GluRIIA<sup>M/R</sup>* subunit. Dominant-negative animals were compared at 30°C to driver controls,  
776 *BG57-Gal4* crossed to WT. mEPSP amplitude was significantly decreased for the dominant-  
777 negative NMJs ( $** p < 0.01$ , Student's T-test). A dramatic decrease in EPSP amplitude also oc-  
778 curred ( $*** p < 0.001$ ) because of a significant decrease in QC in the dominant-negative animals  
779 ( $*** p < 0.001$ ). **(D)** Representative electrophysiological traces. Scale bars as in (B). **(E)**  
780 *GluRIIA<sup>SP16</sup>* animals and WT controls were raised at 30°C. mEPSPs were significantly de-  
781 creased for *GluRIIA* mutants (Student's T-test,  $*** p < 0.001$ , Student's T-test) and QC was sig-  
782 nificantly increased ( $** p < 0.01$ ). EPSP values were also decreased ( $*** p < 0.001$ ) but not to  
783 the same degree as mEPSP amplitudes. **(F)** Representative electrophysiological traces. Scale  
784 bars as in (B).

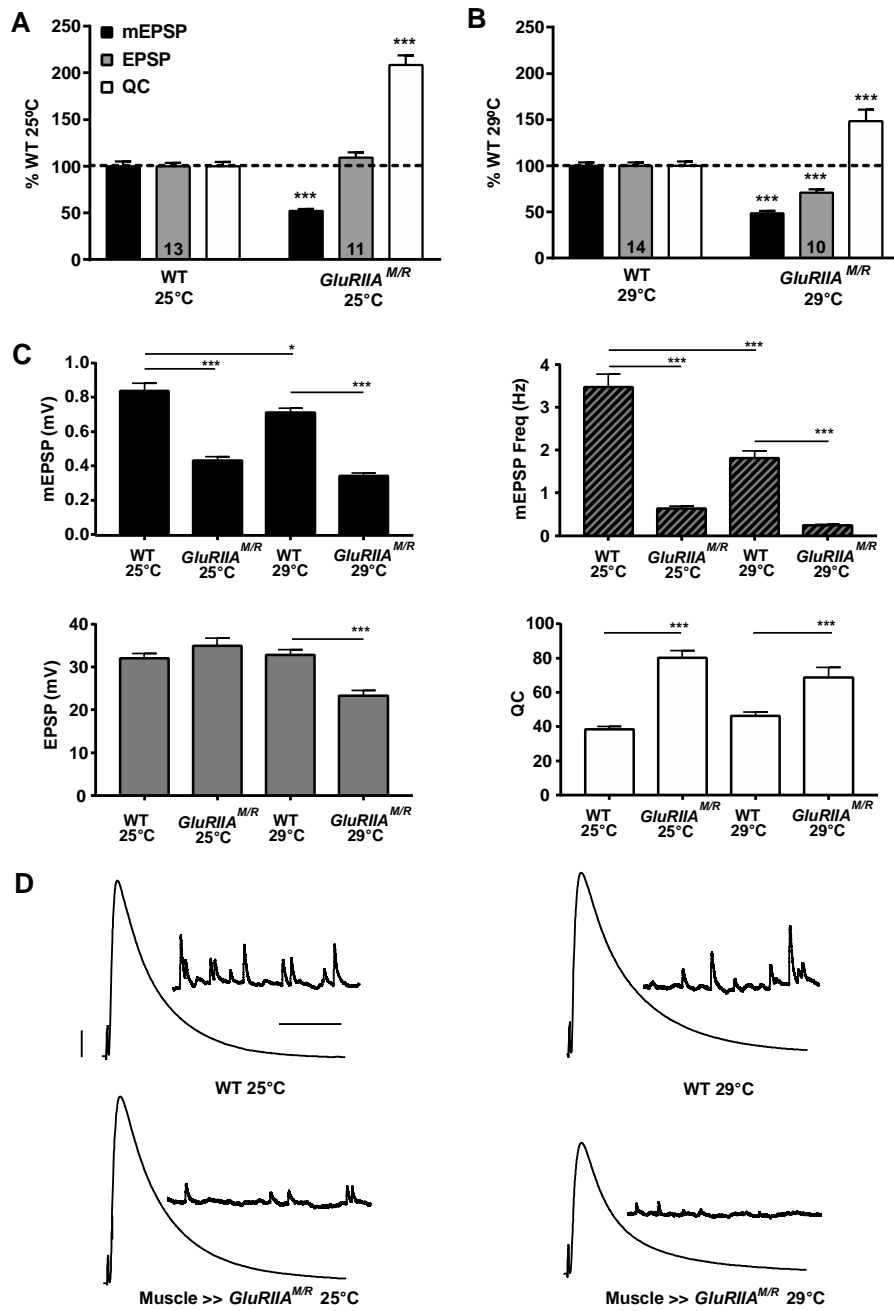
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786 **Table 1. Raw Electrophysiological Data.** Full genotypes and rearing conditions for electro-  
787 physiological data presented in the study. Average values  $\pm$  SEM are presented for each pa-  
788 rameter, with  $n$  = number of NMJs recorded. Values include miniature excitatory postsynaptic  
789 potential (mEPSP) amplitude, mEPSP frequency, excitatory postsynaptic potential (EPSP) am-  
790 plitude, quantal content (QC), muscle input resistance, and resting membrane potential.

791

792 **Figure 1**

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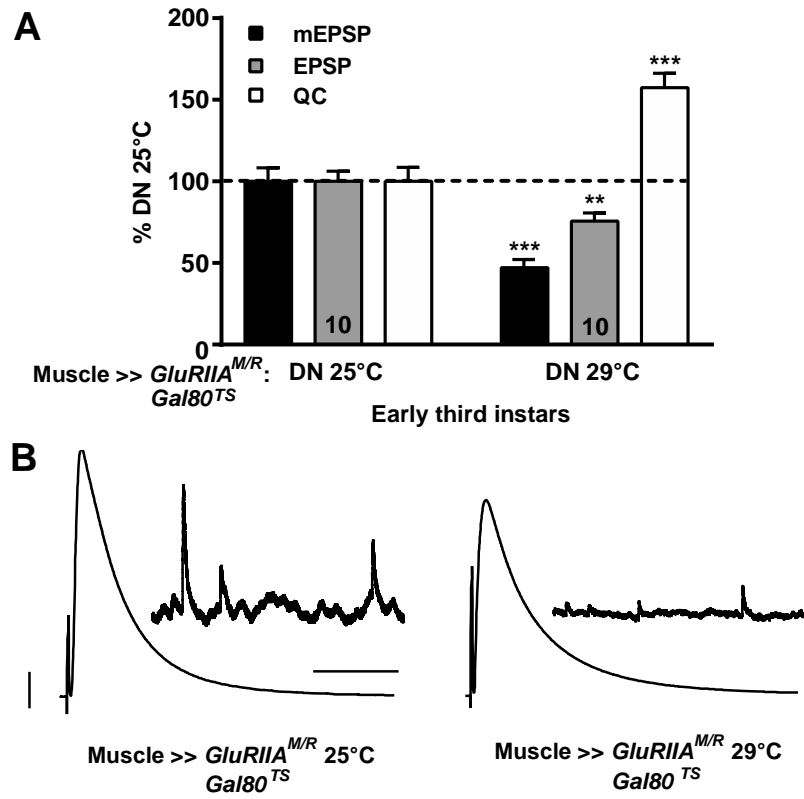
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797 **Figure 2**

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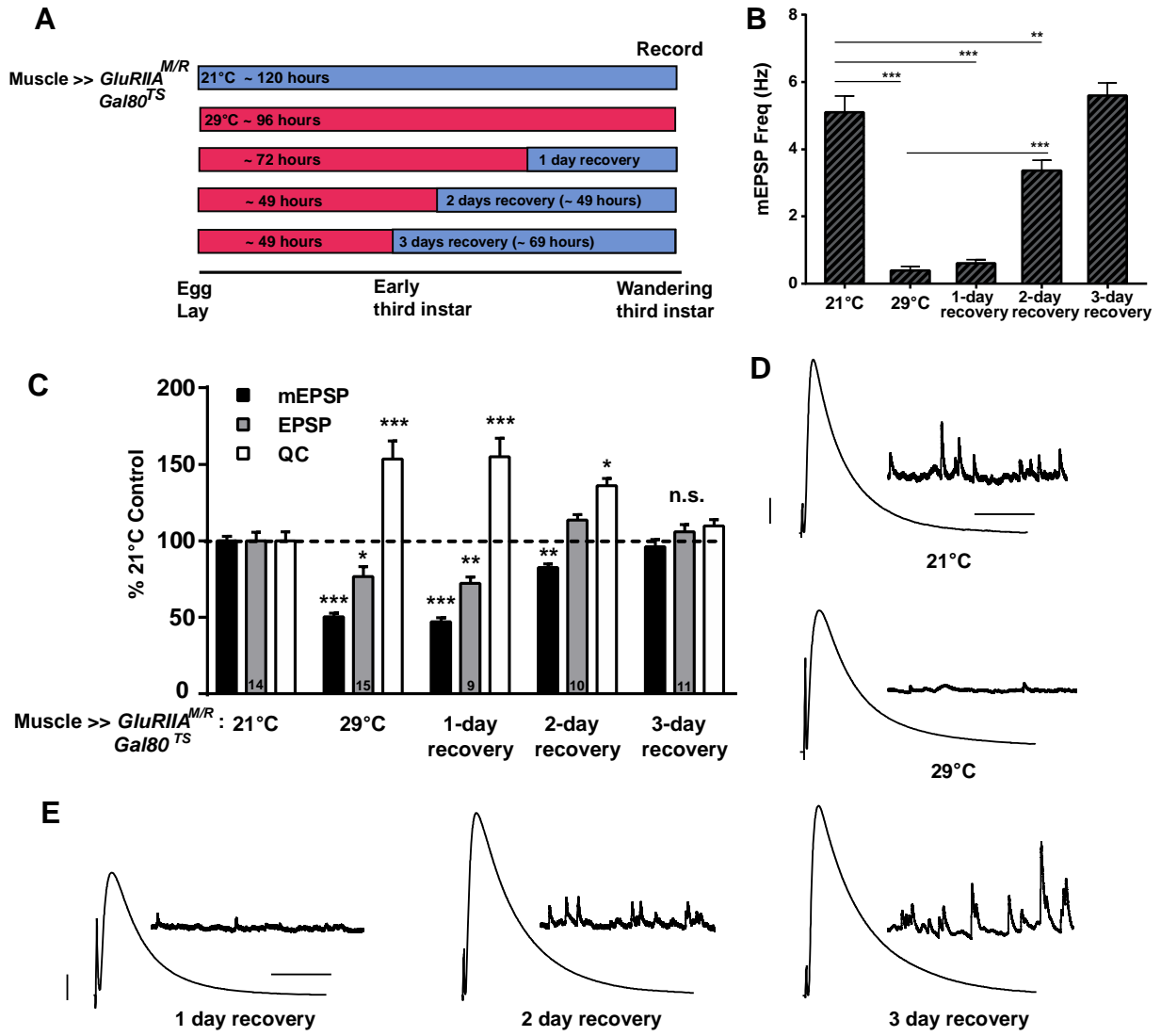


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801 **Figure 3**

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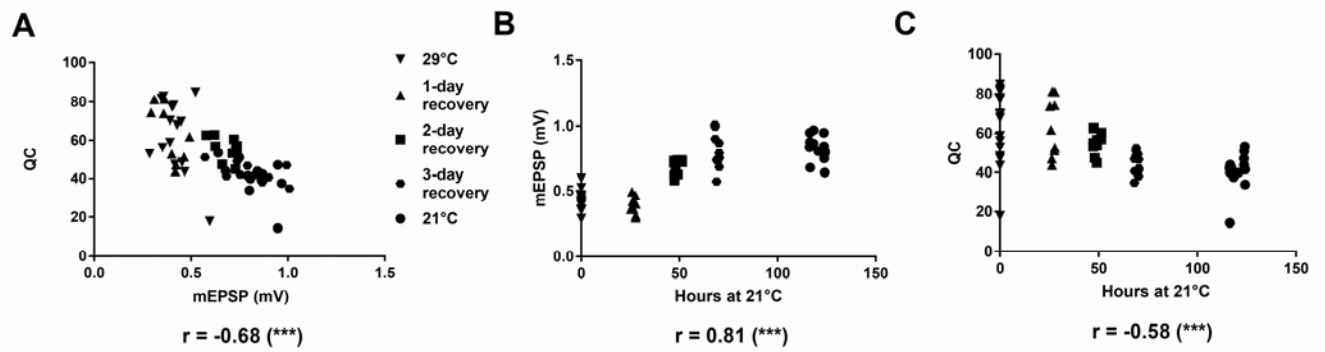
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807 **Figure 4**

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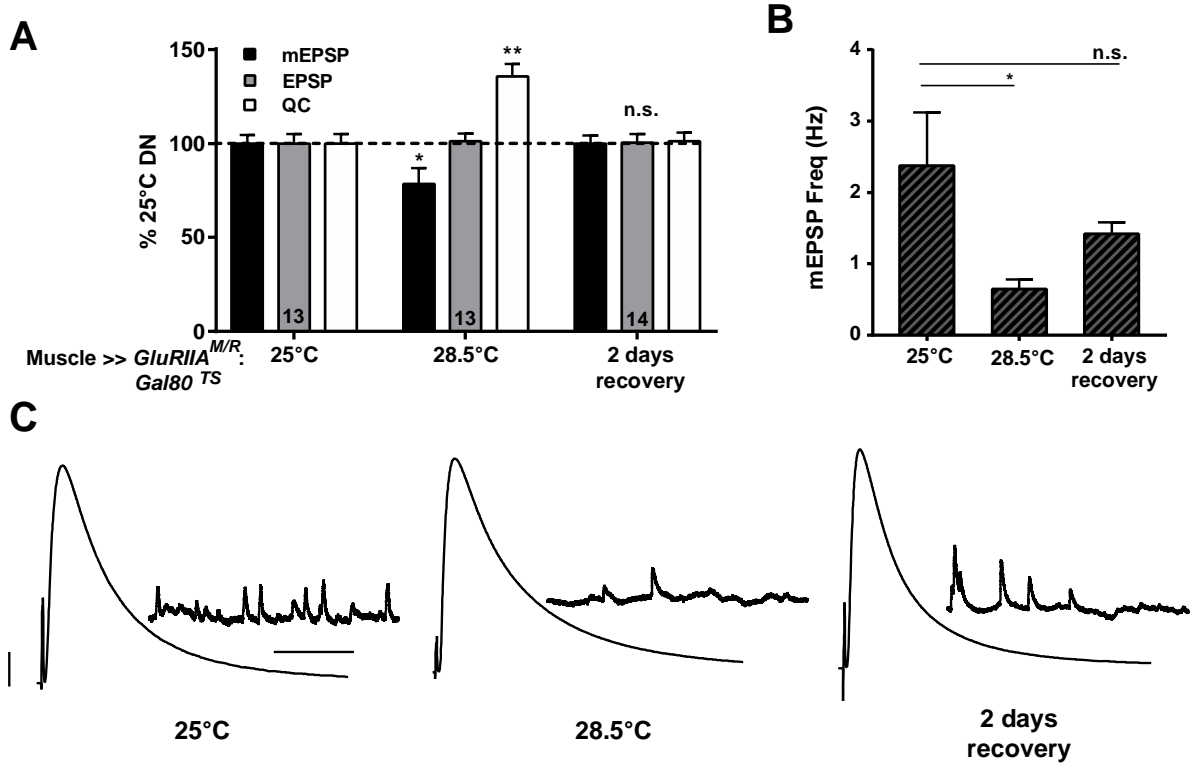
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812 **Figure 5**

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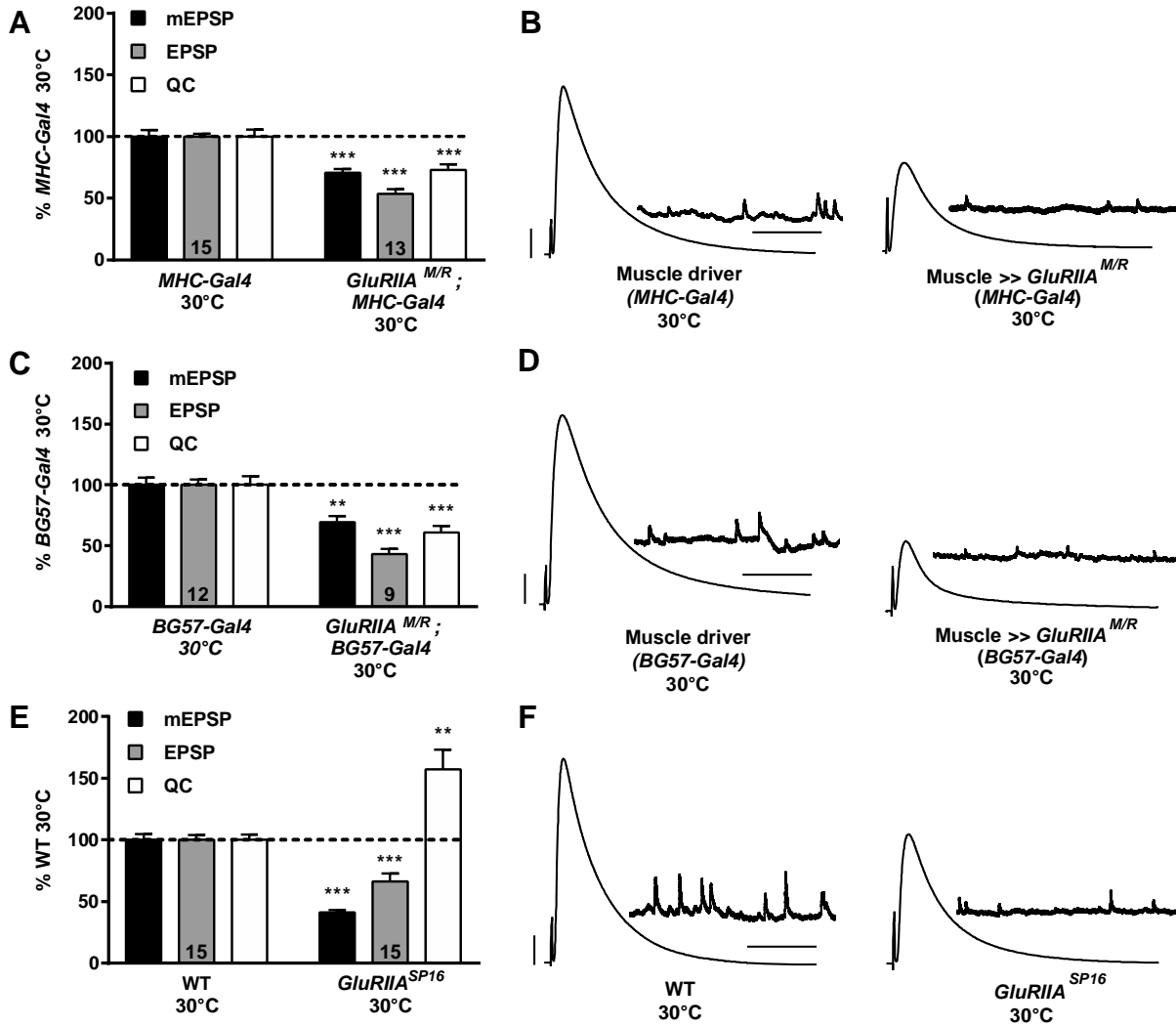
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817 **Figure 6**

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822 **Table 1**

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824

Genotype	Condition	mEPSP (mV)	mEPSP Freq (Hz)	EPSP (mV)	QC	I <sub>h</sub> (mV)	RMP (mV)	n	Figure
<i>W<sup>1118</sup></i> (wild-type)	25°C	0.85 ± 0.04	3.5 ± 0.3	32.0 ± 1.2	38.5 ± 1.7	6.4 ± 0.6	-64.6 ± 1.1	13	1
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/+</i>	25°C	0.44 ± 0.02	0.6 ± 0.1	35.0 ± 1.8	80.3 ± 4.1	5.8 ± 0.5	-69.1 ± 1.2	11	1
<i>W<sup>1118</sup></i>	29°C	0.72 ± 0.03	1.8 ± 0.2	32.9 ± 1.2	46.4 ± 2.2	6.2 ± 0.5	-64.4 ± 0.9	14	1
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/+</i>	29°C	0.35 ± 0.02	0.2 ± 0.0	23.3 ± 1.2	68.9 ± 5.7	5.2 ± 0.2	-65.0 ± 1.1	10	1
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	Early third instar 25°C	1.27 ± 0.1	2.0 ± 0.3	33.2 ± 2.1	27.4 ± 2.4	9.5 ± 0.8	-64.7 ± 2.0	10	2
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	Early third instar 29°C	0.60 ± 0.05	0.4 ± 0.1	25.1 ± 1.4	43.1 ± 2.6	13.6 ± 0.9	-63.0 ± 0.8	10	2
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	21°C	0.83 ± 0.03	5.1 ± 0.5	33.3 ± 1.9	40.7 ± 2.4	5.6 ± 0.4	-66.3 ± 0.8	14	3, 4
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	29°C	0.42 ± 0.02	0.4 ± 0.1	25.6 ± 2.1	62.5 ± 4.8	6.0 ± 0.3	-66.8 ± 1.1	15	3, 4
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	1-day recovery at 21°C	0.39 ± 0.02	0.6 ± 0.1	24.1 ± 1.4	63.1 ± 5.0	5.3 ± 0.3	-66.0 ± 1.1	9	3, 4
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	2-day recovery at 21°C	0.69 ± 0.02	3.4 ± 0.3	37.8 ± 1.2	55.4 ± 1.9	5.3 ± 0.3	-67.7 ± 0.7	10	3, 4
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/GAL80<sup>TS</sup></i>	3-day recovery at 21°C	0.80 ± 0.04	5.6 ± 0.4	35.3 ± 1.5	44.7 ± 1.7	5.5 ± 0.2	-68.4 ± 0.9	11	3, 4
<i>GU/RIIA<sup>W614R/+</sup>; BG57-GAL4/GAL80<sup>TS</sup></i>	25°C	0.79 ± 0.04	2.4 ± 0.7	31.1 ± 1.6	39.5 ± 1.9	5.3 ± 0.4	-68.4 ± 1.1	13	5
<i>GU/RIIA<sup>W614R/+</sup>; BG57-GAL4/GAL80<sup>TS</sup></i>	28.5°C	0.62 ± 0.05	0.6 ± 0.1	31.5 ± 1.3	53.6 ± 3.6	7.2 ± 0.4	-67.0 ± 1.3	13	5
<i>GU/RIIA<sup>W614R/+</sup>; BG57-GAL4/GAL80<sup>TS</sup></i>	2-day recovery at 25°C	0.79 ± 0.03	1.4 ± 0.2	31.3 ± 1.4	39.9 ± 1.8	6.3 ± 0.3	-66.7 ± 1.1	14	5
<i>W<sup>1118</sup></i>	30°C	0.78 ± 0.04	2.4 ± 0.2	33.1 ± 1.4	42.9 ± 1.9	6.8 ± 0.3	-66.2 ± 0.8	15	6
<i>GU/RIIA<sup>S<sup>716</sup></sup></i>	30°C	0.32 ± 0.02	0.8 ± 0.1	21.9 ± 2.2	67.4 ± 6.8	6.0 ± 0.4	-63.0 ± 0.5	15	6
<i>MHC-GAL4/+</i>	30°C	0.47 ± 0.02	1.1 ± 0.1	28.9 ± 0.6	64.3 ± 3.6	4.9 ± 0.2	-63.9 ± 0.9	15	6
<i>GU/RIIA<sup>W614R/+</sup>; MHC-GAL4/+</i>	30°C	0.33 ± 0.01	0.2 ± 0.0	15.4 ± 1.1	47.0 ± 2.8	5.3 ± 0.2	-64.4 ± 0.9	13	6
<i>BG57-GAL4/+</i>	30°C	0.51 ± 0.03	0.6 ± 0.1	31.3 ± 1.4	63.9 ± 4.5	6.6 ± 0.3	-70.2 ± 1.4	12	6
<i>GU/RIIA<sup>W614R/+</sup>; BG57-GAL4/+</i>	30°C	0.35 ± 0.03	0.8 ± 0.7	13.5 ± 1.3	39.1 ± 3.4	6.1 ± 0.5	-65.5 ± 1.6	9	6