Optogenetics in the teaching laboratory: using Channelrhodopsin2 to study the neural basis of behavior and synaptic physiology in *Drosophila*

Stefan R. Pulver[1]* Nicholas J. Hornstein[2], Bruce L. Land[3,4], Bruce R. Johnson[3]

[1]Department of Zoology, University of Cambridge, Cambridge, UK CBQ 3EJ
[2]Department of Biology, Brandeis University, Waltham, MA 02454
[3]Department of Neurobiology and Behavior, Cornell University, Ithaca, NY 14850
[4]Department of Electrical and Computer Engineering, Cornell University, Ithaca, NY 14850
*Corresponding author

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32 **ABSTRACT**

33 Here we incorporate recent advances in *Drosophila* neurogenetics and
34 'optogenetics' into neuroscience laboratory exercises. We used the light
35 activated ion channel, Channelrhodopsin2 (ChR2) and tissue specific genetic
36 expression techniques to study the neural basis of behavior in *Drosophila* larvae.
37 We designed exercises using inexpensive, easy-to-use systems for delivering
38 blue light pulses with fine temporal control. Students first examine the behavioral
39 effects of activating glutamatergic neurons in *Drosophila* larvae, and then record
40 excitatory junctional potentials (EJPs) mediated by ChR2 activation at the larval
41 neuromuscular junction (NMJ). Comparison of electrically- and light-evoked EJPs
42 demonstrate that the amplitudes and time courses of light-evoked EJPs are not
43 significantly different from those generated by electrical nerve stimulation. These
44 exercises introduce students to new genetic technology for remotely
45 manipulating neural activity, and they simplify the process of recording EJPs at
46 the *Drosophila* larval NMJ. Relatively little research work has been done using
47 ChR2 in *Drosophila*, so students have opportunities to test novel hypotheses and
48 make tangible contributions to the scientific record. Qualitative and quantitative
49 assessment of student experiences suggest that these exercises help convey
50 principles of synaptic transmission while also promoting integrative and inquiry
51 based studies of genetics, cellular physiology and animal behavior.

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62 **INTRODUCTION**

63 *Drosophila* neurogeneticists have developed an impressive array of tools
64 for studying the neural basis of animal behavior. In recent years, tissue specific
65 genetic expression systems, particularly GAL4-UAS (3) have been used to
66 ectopically express transgenes that allow for acute, reversible manipulation of
67 neural activity. These new techniques exploit ion channels and vesicle trafficking
68 proteins that are gated by light and temperature (1, 9, 15, 25, 28). This allows
69 researchers to remotely control neural activity in selected cells simply by raising
70 the ambient temperature or shining light on behaving flies.

71 One powerful new tool for acutely activating neurons is the light gated ion
72 channel, Channelrhodopsin-2 (ChR2). Originally isolated from the green algae,
73 *Chlamydomonas reinhardti*, the channel is directly activated by blue light (24).
74 When expressed in neurons, channel opening causes depolarization through a
75 non-specific cation conductance (2, 23) which leads to action potential
76 generation. This technique has been used to depolarize excitable cells in
77 invertebrate (22, 24, 27) and vertebrate (2, 5, 8, 24) preparations for research
78 purposes.

79 'Optogenetic' methods for activating neurons offer attractive options for
80 physiology educators. With the range of genetic tools available in *Drosophila*,
81 teachers can potentially design exercises that explore the neural basis of animal
82 behavior in ways that are not possible in traditional laboratory preparations.
83 These new tools can also be used to make technically difficult preparations more
84 accessible to students. Our goal here is to outline one potential use of *Drosophila*
85 neurogenetics and ChR2 in neuroscience education. Specifically, we show how
86 to use ChR2 to 1) promote quantitative analysis of animal behavior, 2) teach
87 principles of synaptic transmission, and 3) help students learn how to formulate
88 and test their own research hypotheses.

89 Previous work has proposed using the *Drosophila* larval neuromuscular
90 junction (NMJ) to teach students synaptic physiology (16, 33). This glutamatergic
91 synapse yields large excitatory junctional potentials (EJPs) that can be recorded
92 with basic electrophysiology equipment (13,14). However, to successfully record

93 EJPs, students must precisely maneuver both an intracellular electrode and a
94 stimulating (suction) electrode in a very small area. Here, we present inexpensive
95 laboratory exercises that use targeted expression of ChR2 in motor neurons,
96 instead of direct electrical nerve stimulation to activate larval NMJs. Students are
97 exposed to newly developed *Drosophila* neurogenetic tools and learn synaptic
98 neurophysiology. We report feedback on the exercises from two student cohorts
99 across two different years in a neurophysiology laboratory course at Cornell
100 University. Overall, this work and a companion publication (1) lay the foundation
101 for wider use of *Drosophila* neurogenetics in teaching principles of neurobiology
102 and animal behavior.
103
104 **MATERIALS AND METHODS**
105 **Fly lines and animal care**
106 We used a GAL4 driver (OK371-GAL4) that drives expression exclusively
107 in glutamatergic neurons (22) and a UAS construct (UAS-H134R-ChR2-mcherry)
108 from a previous larval locomotion study (25). Virgin OK371-GAL4 females were
109 crossed to UAS-H134R-ChR2-mcherry males. The resulting larvae were grown
110 in darkness at 23-25°C on standard fly media containing 1 mM all-*trans* retinal
111 (ATR, Toronto Research Chemicals, North York, CA). ATR is a co-factor allowing
112 proper folding and membrane insertion of ChR2. Supplementing fly food with
113 ATR is essential for functional ChR2 expression. We previously described the
114 preparation of ATR-containing fly food in video form (10).
115 All fly lines are freely available from S.R. Pulver at the University of
116 Cambridge and/or the Bloomington *Drosophila* Stock Center (OK371-GAL4:
117 http://flybase.org/reports/FBst0026160.html; UAS-H134R-ChR2-mcherry:
118 http://flybase.org/reports/FBst0028995.html). Detailed guidlelines on rearing fruit
119 flies and making genetic crosses are available in previous publications (10, 16).
120
121 **Blue LED control system**
122 Commercially available systems for controlling blue light emitting diodes
123 (LEDs) typically cost >US $300. This could be prohibitively expensive for many

124 teaching laboratories, so we designed two simple, inexpensive alternatives. First
125 we connected an ultrabright blue LED (Luxeon V star, LED Supply, Randolph,
126 VT) to a 700 mA 'BuckPuck' power converter (LuxDrive 3021, LED Supply).
127 When the BuckPuck is directly connected to the analog output from an analog-to-
128 digital converter, light intensity and duration can be controlled with 0-5 V pulses
129 from an external voltage source (10, 25). We attached a small heat sink to each
130 LED (e.g. TO220, Radio Shack) to dissipate heat. To ensure good heat transfer,
131 we placed thermal paste in between the LED and heat sink and glued only the
132 edges of the LED to the bare metal of the heat sink. Total cost of all components
133 is under US$50. A basic wiring plan for this LED controller is shown in Figure 1A.
134 A typical controller is shown in Figure 1C, and an LED mounted on a heat sink is
135 shown in Figure 1D. We controlled timing and light intensity with two commonly
136 available analog-to-digital conversion systems. For demonstration here, we
137 delivered 0-5 V pulses through a Powerlab 4/30 (AD Instruments, Colorado
138 Springs) with Chart 5 data acquisition software (AD Instuments). In the teaching
139 exercises reported below, students controlled the LED through the analog output
140 of a NIDAQ BNC-2110 A-D board (National Instruments, Austin, TX) with the free
141 data acquisition software 'g-PRIME' (21). Both systems were able to control
142 timing and intensity equally well.
143 As an alternative to the above, we also designed a second simple control
144 circuit that can be driven by analog pulse stimulators with low current output.
145 Figure 1B shows a wiring plan for this type of control system. A 74HC04 hex
146 inverter and a 5 KΩ resistor are used to ensure that a standard TTL signal will
147 trigger light pulses. An input protection circuit consisting of two 1N914 diodes
148 protects the hex inverter from a reversed connection and/or electrostatic
149 discharge. The primary advantage of this control circuit is that it does not require
150 analog-to-digital converters and/or data acquisition software. Total cost of all
151 components is under US$70.
152 Unfocused LEDs are not able to deliver the light intensities needed to
153 activate ChR2 in fly neurons. To focus LEDs, we placed a Carl Zeiss 10X
154 dissecting scope eyepiece in front of the LED, and mounted both the light source

155 and eyepiece on magnetized bases suitable for electrophysiology 'rig' tables. The
156 make and model of eyepiece is not critical; any removable eyepiece that can
157 cover the LED is suitable. An LED and heat sink coupled to an eyepiece and
158 attached to a magnetized base is shown in Figure 1E. The complete LED setup
159 on a working electrophysiology rig is shown in Figure 1F. Additional views of LED
160 system components are shown in video form in (10). It is important to note that
161 the light emerging from the LED system outlined here is high intensity and very
162 focused, so it is imperative that students do not look directly into active LEDs.
163
164 **Larval behavior**
165 Animals with ChR2 in motor neurons (OK371-GAL4 / UAS-H134R-ChR2)
166 were grown in two batches: one group was raised on normal fly food, the other
167 on food containing 1mM ATR. We selected 3rd instar individuals from each group
168 and observed behavioral responses to blue light pulses. For demonstration,
169 larval behavior was filmed with a Leica DFC 420 C camera mounted on a Leica
170 MZ16 F Fluorescence Stereomicroscope (Leica Camera AG, Solms, Germany).
171 Blue light pulses (5 s) were delivered by manual control of shutter timing.
172 In classroom exercises, students placed larvae in dissection dishes and
173 delivered light pulses using a mounted LED. Students observed larval responses
174 to blue light and scored responses manually. We did not require students to
175 analyze larval behavior in any particular way. Instead, we encouraged students
176 to devise their own methods for quantifying the effects of blue light stimulation on
177 larval behavior in experimental and control animals
178
179 **Larval dissection**
180 For NMJ electrophysiology, third instar larvae were dissected in a clear
181 Sylgard (Dow Corning, Midland, MI) lined dish containing chilled 'HL3.1'
182 physiological saline (6). HL3.1 consisted of (in mM): NaCl 70, KCl 5, $CaCl_2$ 0.8,
183 $MgCl_2$ 4, $NaHC0_3$ 10, trehalose 5, sucrose 115, HEPES 5, pH 7.15. In this saline,
184 preparations typically remained viable for 1-2 hours at room temperature.

185 Each larva was positioned dorsal side up and 0.1 mm insect pins were
186 placed in the head and tail. Using a pair of microscissors, we made a shallow
187 incision from the posterior pin to the anterior pin. After making the initial cut, we
188 placed one pin into each corner of the animal's body wall and stretched each
189 corner taut. Next, we removed fat bodies and digestive organs, exposing the
190 anterior brain lobes, ventral ganglion, segmental nerves and body wall muscles.
191 In some experiments we removed the central nervous system (CNS), leaving
192 only motor axons and nerve terminals. In other preparations, we dissected away
193 the brain lobes and cut the posterior-most nerves leaving the ventral ganglion. In
194 tightly pinned preparations, this reduces locomotor rhythms, but leaves motor
195 neuron cell bodies, axons and nerve terminals intact (25). See (10) for videos
196 describing the larval dissection.
197

198 **Intracellular recording**

199 Dishes with dissected preparations were first fixed to a plexiglass stage
200 with artist's clay and viewed through a dissecting microscope on a standard
201 electrophysiology 'rig'. We targeted larval muscle 6 (m6; see Fig. 3A) for all
202 intracellular recordings. Recordings were made with sharp glass electrodes (10-
203 20 MΩ, filled with 3M KCl).
204 For the demonstration electrophysiology data presented here, the
205 electrode and headstage were maneuvered with a MP285 micromanipulator
206 (Sutter Instruments, Novato, CA). Voltage signals were amplified with a
207 Neuroprobe amplifier (A-M systems, Sequim, WA). Data were digitized using a
208 Power lab 4/30 and recorded in Chart 5 (ADinstruments, Colorado Springs, CO).
209 Data were analyzed in Spike2 (Cambridge Electronic Design, Cambridge, UK)
210 using custom made analysis scripts (www.whitney.ufl.edu/BucherLab). EJPs
211 were evoked in ChR2 expressing animals with 1, 2.5, 5, and 10 ms pulses (25) to
212 examine the effects of light pulse duration on light-evoked EJPs (lEJPs). We
213 also compared lEJPs to electrically evoked EJPs (eEJPs) by attaching a suction
214 electrode to segmental nerves and delivering 1 ms duration electrical shocks with
215 a model 2100 isolated pulse stimulator (A-M Systems) (see Figure 3A).

216 In teaching exercises, students used Narishige MM-333
217 micromanipulators (Narishige, East Meadow, NY) to maneuver recording
218 electrodes. These micromanipulators offer enough precision to record from larval
219 NMJs, and are substantially less expensive than other research-grade
220 manipulators. Students also used Neuroprobe amplifiers to amplify voltage
221 signals, but used g-PRIME for LED control, data acquisition and analysis (21).
222 The quality of data recorded with teaching lab equipment was equivalent to the
223 demonstration data we present here. In teaching laboratory exercises, students
224 began by giving light pulse durations (10 ms) and intensities (5V into control
225 circuit, ~1 mW / mm^2) that reliably evoked at least 1 IEJP with pulse stimulation in
226 previous work (25). Students were encouraged to design their own experiments
227 and explore the effects of varying intensity, duration and frequency of light pulses
228 on synaptic transmission.
229
230 **Analysis of student evaluations**
231 We test ran these exercises with two different student cohorts in two
232 successive years (Spring semesters, 2009 and 2010) of an undergraduate
233 neurophysiology course (BIONB/BME 4910) at Cornell University. The 2009
234 students completed the exercise in 1 laboratory session; they were
235 undergraduate students from Biology (11), Biological Engineering (2), and one
236 each from Psychology, Mathematic and Human Ecology majors, and first year
237 graduate students from Neurobiology and Behavior (6), and Biomedical (2) and
238 Electrical/Computer (2) Engineering. In 2010 we spread the exercise over two
239 weeks; undergraduate students were from Biology (11), and one each from
240 Biology and Society, Psychology, Biological and Electrical/Computer Engineering
241 majors, and first year graduate students from Neurobiology and Behavior (4)
242 Biomedical Engineering (6) and one each from Electrical/Computer Engineering,
243 Entomology and Psychology. Students worked in groups of 2 or 3 at each
244 physiology rig. Their background in neuroscience ranged from very little (the
245 Engineering students) to a sophomore level class in Neuroscience (biology
246 students), which used the Purves et al. (26) textbook. Student experiences were

247 evaluated qualitatively in 2009; we asked for a 1 page informal opinion on the
248 exercise from each student. In the second year, we quantified student
249 experiences by asking them 12 questions designed to evaluate various technical
250 and conceptual aspects of the exercise. Student responses were measured on a
251 Likert scale (19). All students had previous electrophysiological experience
252 earlier in the semester with exercises from the Crawdad CD (32), including
253 recording synaptic potentials from the crayfish NMJ. NJH and SRP presented
254 background lectures on fly genetics and *Drosophila* NMJ electrophysiology prior
255 to students starting the lab exercises.
256
257 **RESULTS**
258 **Behavioral responses to blue light**
259 Previous work has demonstrated that larval locomotion is inhibited when
260 motor neurons are depolarized with ChR2 activation to fire action potentials (25).
261 To assess whether these effects are robust enough for use in teaching
262 laboratories, we expressed ChR2 in motor neurons (Fig. 2A), then filmed
263 behavioral responses to blue light. OK371-GAL4 x UAS-H134R-ChR2 animals
264 raised on normal fly food were not affected by blue light pulses (Fig. 2B, left and
265 right; Fig. 2D; n = 10). In contrast, genetically identical animals reared on food
266 containing ATR showed immediate, obvious responses to blue light. In ambient
267 light or green light, these larvae usually crawled normally, showing well-
268 coordinated posterior to anterior peristaltic waves of muscle contractions (Fig.
269 2C, left; Supplemental movie 1). In blue light, all body segments contracted at
270 once and peristaltic waves stopped (Fig. 2C, right; Supplemental movie 1). 100%
271 of all animals raised on ATR food showed immediate, strong contraction of all
272 body segments (Fig. 2D, left). Over 90% of these animals were completely
273 paralyzed for the duration of a 5 s light pulse (Fig. 2D, right; n = 12). Paralyzed
274 animals recovered within 5 s following a 5 s light pulse (Supplemental movie 1).
275 In demonstration experiments (shown here), we delivered blue light pulses
276 through a dissecting microscope equipped for fluorescence microscopy. In

277 classroom exercises, we obtained similar results using the LED control system
278 described above.
279 Each student group was encouraged to devise their own methods for
280 measuring ChR2-mediated behavioral effects. One example of a student
281 conceived analysis is shown in Table 1. This student group compared crawling
282 behavior in control and ChR2 expressing animals under ambient and blue light.
283 They measured the frequency of forward peristaltic waves by counting number of
284 waves in a 30 s trial. They also estimated total distance traveled by placing a grid
285 of 1cm x 1cm squares beneath each larva, and measuring the number of
286 squares traveled during the same 30 s trial. Under ambient light, both genotypes
287 showed similar crawling parameters. In the presence of rhythmic blue light pulses
288 (1 sec duration, 0.5 Hz cycle period), controls continued to crawl, whereas
289 animals expressing ChR2 showed no forward peristalsis. Consistent with
290 previous work, behavioral effects were strong at first, but gradually wore off after
291 20-30 s under constant illumination (Data not shown; 25). Several student groups
292 noted that high intensity white light could also elicit behavioral responses in
293 ChR2 expressing animals. Students were therefore encouraged to minimize the
294 intensity of dissection scope lamps during experiments.
295

296 **LIght-evoked EJPs at the larval NMJ**

297 Previous work has shown that the LED system presented here can reliably
298 generate single lEJPs at the larval NMJ (10, 25). We asked students to first apply
299 light pulses of varying durations to the larval preparation (Fig. 3A) and record
300 lEJPS to ensure that they had a working preparation (demonstration examples in
301 Fig. 3C). Next we encouraged them to formulate and investigate their own
302 research questions. Several groups chose to examine how these lEJPs
303 compared to eEJPs at the larval NMJ. They easily recorded lEJPs, but had
304 difficulty successfully stimulating motor nerves to record eEJPs. For
305 demonstration purposes, we repeated this experiment. In the preparation shown
306 in Figure 3A, the central nervous system (CNS) was removed and a suction
307 electrode was placed on a single segmental nerve. Nerve shocks (1 ms) reliably

308 evoked single eEJPs. Consistent with previous work, as stimulus intensity
309 increased, a second motor unit innervating muscle m6 was recruited, leading to a
310 stepwise increase in eEJP amplitude (Fig. 3B). One ms blue light pulses failed to
311 evoked lEJPs in 7 out of 7 preparations, but 2.5, 5, and 10 ms light pulses
312 evoked lEJPs in most preparations (2.5 ms: 5 / 7; 5 ms: 7 / 7; 10 ms: 7 / 7
313 experiments). lEJP and low threshold eEJP amplitudes and time courses were
314 not significantly different (Fig. 3B-F; $p > 0.05$, One way ANOVA with Tukey-
315 Kramer post-hoc test).

316 In previous work, lEJPs have been measured in preparations in which
317 motor neuron cell bodies were present and ventral ganglion circuitry was intact
318 (25). Several student groups chose to study lEJPs in this type of preparation (a
319 schematic is shown in Fig. 4A). In demonstration experiments, 1 ms electrical
320 pulses recruited both motor units with amplitudes and time courses similar to
321 those seen in reduced nerve-muscle preparations (data not shown). With the
322 ventral ganglion intact, we reliably evoked single low threshold lEJPs with light
323 pulse durations as short as 1 ms (Fig. 4B). Longer light pulses evoked
324 summating trains of EJPs (Figure 4B,C). Increasing light pulse duration did not
325 affect the amplitudes of leading eEJPs (Fig. 4D).

326 In several preparations with intact ventral ganglia, (3 / 7), short light pulses
327 evoked a single lEJP, followed by a long (1 - 5 s) train of spontaneously
328 generated EJPs (Fig. 4E). In these experiments, lEJPs were similar in amplitude
329 and time course to spontaneous EJPs (Figure 4F). Trains of spontaneously
330 generated EJPs were not seen in preparations in which the ventral ganglion had
331 been removed. In classroom experiments, several groups noted that in
332 preparations with intact ventral ganglia, high intensity white light pulses from
333 dissection lamps could trigger trains of lEJPs.

334 An example of data collected during a student initiated classroom project
335 is shown in Figure 5. This particular group recorded lEJPs in response to paired
336 pulses of blue light (Fig. 5A). They then calculated facilitation ratios (EJP_2
337 amplitude / EJP_1 amplitude) at various stimulation intervals (Fig. 5B) to compare
338 with published descriptions of short-term plasticity at the larval NMJ. The

339 students used off-line analysis tools in G-Prime to compensate for summation at
340 short stimulus intervals. Specifically, they fit an exponential curve to the
341 repolarizing phase of leading EJPs and used that as a baseline to estimate
342 trailing EJP amplitudes. This allowed them to accurately estimate facilitation
343 ratios even at stimulus intervals where summation dominated in the synaptic
344 responses. The students' results suggest the presence of short-term facilitation
345 at stimulus intervals <1 s.
346
347 **Student evaluations**
348 In the first year qualitative evaluation, student reviews of the exercises were
349 generally favorable. The students were excited to be working with a novel
350 research preparation, they enjoyed the integration of behavior and physiology,
351 and they seemed to be inspired by the idea of using genetics to remotely control
352 neural activity. From a practical point of view, students liked being able to see
353 light-evoked muscle contractions in dissected preparations; it helped them target
354 healthy muscle cells for intracellular recording. In the first year, students
355 complained that 1) the LED control system was not 100% reliable, 2) 1 week was
356 too short to complete the exercise, and 3) there was not enough time allocated
357 for exploring their own research questions.
358 Before running the exercises in year two, we corrected problems with the
359 LED control system and allocated a second week for student exploration. After
360 the exercises, we quantitatively evaluated student reactions. Figure 6 shows
361 student responses (n = 21) to 6 questions designed to rank technical features of
362 the exercises. While some students had difficulty clearly seeing muscle fibers for
363 electrode penetration (Fig. 6D), on the whole, students were satisfied with the
364 technical features of the exercises (Fig. 6 A, C, E). The students also liked
365 starting the lab with behavioral analysis (Fig. 6B), and appeared to understand
366 and be excited about what they were doing (Fig. 6F). Figure 7 shows student
367 responses to an additional 6 questions aimed at evaluating how effective these
368 exercises were at conveying biological concepts and promoting interest in
369 biological research. Students indicated that these exercises helped them

370 understand principles of synaptic transmission (Fig. 7A) while also stimulating
371 interest in studying neural mechanisms of behavior and genetics (Figs. 7B, C).
372 Students were extremely excited about using new optogenetic technology and
373 doing experiments that have not yet been done by researchers (Fig. 7D). Overall,
374 the exercises helped students learn how to implement the scientific method and
375 heightened student interest in pursuing careers as research scientists (Fig. 7E,
376 F).
377
378 **DISCUSSION**
379 **Behavior experiments**
380 In a teaching exercise, it is important that any behavior phenotypes being
381 studied are robust. We reasoned that activating glutamatergic neurons with
382 ChR2 might produce phenotypes appropriate for teaching labs. Glutamate is the
383 primary neurotransmitter at neuromuscular junctions in *Drosophila* (13, 14).
384 Demonstration and student data (Fig. 2; Table 1) show clearly that despite
385 longer-term adaptation (25), activation of glutamatergic neurons with ChR2 leads
386 to an immediate and dramatic decrease in larval locomotion. Quantification of
387 student feedback suggests that it was instructive to start the exercise by
388 examining ChR2 mediated behavioral responses (Fig. 5B), thus providing a
389 behavioral context for the following physiology. This is probably because the
390 behavior responses are so unambiguous; they produce immediate positive
391 reinforcement for students early on in the exercise.
392 Activating glutamatergic neurons provides a reliable and easily
393 interpretable phenotype (motor neuron activation = muscle contraction = tetanic
394 paralysis). However, these experiments also provide a solid jumping off point for
395 additional behavioral studies aimed at analysis of other ensembles of fly neurons.
396 With the genetic tools currently available in *Drosophila*, students can remotely
397 stimulate a variety of transmitter systems and neuronal subpopulations. For
398 example, GAL4 drivers currently exist for labeling various aminergic systems
399 (28); peptidergic cells, (31) and cholinergic neurons (27). Other drivers target the
400 peripheral nervous system and identified sensory cells (11, 30). To date, the

401 functions of some identified neuronal populations have been examined with
402 ChR2 (12, 25, 28, 29, 34), but a large and ever growing number of GAL4 lines
403 (and by extension, hypotheses) remain to be tested.
404
405 **Electrophysiology experiments**
406 Consistent with previous work (25), in demonstration experiments, we
407 reliably evoked lEJPs in reduced preparations that consisted only of motor
408 axons, nerve terminals and muscles with stimulus durations of 2.5-10 ms. When
409 evoking EJPs with electrical stimulation, researchers typically use 100 μs to 1 ms
410 duration stimuli (14, 33). Critically, the lEJPs recorded with longer stimulation
411 times were essentially identical to those evoked by 1 ms electrical stimulation of
412 a single low threshold motor unit innervating m6 (most likely the 'RP3' motor
413 neuron, 18, 20). Furthermore, increasing light pulse duration did not affect single
414 lEJP parameters. These results suggest that EJPs resulting from ChR2 initiated
415 action potentials (APs) are not essentially different from EJPs evoked by
416 traditional nerve stimulation. There was only one obvious difference between the
417 two methods of evoking EJPs: using ChR2, we were not able to recruit both
418 motor units innervating m6. One possible explanation for this result is simply that
419 our LED system cannot generate high enough intensity blue light to trigger an AP
420 in the motor unit with the higher threshold. A second possibility is that ChR2
421 expression in the two motor neurons is not uniform. The strength of GAL4
422 expression often varies among cell types within an expression pattern (S. R.
423 Pulver, personal observations). If GAL4 expression is relatively weak in high
424 threshold motor neurons, then those cells would have fewer functional ChR2
425 channels and would, in turn be less responsive to blue light than other ChR2
426 containing motor neurons. The use of higher power LEDs and/or alternative
427 motor neuron GAL4 drivers could help resolve this issue.
428 In our second set of demonstration experiments, we found that leaving the
429 ventral ganglion intact lowered the effective stimulus duration needed to evoke
430 EJPs. This could be a consequence of having intact motor neurons (dendritic
431 regions, cell bodies, and initial spike generation zones) in the ventral ganglion

432 exposed to blue light. It could also be caused by activation of excitatory
433 glutamatergic interneurons, which in turn, activate motor neurons through
434 synaptic pathways. Regardless, the leading lEJPs in these CNS-nerve-muscle
435 preparations were similar in amplitude and duration to lEJPs in experiments with
436 only nerve and muscles present.

437 One prominent feature of preparations with intact ventral ganglia was that
438 they generated multiple EJPs in response to single light pulses with durations
439 longer than 2.5 ms. In addition, in about half the preparations, short light pulses
440 triggered long lasting trains of spontaneously generated lEJPs. From a teaching
441 perspective, these features provide students and educators with opportunities for
442 further exploration. For example, students can easily examine basic synaptic
443 integration when motor neurons fire high frequency bursts and postsynaptic
444 potentials summate; students can also compare lEJPs and spontaneously
445 generated EJPs without the use of stimulating electrodes.

446 In classroom exercises, students recorded EJPs from different body wall
447 muscles. They were encouraged to target any muscles that contracted in
448 response to light pulses (as opposed to specifically targeting only m6). While this
449 resulted in heterogeneity across student results, it also increased the chances of
450 students obtaining usable data, because many had difficulty visualizing individual
451 muscles for electrode penetration (Fig. 6D). Opportunistically targeting muscle
452 areas that contract with light stimulation facilitated student success. For example,
453 all student groups (11 groups / two lab sessions) from our 2010 cohort recorded
454 lEJPs. Once they successfully recorded EJPs, most students focused on
455 examining short-term synaptic plasticity at the larval NMJ (Figure 5). They were
456 aided by a suite of powerful software tools to analyze the dynamics of synaptic
457 transmission. The data analysis program, g-PRIME
458 (http://crawdad.cornell.edu/gprime/), has been optimized and student tested for
459 analyzing many aspects of synaptic transmission at the crayfish NMJ (21). These
460 freely available analysis tools can be immediately and directly applied to
461 analyzing synaptic transmission in *Drosophila*.
462

463 **Dissection for electrophysiology experiments: coping with small size**

464 The largest drawback to the *Drosophila* NMJ electrophysiology
465 preparation is its small size. Because of this, students have difficulty doing the
466 larval dissection. In particular, they often cannot make a clean initial posterior to
467 anterior cut with the spring scissors typically provided in teaching laboratories
468 (10). We have found two solutions to this problem. One option is for teachers and
469 teaching assistants (TAs) to prepare the dissections ahead of time and provide
470 preparations 'on the fly' during a 3-4 hour lab class. With high quality scissors
471 and a few practice sessions, experienced TAs (and students) can typically
472 complete a dissection in under 5 minutes. The second approach is to follow a 'try
473 one, then get one free' policy. Student groups try the dissection once, and if they
474 do not see light evoked muscle contractions, they receive a fresh preparation
475 from an instructor. Most preparations will provide some data unless large areas
476 of the body wall are obviously damaged. Scotch Tape™ placed on the under
477 surface of Sylgard lined petri-dishes diffuses transmitted light and increases
478 contrast to more easily visualize target muscles.
479

480 **Practical advantages of using ChR2**

481 A major advantage of using ChR2 is that students are able to evoke lEJPs
482 without the use of suction electrodes. Students (and researchers) often have
483 difficulties maneuvering and operating suction or other stimulating electrodes in
484 small working areas, especially with the larval fly prep. Eliminating the need for a
485 suction electrode potentially eliminates a major source of frustration in the
486 teaching laboratory. Before our fly laboratory sessions, the BIONB/BME 4910
487 students spent 2 weeks studying synaptic transmission at the crayfish NMJ. The
488 students used the same equipment as used in our study and had the same
489 primary instructor (BRJ); use of suction electrodes in the crayfish preparation
490 was required. This gave us the opportunity to test the hypothesis that evoking
491 EJPs with ChR2 in *Drosophila* was technically easier for students than traditional
492 suction electrode stimulation in crayfish. Indeed, about 75% of the students
493 agreed that using ChR2 to evoke lEJPs at the larval NMJ was easier than using

494 a suction electrode at the crayfish NMJ (Figure 6C). This suggests that the ChR2
495 based exercises demonstrated here offer a technical advantage over at least one
496 traditional NMJ teaching preparation.
497 A second practical advantage of using ChR2 is that students can get
498 continuous feedback on the health of their preparations and where to insert
499 intracellular electrodes. In dissected preparations, shining blue light on a larval
500 CNS expressing ChR2 causes visible muscle contractions. Therefore, if students
501 see light-evoked contractions, they know that their preparation is healthy, and in
502 what muscle area to insert an electrode, even if individual muscle fibers are not
503 distinguishable. Since all motor neurons express ChR2, students can target
504 muscles in any healthy body wall segment of the larvae for intracellular
505 recording.
506 We noticed that many students had difficulty identifying muscle cells for
507 penetration with recording electrodes (Fig. 6D). Our student evaluations point to
508 a solution to this problem: simply being able to see light-evoked muscle
509 contractions in dissected preparations helped over 90% of students target
510 individual muscles for successful recordings (Figure 6E). We also noted that
511 seeing these contractions appeared to galvanize students to continue trying to
512 get intracellular recordings even in the face of frustration caused by technical
513 difficulties.
514
515 **Outlook for student-led research**
516 The ability to optogenetically evoke EJPs at the larval NMJ opens multiple
517 avenues for further exploration and independent student projects. For example,
518 students can explore in depth fundamental features of ChR2 mediated synaptic
519 transmission and its plasticity, including facilitation, summation, post-tetanic
520 potentiation and depression. They can also examine how these properties vary
521 among identified muscles in larvae (something that has never been done
522 systematically by researchers). Furthermore, since miniature EJPs (mEJPs) are
523 visible in m6 (13,14) students can estimate the quantal content of lEJPs (i.e. lEJP
524 amplitude / mEJP amplitude; 4). Finally, students can also examine how acute

525 application of neuromodulatory substances (i.e. neuropeptides and biogenic
526 amines) affect synaptic transmission at the larval NMJ. Overall, many
527 fundamental experiments have yet to be performed using optogenetic methods to
528 evoke lEJPs in fly larvae; therefore, any student projects would be breaking new
529 ground, not just repeating previous work.
530 Students were clearly motivated by this laboratory exercise. They felt it
531 helped them understand communication within the nervous system, and it
532 enhanced their interest in the intellectual background material (Fig. 7 A-C).
533 Perhaps more importantly, almost all (94%) expressed excitement that they could
534 potentially do novel experiments that have not yet been done by researchers
535 (Fig. 7D). This lead most of them to express a positive interest in practicing the
536 scientific method as students, and even to consider a career in research (Figs. 7
537 E, F).
538
539 **CONCLUSIONS**
540 Here we present inexpensive methods for remotely activating neural circuits in
541 freely behaving *Drosophila* larvae with ChR2. We also show how to record ChR2
542 mediated EJPs at the larval NMJ and show that they are equivalent to EJPs
543 evoked by traditional electrical stimulation. These teaching exercises give reliable
544 results with minimal effort and expense. More importantly, they generate
545 avenues for further research and give students and educators the means to
546 explore them independently.
547
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560

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659

660 **FIGURE / TABLE LEGENDS**

661 **Figure 1:** LED control systems. A) Diagram of control system used in teaching
662 exercises. Connections between LED, ‘Buckpuck,’ BNC connector, and power
663 adapter are indicated. B) Equivalent diagram for control system designed for
664 analog pulse stimulators and TTL signals with low current output. C) Typical LED
665 control system (based on diagram in A) showing Buck Puck, BNC connector, and
666 wiring. Housing is made from an empty pipette tip holder box. D) LED mounted
667 on heat sink. Rolls of electrical tape are placed around the LED to prevent the
668 microscope eyepiece from crimping wires supplying power to LED. E) LED and
669 heat sink mounted to eyepiece and attached to magnetized base. F) LED system
670 in place on a working electrophysiology rig.
671

672 **Figure 2:** Activation of glutamatergic neurons with ChR2 causes tetanic paralysis
673 in larvae raised on food containing ATR. A) Schematic of genetic crossing
674 scheme and larval rearing. B) 3rd instar larva raised on food without ATR.
675 Locomotion and body posture under ambient light is the same as that under blue
676 light. B) 3rd instar larva raised on food containing 1 mM ATR. Locomotion is
677 unimpaired under ambient light. Under blue light all body segments contract and

animal stops crawling. C) Pooled data: animals raised without dietary ATR do not respond to blue light, while 100% of animals expressing ChR2 show contractile responses to blue light (left; $n = 10$). 92% of these animals are paralyzed for the duration of a 5 s light pulse (right; $n = 12$).

Figure 3: Comparison of light and electrically evoked EJPs (lEJPs and eEJPs, respectively) in the absence of motor neuron cell bodies and ventral ganglion circuitry. A) Schematic of a dissected larval preparation. Brain and ventral ganglion are removed. A single segmental nerve is stimulated via suction electrode. Muscle 6 (m6) is targeted for recording. B) Long time-base recording showing a typical experiment. One motor unit is recruited with the lowest stimulus voltage. An additional motor unit is recruited as electrical stimulus intensity is increased. lEJPs are evoked by 2.5-10 ms light pulses. C) Expanded time-base views of eEJPs and lEJPs shown in (B). D-F) lEJPs show amplitudes and time courses which are not statistically different from eEJPs evoked by the low threshold motor unit ($F > 0.05$, One-way ANOVA). Data from 1 ms light pulses are not shown because they did not evoke lEJPs in any preparations. In pooled data, resting membrane potentials were between -40 and -55 mV. Resting membrane potentials were not significantly different across stimulation types ($F > 0.05$, One-way ANOVA, data not shown). Pooled data are presented as mean +/- S.E.M. Asterisk indicates significant difference compared to all other conditions ($p < 0.05$, One-way ANOVA with Tukey-Kramer posthoc test).

Figure 4: Comparison of lEJPs and eEJPs with motor neuron cell bodies and ventral ganglion intact. A) Schematic of a dissected larval preparation, showing brain, ventral ganglion (Vg) segmental nerves and an intracellular electrode in the m6 muscle. The brain is removed, but ventral ganglion is intact. B) EJPs in response to a 1 ms electrical stimulus, and four different blue light pulse durations. Electrical stimulus intensity has been adjusted to activate both motor units innervating m6. Note multiple summating lEJPs after longer light pulse durations. C) Number of EJPs for each light pulse duration. D) Increasing light

709 pulse duration does not affect the amplitudes of leading lEJPs. E) Short light
710 pulses can trigger long trains of spontaneous EJPs. A 1 ms light pulse (arrow)
711 triggers a single EJP in m6 (1) followed by a train of endogenously generated
712 EJPs (2, 3). F) The lEJP is similar in amplitude and duration to the spontaneous
713 EJPs. Data in B and E, F are from two different animals. In pooled data, resting
714 membrane potentials were between -40 and -55 mV. Leading EJP amplitudes
715 and resting membrane potentials were not significantly different across
716 stimulation types ($F > 0.05$, One-way ANOVA). Pooled data are presented as
717 mean +/- S.E.M.
718
719 **Figure 5:** Example of student-initiated electrophysiology experiment: analysis of
720 short-term plasticity at the larval NMJ. A) A pair of lEJPs evoked by 20 ms light
721 pulses spaced 40 ms apart. Arrows indicate lEJPs. To compensate for additive
722 summation at short stimulation intervals, an exponential curve (grey) is fit to the
723 repolarizing phase of the first EJP. The amplitude of lEJP2 is determined by the
724 difference between its peak voltage and the exponential fit voltage at the time of
725 peak voltage. B) Paired pulse facilitation indices over a range of stimulation
726 intervals (black squares). Data are fit to an exponential decay equation.
727 Calculated long-term facilitation ratio is 0.8+/-4 (95% confidence interval). Data
728 are from a single NMJ. All experimental design, data collection, analysis, and
729 figure preparation carried out by students.
730
731 **Figure 6:** Student evaluation of the technical aspects of the ChR2 behavior and
732 physiology exercises. A-F) Responses to 6 queries (shown above each plot)
733 ranked on Leikert scale; n = 21 students.
734
735 **Figure 7:** Student evaluation of the conceptual and motivational aspects of ChR2
736 exercises. A-F) Responses to 6 queries (shown above each plot) ranked on
737 Leikert scale; n = 21 students.
738

739 **Table 1:** Example of student-initiated behavioral analysis. Students counted
740 number of peristaltic waves and distance traveled during 30 s trials in control (no
741 ChR2 expression, n = 2) and experimental animals (ChR2 expressed in
742 glutamatergic neurons, n = 2). In both groups, locomotion was measured in
743 ambient light and in the presence of rhythmic (1 s pulses, 0.5 Hz) blue light
744 pulses.
745
746 **Supplemental Movie 1:** Activation of glutamatergic neurons with ChR2 causes
747 tetanic paralysis in larvae. Larvae (genotype: OK371-GAL4 / UAS-H134R-ChR2)
748 crawl freely in ambient and green light, but stop moving in blue light.
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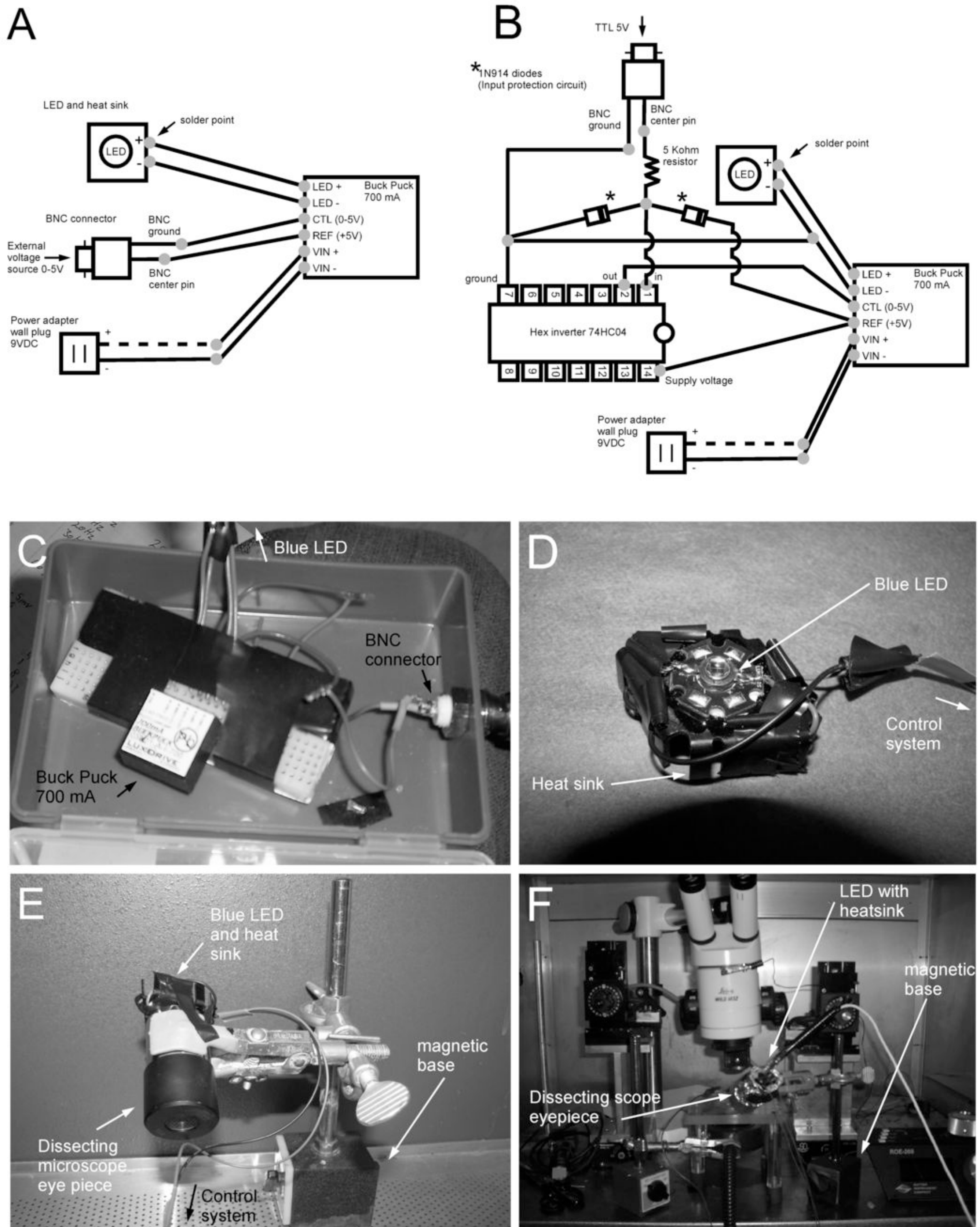
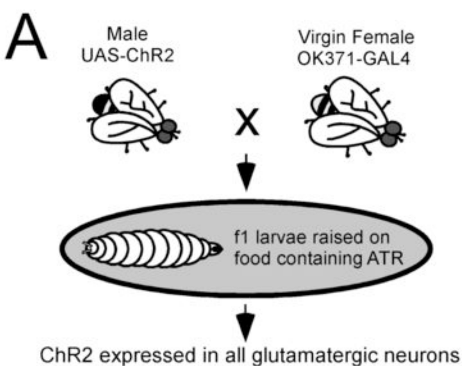
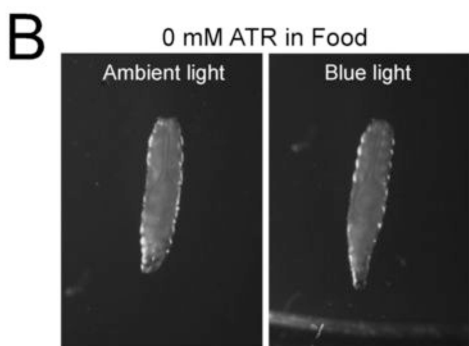
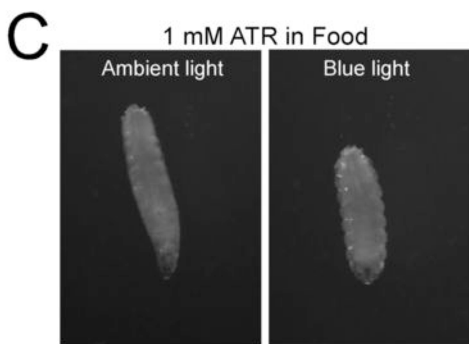


Figure 1







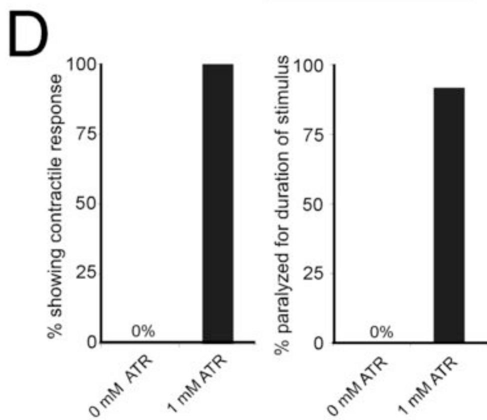


Figure 2

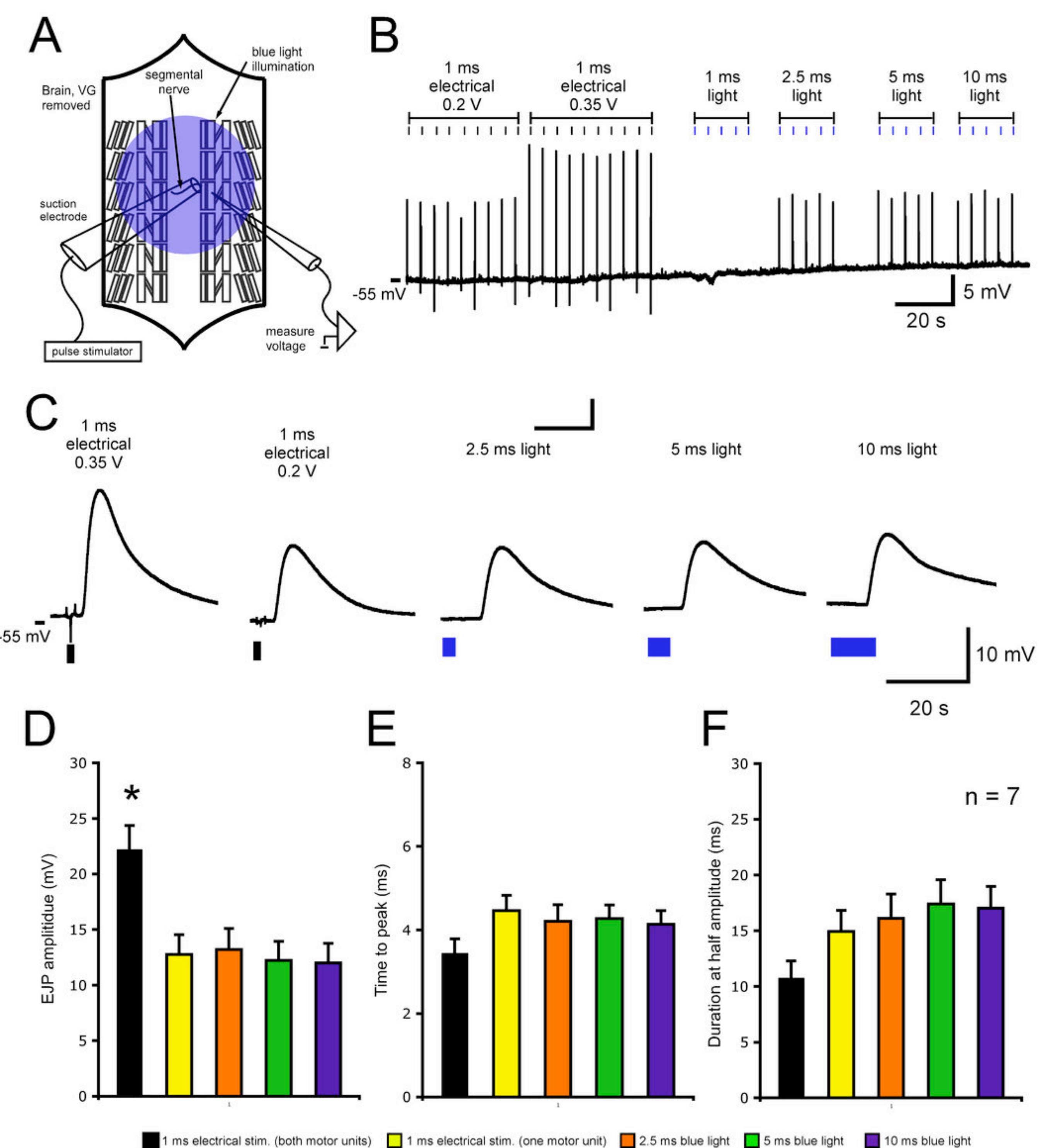


Figure 3

A

blue light illumination
Brain
Vg
segmental nerves
suction electrode
pulse stimulator
measure voltage

B

1 ms electrical 0.33 V
1 ms light
2.5 ms light
5 ms light
10 ms light
-49 mV
5 mV
40 ms

C

EJPs after light pulse
0
1
2
3
4
5

D

n = 6
1st EJP amplitidue (mV)
0
5
10
15
20
25
30

1 ms electrical stim. (both motor units)
1 ms blue light
2.5 ms blue light
5 ms blue light
10 ms blue light

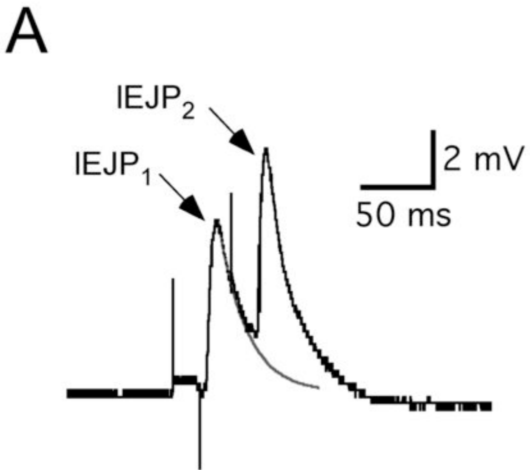
E

1
2
3
-40 mV
3 mV
5 ms

F

1
2
3
overlay
3 mV
20 ms

Figure 4



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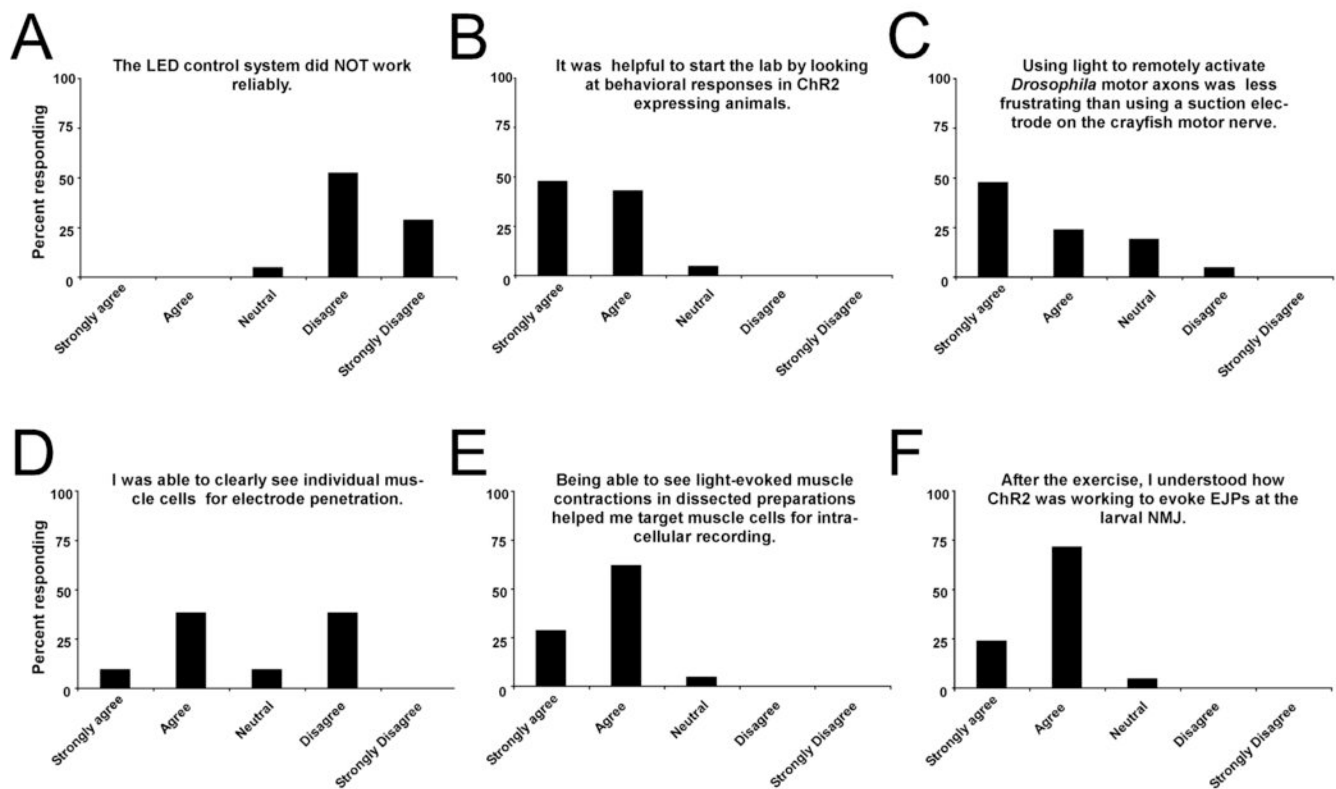

Figure 6

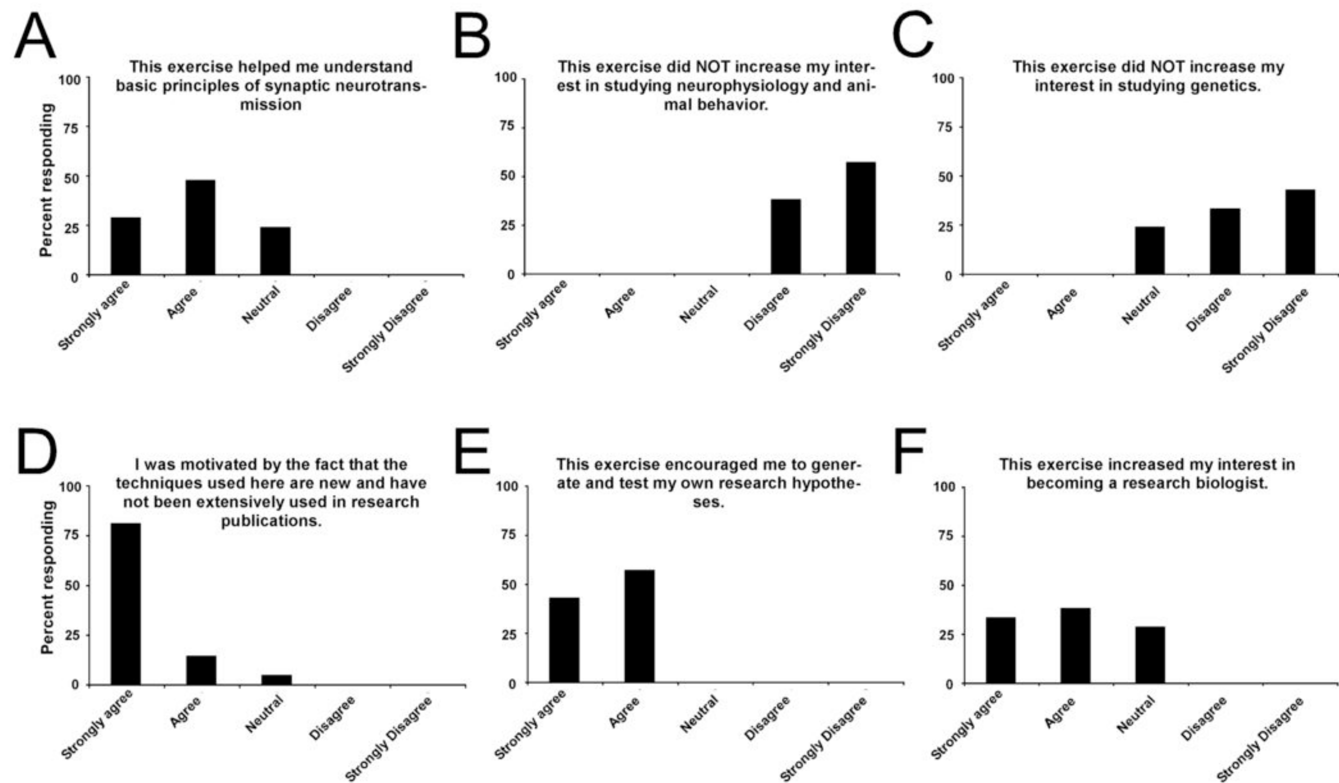

Figure 7

Group	Group A (No ChR2)		Group B (ChR2 Expression)	
Trial #	Trial 1	Trial 2	Trial 1	Trial 2
A: Control Condition – No Blue Light Stimulation				
Number of Peristaltic Waves	21	24	23	23
Total Distance Traveled (# of squares)	8	12	12	14
B: Experimental Condition – 1 sec Blue Light Pulses				
Number of Peristaltic Waves	19	21	0	0
Total Distance Traveled (# of squares)	12	13	0	0

Table 1