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Considerations in repetitive activation of light sensitive ion channels for long-term studies: Channel rhodopsin in the *Drosophila* model

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ABSTRACT

Optogenetics is a technique used in various animal models and holds a potential for therapeutic possibilities in mammals. There are technical issues with the use of light sensitive ion channels: reproducible effects over time, controlling where the non-native proteins are targeted within the cell and changes in the biophysical properties of the cells they are expressed in. We used a variant of channel rhodopsin (ChR2-XXL) and targeted expression in neurons of larval *Drosophila* to investigate the acute and chronic activation, with light pulses, of the channels on synaptic function. The rhodopsin channel modifier all trans retinal (ATR) also plays a role in the sensitivity of the channel to light. Periods of acute, repetitive, and pulsatile blue light exposure over larval development produced attenuated responses. These blue light sensitive ion channels, with ATR, show accommodation and produce an electrical refractory period in inducing synaptic responses. The biological significance and aim of this study is to demonstrate that in controlling particular neurons or neuronal circuits with optogenetics, over time and throughout development, one will have to understand the dynamic nature of activating and silencing the light sensitive channels as well as the biophysical effects on neuronal activity.

1. Introduction

The advent of optically stimulating exogenous ion channels and ion pumps, expressed in specific neurons, allows one to augment neural circuits without altering non-specific neurons or introducing systemic agents (Banghart et al., 2004; Fiala, 2013; Klapoetke et al., 2014; Towne and Thompson, 2016). The rapid growth and heightened attention in the experimental use of optogenetics in various animal models, worms, insects, rodents (Nagel et al., 2005; Hornstein et al., 2009; Titlow et al., 2015; Riemensperger et al., 2016; Giachello and Baines, 2017) and even humans (Scholl et al., 2016; Sengupta et al., 2016; Towne and Thompson, 2016) is demonstrating great promise for manipulating activity in various types of tissue (Quinn et al., 2016; Zhu et al., 2016; Malloy et al., 2017). In order to advance the field and uncover the potential therapeutic uses, the limitations, and the ability to finely tune the activation or silencing of optically sensitive ion channels must be understood (Bender et al., 2016; Blumberg et al., 2016). The ability to activate or inactivate ion channels rapidly and to control for specified cellular expression is an advantage of this technique (Gunaydin et al., 2010; Deisseroth, 2015). In addition, the ability to prod neurons deep within the brain with flexible optical fibers (Bass et al., 2010; Danjo et al., 2014) or the use of triggering channel rhodopsins by bioluminescence (Birkner et al., 2016) add to the tractability of optogenetics. However, there are some struggles researchers are facing with the use of these associated techniques (Dawydow et al., 2014; Deisseroth, 2014, 2015; Gradinaru et al., 2007; Grosenick et al., 2015; Lee et al., 2014). With increased experimental investigations, these issues will likely be resolved.

The complexity and accessibility of the central neural circuits complicates controlling some of the factors accounting for the variability in responses. It is also difficult to measure quantal events in intact CNS preparations at postsynaptic contacts. Thus, in examining activity dependent influences of synaptic transmission by optogenetic approaches we have focused on the larval *Drosophila* motor unit and obtaining synaptic measures at neuromuscular junctions (Pulver et al., 2009; Majeed et al., 2016). The larval *Drosophila* neuromuscular junction (NMJ) allows ease in measures of quantal events and evoked synaptic transmission under various experimental conditions. This animal model is excellent for investigating mechanism of synaptic development (Nose, 2012).

All trans retinal (ATR), a compound used to help in promoting the ion conductance and preventing the degradation of channel rhodopsin, is used in animal models in which the organisms does

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not naturally produce ATR; thus, it is supplemented in the diet (AzimiHashemi et al., 2014; Hegemann et al., 1991). The need to use ATR produces additional variables. The concentration used, the potential degradation over time in the presence of light, how the cell metabolizes the compound over time, and the impact on the channels rhodopsins themselves are all factors that need to be considered when utilizing optogenetics in experimentation. The ChR2-XXL variant is highly sensitive to blue light and does not require additional ATR supplementation in the diet for activation of cells. Therefore, we have tested the efficacy of responses with and without a controlled concentration of ATR to aid in understanding the impact on cellular and organismal function in order to refine this technique.

In this report, we highlight goals we are pursuing and experimental issues we have come across with the use of optogenetics in activating neurons throughout development in *Drosophila* larvae. We have uncovered reproducible but unexpected outcomes in particular experimental paradigms and here we share these outcomes and discuss our interpretations and their implications for future experimentation. We illustrate with electrophysiological and behavioral approaches, that conditioning whole animals with the optogenetic technique might provide some misleading results if the physiology is not directly measured. Thus, if one is to alter neural circuits that may or may not be activated, electrophysiological measurement and stimulation paradigms are necessary to be experimentally determined.

Our long-term goal is to develop a means to repetitively and consistently activate neurons over the long term, throughout key developmental periods in neural development of animals. However, in pursuing this goal a number of novel experimental findings have awakened us to some issues in the larval *Drosophila* model. Thus, for the benefit of other researchers we highlight a few of the concerns we have had in repetitively exciting the channel rhodopsins in neurons spanning from minutes to days, and biophysical changes while electrically stimulating neurons during and after activation of channel rhodopsin.

2. Methods

2.1. Drosophila *lines*

The filial 1 (F1) generations were obtained by crossing females of UAS-ChR2-XXL (BDSC stock # 58374) with males of D42-GAL4 (BDSC stock#8816). The parental lines were also examined for the effect of light sensitivity for behaviors and electrophysiological studies. *Drosophila* were raised on a mixture of cornmeal-agar-dextrose. The general maintenance is described in Campos-Ortega and Hartenstein (1985). The D42 strain was used as a proof of concept since it is known to be expressed highly in motor neurons (Yeh et al., 1995; Nitz et al., 2002) but also some expression in sensory neurons (Sanyal, 2009). When the ChR2-XXL expressing neurons are targeted the result is body wall muscle contraction. This leaves the larvae in a state of paralysis.

2.2. Preparation of fly food supplemented with ATR

All trans retinal (ATR; Sigma-Aldrich, St. Louis, MO, USA) was diluted in 5 ml of standard fly food to a final concentration of 400 μM and it was protected from light with aluminum foil. For control experiments, larvae were cultured in food that only contained the solvent (absolute ethanol) in fly food. All the animals were reared in vials with the same cornmeal–agar-dextrose-yeast medium (modified from Lewis, 1960). Food without added retinal is likely devoid of retinal as this food is cooked and made into a fly media for culturing the flies and larvae. Considering the food is boiled it is unlikely if any retinal did exist in the dried corn meal would be able to remain active since the compound is heat liable.

2.3. Larval behavior

Locomotion behavior was assessed by placing larvae on an apple-juice 1% agar plate (Majeed et al., 2016). The larvae were left for one minute to acclimate to their new environment. Body wall movements were recorded while being exposed to a dim white light and when exposed to diffuse blue light from an LED mounted in a soda can (see Titlow et al., 2014). The locomotion activities were recorded with a webcam (WEBCAM HD4110, Hewlett-Packard Company, Palo Alto, CA) and stored on a computer. The activity was recorded at 25 frames per second for various experimental paradigms (see Results).

2.4. Electrophysiology

The synaptic responses at the larval *Drosophila* NMJs were recorded by standard procedures (Lee et al., 2009) with stimulation at 0.5 Hz as described in the Results section. All the experiments were performed at room temperatures (20–21 °C). The excitatory post synaptic potentials (EPSPs) were measured by intracellular recordings with a sharp glass electrode (3 M KCl) and AxoClamp-2 B amplifier (Molecular Devices, LLC. 1311 Orleans Drive, Sunnyvale CA, USA). Stimulations were made with a Grass S88 dual stimulator (Natus Neurology Incorporated, Middleton, WI, USA). Preparations were used immediately after dissection. Electrical signals were recorded online to a computer via a PowerLab/4 s interface (ADI Instruments, Colorado Springs, CO, USA). The larval *Drosophila* preparations were dissected as previously described (Li et al., 2001) for early 3rd instars. The CNS was left intact for studies as expression is likely high in the cell body and axons, as compared to the isolated nerve terminal.

The modified HL3 saline was used for physiological measures (Stewart et al., 1994) at a pH of 7.1 (de Castro et al., 2014). Saline solution (in mM): 1.0 $CaCl_2{\cdot}2H_2O$, 70 NaCl, 20 $MgCl_2$, 5 KCl, 10 $NaHCO_3$, 5 trehalose, 115 sucrose, 25 5*N*,*N*-bis(2-hydoxyethyl)-2-aminoethanesulfonic acid (BES). All chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All measures were made in muscle 6 of segments 3, 4 or 5.

2.5. Blue light exposures

The blue light (470 nm wavelength, LEDsupply, LXML-PB01-0040, 70 lm @ 700 mA) was provided by a high intensity LED. The photon flux (number of photons per second per unit area) was measured with a LI-COR (model Li-1000 data Logger, LDL 3774; LI-COR from Lincoln, Nebraska, USA) which produced around 103 $\mu Mol\ s^{-1}\ m^{-2}$ per μA (or 22.24 $\mu W\ mm^{-2}$) on the surface of the dish for the behaviors and on the dissected preparations around 133 $\mu Mol\ s^{-1}\ m^{-2}$ per uA (or 28.9 $\mu W\ mm^{-2}$).

The exposures during the developmental conditions were around 50 $\mu Mol\ s^{-1}\ m^{-2}$ per μA (or 10.87 $\mu W\ mm^{-2}$) at a distance of about 12 cm from the light source to the larvae. The larvae were distributed in the food so only a thin layer (about body thickness) would occur to continuously expose the larvae to the light. The food needed to be dampened with water about every 12 h to keep it from drying out. The exposure during development occurred by taking early 2nd instar larvae and either placing them in food with or without ATR and either exposing the dish to blue light or maintaining the dish in total darkness. The light was timed to provide 30 s of continuous blue light followed by 30 min of darkness for 48 h. The ability to control the light stimulation was managed by Arduino system (2015 Arduino, LLC.); https://www.arduino.cc/).

2.6. Statistical analysis

All data are expressed as raw values or mean ± SEM. A paired Student's *T*-test (before and after), ANOVA or a rank sum pairwise test was used to analyze changes in behavior or electrophysiological responses after changing bath conditions or stimulating with blue light. Since the groups are not normally distributed (i.e., a number of zeros in some groups) and having different sample sizes we used a Dunn's Test or a sign rank sum test for trends among preparations.

3. Results

The initial approach to determine if intact larvae would show repetitive behavioral responses to pulses of blue light was performed by monitoring crawling behavior before, during, and after a series of repetitive light pulses. The light pulses consisted of 30 s of blue light followed by 10 min of very dim white light which allowed the camera to monitor body movements. The time it took larvae to initiate a full body contraction after the blue light was turned off was used as an index in reproducibility of a behavioral response. The effect of supplementing the food with ATR was also examined by comparing larvae which were raised with and without food containing ATR (400 μM). In addition, parental lines (D42-GAL4 and ChRXXL-UAS) were also examined with and without exposure to ATR. The larvae were placed in the associated feeding conditions from early 2nd instar stage and left for 48 h in the dark. The larvae reached an early 3rd instar stage by 48 h. The developmental time is slightly slowed in the presence of ETOH solvent in the ATR mixture and. ETOH was added to food not containing ATR to control for the ETOH exposure.

The time to initiate a body wall contraction after 30 s of blue light exposure and 10 min of dim white light (Fig. 1A) indicated that ATR fed larvae were slower to initiate movements. Over time the ability to move was faster in larvae not exposed to ATR. This is illustrated by representative movement maps, which depict the first 10 min of dim white light exposure after blue light exposure for 3 larvae in each condition (Fig. 1B). Upon blue light exposure, the larvae with and without ATR all showed strong body wall contractions creating a paralysis, which generally persisted the entire 30 s of blue light exposure (Fig. 1C; N = 10, $P \leq 0.05$, *T*-test and ANOVA compared to with or without ATR as well as to parental lines). The parental lines with or without ATR showed no reduction in movement when exposed to blue light or white light. Thus, the time to initiate a movement is shown as time zero. To compare the effect of longer periods of blue light exposure, an entire hour was used followed with one hour of very dim white light. The blue light exposure was subsequently repeated a second time. The time to initiate a body wall movement was also measured, and as for the shorter blue light pulses, the ATR fed larvae took longer to move compared to the ones lacking ATR (Fig. 1D, N = 10, $P \leq 0.05$, *T*-test). The parental lines again showed no effects even to the longer blue light exposure (each group N = 10).

In being able to manipulate a developing neural circuit, or a developed neural circuit over long periods of time, it is of interest to determine if the optically activated responses are consistent in the outcome of the behavioral responses. Thus, we exposed larvae from 2nd to wandering 3rd instar to 30 s of blue light and gave 30 min of time in the dark prior to repeating the blue light exposure. This paradigm was repeated for 48 h on larvae with and without ATR mixed with the food (Fig. 2A1). We switched out the food after 24 h of feeding to control for the pulses of blue light possibly inactivating the ATR. A few preliminary trails indicated more pronounced responses in the larvae after 48 h to blue light when the food was replaced after 24 h but this phenomenon was not carefully

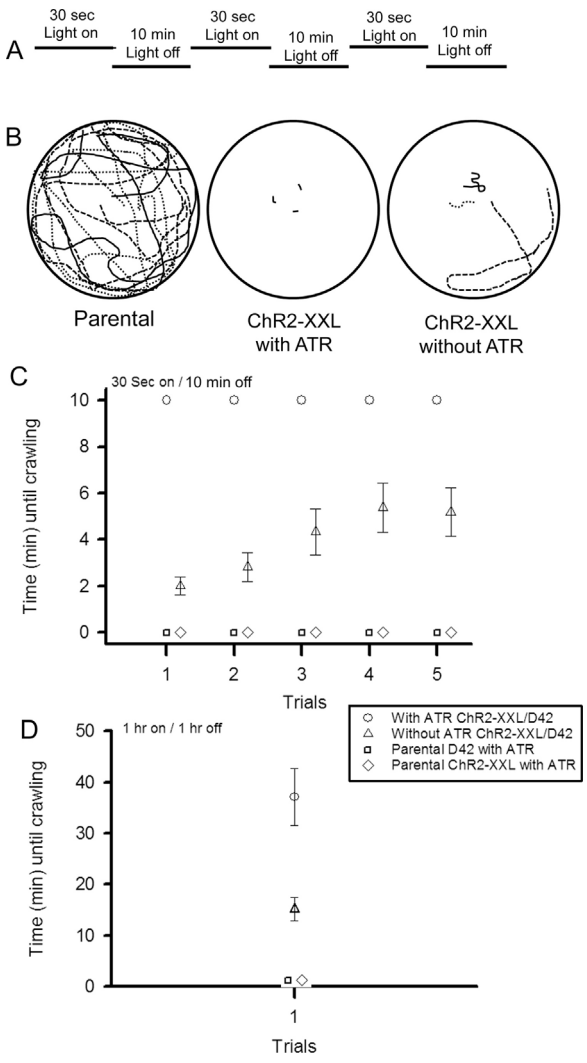

Fig. 1. Locomotion of larvae after exposure to blue light to examine the effect of feeding ATR and expression of ChR2-XXL channels in motor neurons. (A) The acute exposure paradigm to blue light followed by very dim white light (i.e. labeled as dark) with just enough light to visualize the larvae with the camera. (B) The movement pattern of 3 larvae during the first 10 min dark period in the series of light/dark exposures. The larvae fed ATR and expressing the ChR2-XXL would not initiate a full body wall contraction within the 10 min. The larvae expressing the channel but not fed ATR only slightly moved in the same time period, whereas the parental strain of ChR2-XXL and fed ATR showed no inhibition to movement in blue light or the dark. (C) The time to initiate the first full body wall contraction is shown for the each of the 10 min dark periods in the light on/off series. The D42/ChR2-XXL larvae fed ATR remained paralyzed for each of the 10 min dark periods whereas the D42/ChR2-XXL larvae lacking ATR took a little longer to start moving with each exposure. The parental lines (D42 and ChR2-XXL) fed ATR showed no hesitation to keep moving after the blue light was turned off. (D) The effect of 1 h exposure to blue light and then without the blue light resulted in the first occurrence of a complete body wall contraction having a longer lasting effect on D42/ChR-XXL larvae not fed ATR than for the 30 s exposures. The D42/ChR-XXL larvae fed ATR took about 40 min as compared to 15 min for those lacking ATR to start moving. (N = 10 mean+/−SEM).

documented; however, we kept with standardizing the exchange to maintain consistency in the experimental conditions presented herein.

The behavioral experiments were performed to determine the time to initiate body wall contractions for the larvae exposed to blue light pulses during the previous 48 h. A comparison for larvae fed ATR and those without ATR was revealing. The same testing paradigm was used for the ones not exposed to the conditioning light pulses (Fig. 2A2) The ones fed ATR did not move within the 10 min in dim white light (labeled dark) after the 30 s exposures of

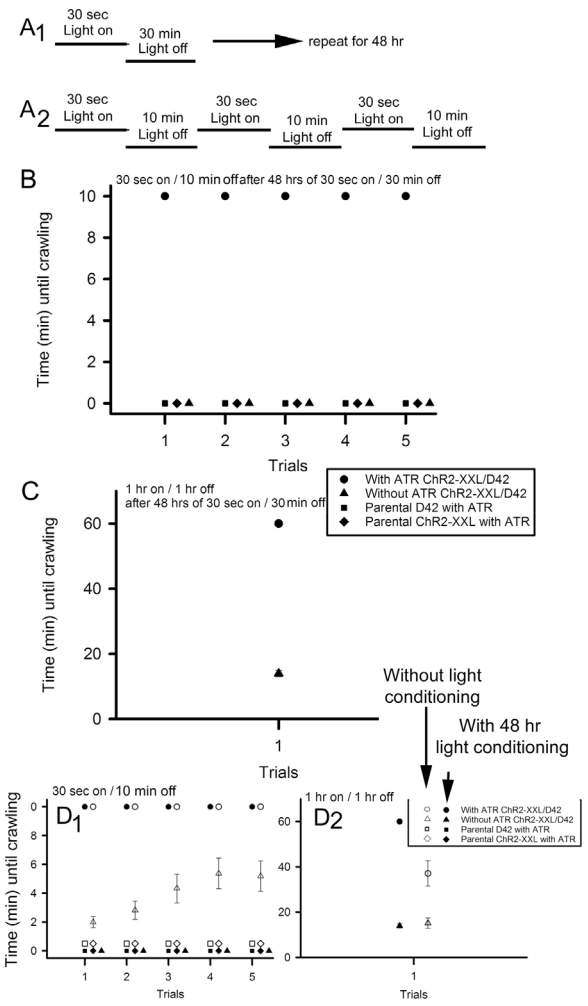

Fig. 2. The effect of blue light pulses during larval development (early 2nd instar to 3rd instar) on the locomotion of larvae with subsequent acute exposures to blue light. This examines the effect of feeding ATR and expression of ChR2-XXL channels in motor neurons (D42) over time. (A1) The 48 h conditioning paradigm followed by the acute exposure paradigm (A2) for the behavioral analysis conducted as for the acute only blue light exposures. (B) The time to initiate the first full body wall contraction is shown for the each of the 10 min dark periods in the light on/off series. Only D42/ChR2-XXL larvae fed ATR remained paralyzed for each of the 10 min dark periods; whereas, the D42/ChR2-XXL larvae not fed ATR and the parental lines (D42 and ChR2-XXL) fed ATR showed no hesitation to keep moving after the blue light was turned off. (D1) The comparison of the responses to initiate movement with and without the pre-conditioning over development to the acute blue light exposures of 30 s on and 10 min off. (D2) The effect of 1 h exposure to blue light and then with the blue light turned off for the first occurrence of a complete body wall contraction had longer lasting effect on D42/ChR-XXL larvae with the pre-light conditioning treatment of the 48 h then the acute only light ($N = 10$, $P \leq 0.05$, *T*-test). The D42/ChR2-XXL not fed ATR over the 48 h showed no difference than larvae without the pre-conditioning treatment. ($N = 10$, data expressed as mean +/−SEM).

blue light (Fig. 2B; $N = 10$, $P \leq 0.05$, *T*-test). When an hour of blue light exposure was used to assess behavioral responses, the larvae lacking ATR in their diet did not remain contracted the entire duration of the hour, in contrast to those fed ATR (Fig. 2C; $N = 10$, $P \leq 0.05$, *T*-test). In addition, the repetitive 30 s blue light exposure and 10 min of dim white light indicated the larvae were able to recover quicker with subsequent light exposures. One might assume that the responses would have already plateaued from the previous 48 h of light pulse conditioning. The robust contractions with the first few blue light exposures during the behavioral test might be due to the fact that the conditioning blue light was at 50 $\mu Mol\,s^{-1}\,m^{-2}$ per μA (10.87 $\mu W\,mm^{-2}$), whereas for the behavioral test on the agar dishes the blue light was around 133 $\mu Mol\,s^{-1}\,m^{-2}$ per μA (28.9 $\mu W\,mm^{-2}$). For ease in comparing the treatments of blue light and the effect of feeding ATR the combined responses are shown in Fig. 2D1 for the 30 s light pulses and 1hr exposures are in Fig. 2D2. When the larvae were placed in complete darkness for a few hours, all larvae move to new locations so even the hour exposure to blue light was not toxic to the larvae fed ATR and dark adapted (i.e. the conditions most sensitive to the blue light behavioral test).

To address the ability to repetitively and consistently activate channel rhodopsin proteins with blue light pulses, EPSPs in the muscles were monitored. The exposed filleted larvae bathed in physiological saline were exposed to 10 s long periods of blue light with 10 min of dark to recover. This was repeated 3 times. The effect of supplementing the food with ATR and prior exposures to blue light pulses during the previous 48 h was also addressed in the electrophysiological assays. Since we used the D42-GAL4 line some sensory neurons may also be activated in this assay.

Larvae raised with ATR (48 h) and kept in complete darkness had an unusual EPSP response to the light pulses. The majority of the initial exposures produced a strong burst of activity during the 10 s exposure but would cease producing large EPSPs (which are due to action potentials within the motor neurons) within the 10 s light stimulating period. Each of the 3 subsequent light exposures with 10 min dark periods are shown (see Figs. 3A1–A3). The subsequent pulses of blue light might or might not result in the evoked EPSPs ceasing within the 10 s of blue light. Notice the 3rd subsequent 10 s light pulse did not evoke a response in the axon to initiate action potential and only small miniature quantal events were observed (see Fig. 3B). However, after the evoked EPSPs would stop, the motor nerve would remain inactivated for about 2 or 3 min followed by a renewed burst of activity which would persist for 2–5 min (Fig. 3B). During the dark period after the blue light was shown, small quantal events could be observed which would dampen in frequency in the 10 min of dark exposure. The trend in the 10 s blue light exposures produced 3 out of 5 larvae to show the phenomenon of limiting evoked responses before the 10 s of blue light exposure was over. In addressing if a longer exposure to ATR from 1st instar to 3rd (7 days) and a longer exposure to blue light while measuring evoked EPSPs (20 s), in both larvae kept in dark as well as exposed to 10 s blue light on and 30 min off for the full 7 days, was examined. Representative responses shown in Fig. 3C1 also illustrated that the light induced evoked EPSPs stop before the light exposure is over. However, many small quantal events continued (Fig. 3C2). The frequency of these spontaneous quantal occurrences was not consistent from larvae to larvae or even within a series of the trials within a preparation. However, 6 out of 7 larvae raised in the dark demonstrated complete quiescent in evoked EPSPs (sign rank $N = 7$, $P \leq 0.05$). In addition, these small quantal events would be masked by the larger evoked events when they occur. Larvae exposed to blue light conditioning for 7 days all produced evoked EPSPs when tested and none showed a cessation of the evoked responses within the 20 s blue light exposure (sign rank $N = 6$, $P \leq 0.05$).

The larvae without supplemented ATR for 48 h, but also raised in the dark, exhibited slightly different responses. The evoked EPSPs would continue throughout the 10 s blue light exposures and would sometimes continue for 1–2 min after the blue light was turned off (Fig. 4A1–A3). Each of the 3 subsequent light exposures with 10 min of dark is shown (Fig. 4A1–A3). To control for the possibility of blue light itself stimulating the motor nerves or central neurons the D42-Gal4 parental line fed ATR for 48 h was also examined. The parental line did not show any response to the blue light exposures and the larvae appeared healthy as the spontaneous events occurred at a relatively consistent frequency with and without blue light exposures (Fig. 4B1, sign rank $N = 6$, $P \leq 0.05$). An enlargement of a

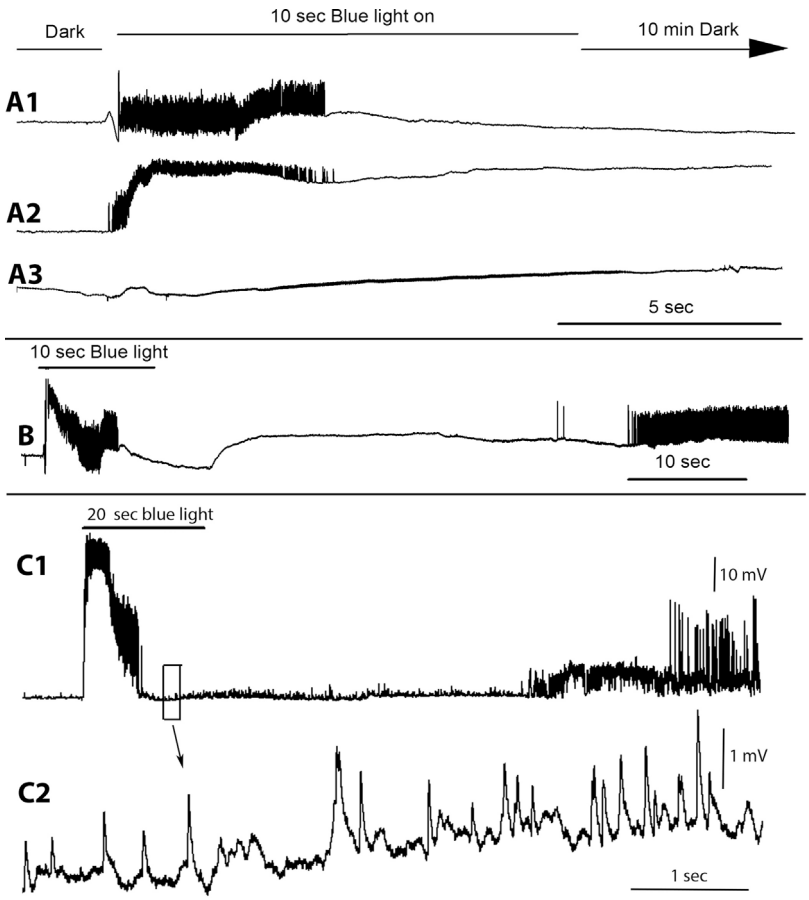

Fig. 3. The synaptic responses obtaining in the larval body wall muscles during acute blue light and dark exposures to examine differences in larvae expressing ChR2-XXL and feeding ATR. (A) D42/ChR2-XXL larvae fed ATR showed a pronounced response to blue light with a burst of excitatory postsynaptic potentials (EPSPs). However, the light induced action potentials within the nerve was apparently not maintained throughout the 10 s light exposure. Even after the subsequent 10 min dark exposure with the 2nd or 3rd dark/light series (see A1-1st, A2-2nd, A3-3rd blue light exposures) the action potential induced burst of EPSPs was not maintained for the 10 s. In fact, in this representative example the 3rd light exposure (A3) did not evoked and large EPSPs. In this paradigm the light induced evoked responses would be turned off within the 10 s light pulse and even during the dark period for 1–2 min before spontaneously starting up again (B). In examining for more robust responses, larvae raised from 1st instar to 3rd in ATR (7 days) and examined with 20 s of blue light produce rapid EPSPs which lasted a few seconds before they ceased still while being exposed to blue light. This is illustrated in the period in during light exposure but a refractory period in evoking EPSPs during the light exposure (C1). Even though evoked EPSPs were not induced small quantal shaped synaptic events would rapidly occur (C2) during this period.

quantal event within the trace shown in Fig. 4B1 is highlighted in Fig. 4B2.

The long-term 1 h exposures of blue light for the larvae fed ATR and lacking ATR revealed a similar response; however, the neural activity would remain for the full hour. The start of the 1 h exposure is shown in Fig. 4C1 and the end of the 1 h is shown in 4C2. In this particular experimental paradigm, after 1 h of blue light, an hour of dark was maintained followed by a second blue light exposure (Fig. 4C3). The ATR fed larvae would also exhibit the initial burst and shut down followed by resumed firing. In one case, the firing pattern was relatively constant at 10 Hz for the entire hour for a larva exposed to food without ATR. The larvae lacking ATR and exposed to the blue light for 1 h did not display the initial refractory period of stopping the light induced responses (Fig. 4D1), and were able to maintain the evoked EPSPs for the entire hour. The end of the hour of blue light exposure and subsequent dark exposure is shown in Fig. 4D2.

We examined if the refractory period in the light induced evoked responses was related to the frequency of the evoked EPSPs. The maximum peak frequency of evoked EPSPs and the occurrence of a refractory period occurring within the 10 s light exposure did not reveal a particular frequency at which the evoked responses would stop occurring. The peak frequencies would be as high as 60 Hz but there was no correlation with the cessation of the light evoked EPSPs due to the same high frequency being obtained in larvae which did not show a refractory period in light induced EPSPs.

The exposure to pulses of blue light (30 s blue light, 30 min dark repetitively cycled) throughout development (early 2nd instar to 3rd) for 48 h prior to electrophysiological testing with larvae fed ATR and without ATR also showed differences in the EPSPs when exposed to the 10 s of blue light. The larvae exposed to blue lights for 48 h did not show the rapid bursts and shut down within the 10 s flashes of light (Fig. 5A). The prevalence of this response was consistent in each of the 5 larvae examined. The larvae lacking ATR but conditioned for the 48 with light pulses did respond with evoked EPSPs to the tested 10 s of blue light and maintained the evoked EPSPs for the 10 s (5 out of 5) and would cease evoking EPSPs after the blue light was shut off. Two different larval responses are shown in Fig. 5B1 and B2.

Larvae fed ATR and kept in the dark for 48 h showed a burst of evoked EPSPs and then become quiescent within 10 s of blue light exposure. Afterwards the EPSPs would spontaneously reoccur while in the dark. Thus, it appeared that the motor nerve was possibly in an electrical refractory period or that the nerve terminal was not able to provide evoked vesicle fusion. The time varied among each of the larvae but within the range of 50–90 s before spontaneous activity reappeared. Thus, we examined this by en passant stimulation of the segmental nerve roots to the segment in

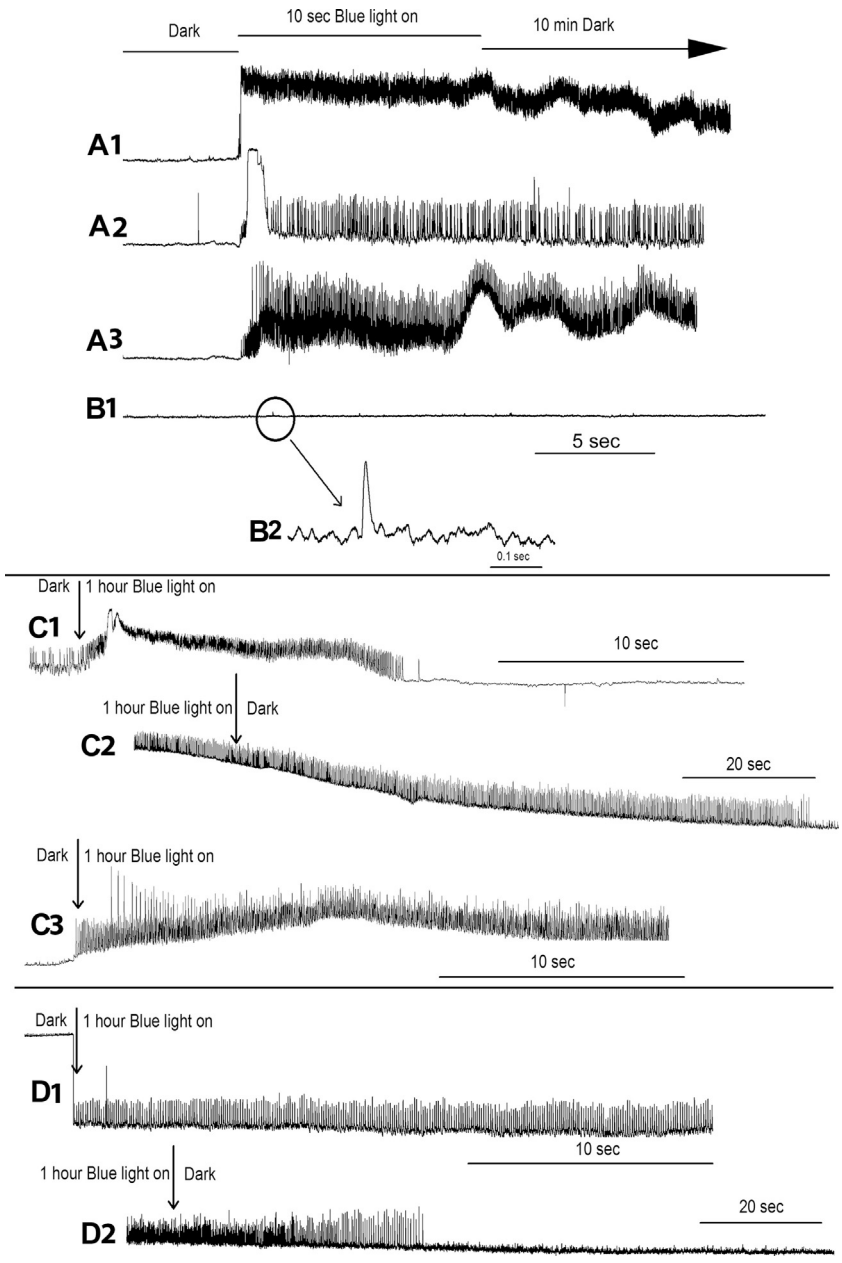

Fig. 4. The synaptic responses obtaining in the larval body wall muscles during acute blue light and dark exposures to examine differences in larvae expressing ChR2-XXL without feeding ATR. (A) The D42/ChR2-XXL larvae lacking ATR would continue to demonstrate light induced action potential evoked EPSPs throughout the 10 s blue light exposure. However with the series of 10 s blue light and 10 min dark each of the 3 recorded subsequent trials varied (see A1–A3). (B) The D42 parental line fed ATR was used to examine the effect of the blue light on the preparation in the absence of the light sensitive rhodopsin channels. No light induced EPSPs were observed and the occasional spontaneous quantal event (B2) observed prior to blue light or with blue light exposures had no noticeable differences in the frequency of occurrences. (C) D42/ChR2-XXL larvae fed ATR and exposure to 1 h of blue light while recording from the muscle with an intracellular electrode demonstrated the similar burst of EPSPs followed by a light induced refractory period with EPSPs resuming within the hr of constant blue light (C1). When the blue light is turned off (complete darkness) the EPSPs would remain firing for some time afterwards (C2). Notice after 1 h of darkness and re-exposure to blue light the evoked EPSPs now remained present without demonstrating the light induced refractory period (C3). (D) The D42/ChR2-XXL lacking ATR over the 48 h did not show the light induced refractory with exposure to blue light and would even maintain light induced evoked EPSP for the whole hour of blue light exposure (D1). Upon subsequent dark exposure the firing usually ceased relatively quickly (D2). Scale bars are shown in traces for C-D and these do not refer to light are dark exposure times.

which the optically evoked EPSPs were being measured. The motor nerve was stimulated at 0.5 Hz and the evoked responses were monitored before, during and after the blue light pulse. In larvae fed ATR, but maintained in the dark, the electrically evoked EPSPs were able to be induced while the light evoked EPSPs were reduced in amplitude or stopped while still being exposed to the blue light or right after the blue light was turned off (Fig. 5C). Thus, the nerve is still electrically active even though the EPSPs were not maintained fully in amplitude during the blue light exposure. In general, the responses were mixed. In one larva, the electrically evoked EPSP grew smaller over the light pulse and afterwards the electrically evoked EPSPs became larger and regained the same amplitude as to pre-light exposure (Fig. 5C). The very light sensitive larvae (i.e., fed ATR) demonstrated in 6 out of 8 larvae that the electrically evoked EPSPs stopped occurring when the light induced EPSPs also stopped. However, very small quantal events would still occur at a high frequency. After some time in the absence of the blue light, the electrically evoked EPSPs would start to appear sometimes gradu-

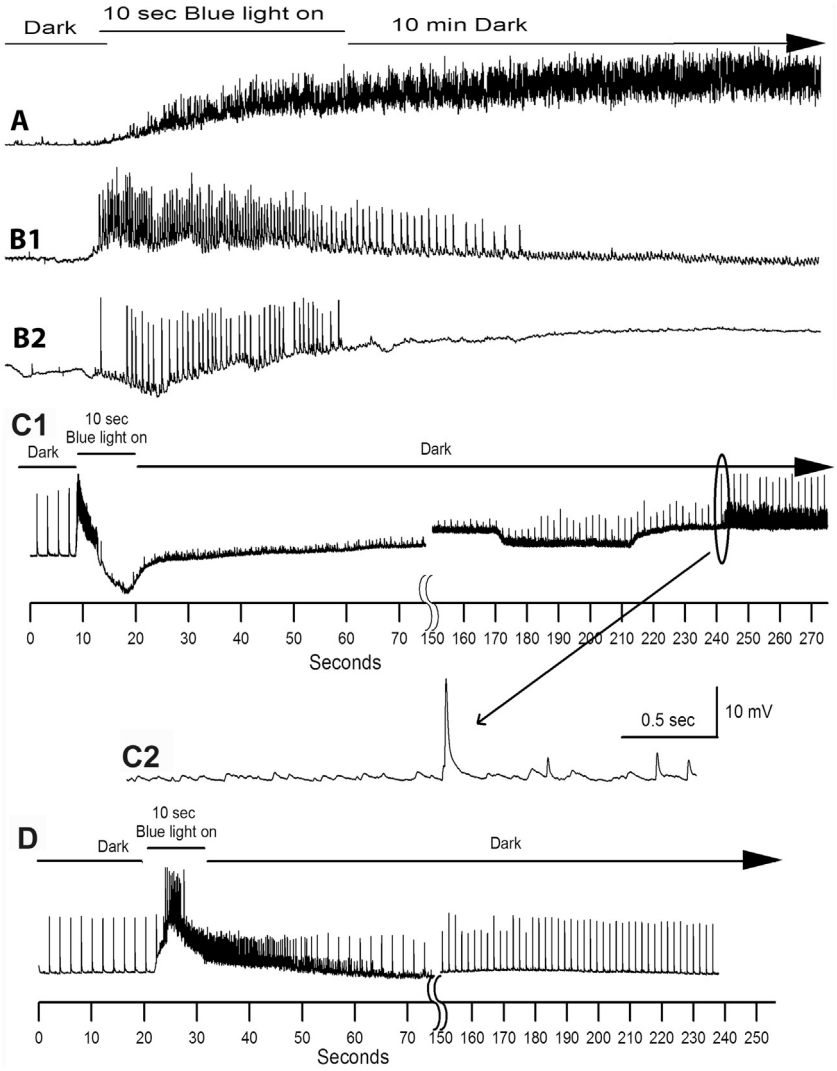

Fig. 5. The synaptic responses obtaining in the larval body wall muscles during acute blue light and dark exposures to examine the effect of repetitive light pulses over development (48 h early 2nd instar to 3rd instar) and the presence of ATR. (A) The D42/ChR2-XXL larvae fed ATR and pre-exposed to blue light pulses (30 s blue light–30 min dark repeated for 48 h) showed evoked EPSPs throughout the 10 s blue light exposures and the evoked EPSPs were maintained for a short while after the blue light was turned off. (B)The D42/ChR2-XXL larvae lacking ATR but also pre-conditioned for 48 h with blue light pulses would also show light induced evoked EPSPs which would cease quickly when the blue light was off. Two different preparations are shown (B1 and B2) for the relatively weaker response as compared to the larvae fed ATR. The light induced refractory period in activating ChR2-XXL for electrical stimulation is related to the sensitivity of the preparation. The segmental nerve is stimulated at 0.5 Hz prior, during and after the light and dark conditions in order to electrically evoked action potentials in the motor neurons to initiate EPSPs. (C) The D42/ChR2-XXL larvae fed ATR which demonstrated the light induced depression of evoked EPSPs also depressed electrically stimulated evoked EPSPs. The electrical evoked EPSP started to rise after almost 2 min of being in a relative refractory period (C1). The electrical evoked responses demonstrated rapid rise indicating the regaining of synchronized vesical fusion in the presynaptic terminal (C2). The D42/ChR2-XXL larvae lacking ATR which exhibited less of a response to blue light did not demonstrate as great of a relative refractory to the electrically evoked EPSPs although there was usually some reduction in the evoked EPSP amplitude (D).

ally increasing in amplitude. The rise time of the electrically evoked responses would first appear with a slow rise and then a rapid rise time (Fig. 4C2). The larvae lacking ATR but being electrically stimulated did not demonstrate the absolute refractory response but did demonstrate reduced electrically evoked EPSP responses initially. They would regain the evoked EPSP amplitude over time (Fig. 4D). This is not because the muscle is not able to respond to glutamate release since the light induced EPSPs are still occurring and eliciting large EPSPs. In addition, the size of the light induced quantal events, after the light exposure, is robust. Indicating that the postsynaptic receptors are not desensitized by the bursts of the EPSPs. Also, it appears the ChR2-induced electrical refractory period is ATR-dependent. Frequently recorded minis (ie. spontaneous quantal events) would suggest there is residual Ca^{2+} remaining within the nerve terminals.

4. Discussion

In this study, we have shown that repetitive short bursts (30 s) of light activating the channel rhodopsin variant, ChR2-XXL, expressed in neurons in intact larvae, can produce behavioral adaptation over time. When the larvae are fed ATR the responses are robust even over an hour of exposure with little accommodation. Over a period of 48 h with short bursts of light (30 s on, 30 min off) there is less responsiveness of the larvae to remain contracted when exposed to acute blue light pulses. The behavioral responses are mirrored in the electrophysiological measures at the NMJs with the presence of light evoked large EPSPs and smaller quantal sized EPSPs. A surprise novel finding was that the nerve would become electrically unexcitable when the nerve demonstrated a reduction in evoking large and synchronized EPSPs with light. Even though large EPSPs could not be electrically induced there were still

spontaneous quantal events occurring. The inability to electrically stimulate the nerve during the optically induced refractory period, as well as the likelihood of the light inducing the refractory period, was related to the sensitivity of neuron to blue light. Larvae which were fed ATR showed more pronounced refractory periods relative to larvae lacking ATR in their diet or larvae exposed to light pulses over a 48 h or 7 days of a conditioning window.

As demonstrated by Pulver et al. (2009) in acute studies of *Drosophila* larvae expressing channel rhodopsin in glutamatergic neurons (motor neurons and interneurons; OK371-Gal4/UAS-ChR2 or UAS-H134RChR2 lines), pulses of blue light produces less accommodation than the constant blue light exposed over a few minutes for initiating nerve induced large EPSPs. In addition, Pulver et al. (2009) reported the more sensitive H134RChR2 variant as compared to ChR2 resulted in prolonged EPSP activity following light stimulation. We report similar findings with ChR2-XXL but we also examined the difference of feeding ATR or not on the sensitivity of the lines. Larvae maintained in the dark and fed ATR produced a very strong response in a burst of EPSPs but the EPSPs would stop occurring within the 10 s period still being exposed to blue light or the first period of the hour exposure. However, the less sensitive larvae (not fed ATR) would generally remain active, producing light induced nerve evoked EPSPs throughout the 10 s, and even hour-long, light exposure. The differences in sensitivities of channel rhodopsin strains gives the advantage of a range to use, but also opens many questions about differences in accommodation of the frequency of EPSPs and an apparent refractory period of the nerve which can occur for evoked nerve induced EPSPs. The high occurrences of small single quantal events, when the nerve is in a light induced refractory period, provides evidence that the postsynaptic receptors are not desensitized, thus providing additional evidence that the abolishment of EPSPs is due to pre-synaptic absolute refractory. Also, since the amplitude of EPSPs gradually recovers fully over time and the rise time is shorter, this may suggest more about how the presynaptic vesicles are being synchronized for electrical evoked voltage gated calcium channels. It is likely that the influx of Ca^{2+} through the ChR2 may indeed induce the voltage gated plasma membrane Ca^{2+} channels to open and even increase the Ca^{2+} load in the presynaptic terminal (Lin et al., 2009).

When the larvae are recovering from blue light pulses they have a distorted locomotion (i.e., wobbling from side to side while trying to crawl forward) at first and then followed by increased coordination in the wave of segmental contractions over the body. This may be a central effect or an indication of the functional recovery at the NMJs. One might expect when the electrophysiological responses of the light induced refractory in EPSPs occurs that the larvae would start moving and crawling instead of remaining in a contracted state. If we had not shown that motor neurons were also in an electrical refractory period from being stimulated, the behaviors response might have been at odds with only measuring the light induced EPSPs. This result opens a new avenue of investigation into the biophysical properties of neurons being electrically excitable in functional neural circuits when inducing light sensitive channels. It is possible that cell bodies or even axons with different diameters may have varying thresholds of being activated when channel rhodopsins are activated due to varied input resistance of the cells., Also a larger surface area of the cell membrane which may provide for a higher density of channel rhodopsin proteins (Arlow et al., 2013). Fictive locomotion patterns measured in isolated larval *Drosophila* CNS, with genetically encoded Ca^{2+} indicators, demonstrated left-right asymmetry across segments (Pulver et al., 2015). This previous study suggested the asymmetry may arise to the larvae initiating a turning behavior. A follow up study would be to measure the Ca^{2+} flux and conditions presented in this study to determine if large alterations in symmetry occur while some neurons are in electrical refractory and possibly during an uncoordinated crawling behavior.

Despite not being able to electrically excite the motor neuron during the light induced refractory period there is a high frequency of the quantal events in a non-synchronized manner. This suggests channel rhodopsins are likely within the nerve terminal or pre-terminal membrane allowing Ca^{2+} to enter the terminal continuously while the blue light is on. The continuous occurrence of quantal events with the nerve in an electrical refractory period is probably due to the nerve's inability to completely reset the $[Ca^{2+}]_i$ with pumps and exchangers in the plasma membrane and/or ER (Mattson et al., 2000). The reduction in the rate of the quantal events over time is also an indication that the terminal is able to reach the homeostatic level of a resting state. However, this is deceiving since in a minute after the light induced refractory period is over, the motor nerve starts to fire again with a barrage of nerve evoked EPSPs in larvae raised in the dark with supplemented ATR. This could be examined by blocking calcium membrane pumps and the Na^{+}-Ca^{2+} exchanger to determine there is a prolonged action resetting the refractory period after activating the channel rhodopsins. The intact larvae in the behavioral tests likely remain contracted during the light refractory period due to the fact that the quantal events are occurring at a high rate in many NMJs and can depolarize the muscle enough to keep a Ca^{2+} load within the skeletal muscles. A high rate of randomized spontaneous quantal events is able to induce muscle contractions in larval *Drosophila* (Majeed et al., 2015).

It appears there is more complexity occurring in the properties of ion channels with a light induced refractory period and spontaneity of neural activity rather than just activating channel rhodopsin. The underlying causes to the light induced refractory remain unresolved. This finding compounds the difficulty in knowing how neural circuits within deep brain regions, laden with light sensitive channels are responding to synaptic events depending on the degree of light sensitivity or timing of the light exposures. The activity within a circuit may also influence the response of the neurons to activating light sensitive channels (Adamantidis et al., 2011). Thus, it will be an exciting challenge as the field moves forward to assess the light sensitive channels to intrinsically change neural activity under different conditions within behaving animals, and at the same time not to dampen the neural activity as the goal is to excite the circuit and vice versa.

The habituation to the onset of movements over time from the repetitive light pulses might be explained by either a reduced sensitivity to the light or the possibility that the neurons regulated a compensatory mechanism to the repetitive activity. However, in measuring the evoked EPSPs with repetitive light pulses every 10 min with 10 s light exposures revealed that the responses were less robust following each exposure. This was particularly evident for the larvae fed ATR. The larvae deprived of ATR also showed a reduced response over time but this reduction was not as pronounced. Thus, the electrophysiological responses would indicate that the behavioral habituation is due to the motor neurons not being as responsive to the blue light over time. As for mechanisms of the habituation, it would appear the ATR molecule itself or the associated channels result in some reduced response with repetitive optical stimulation. It is possibly the ATR or the ATR-channel complex undergoes a conformation change and the dark adaption time of 10 min is not long enough for new channels and ATR to be incorporated in the membrane. It is possible that a less sensitive channel rhodopsin constructs, or even ChR2-XXL expression without the addition of ATR in preparations which do not synthetize ATR, would produce more consistent responses for repetitive light exposures. The use of rhodopsin constructs which are less sensitive to light or different wavelengths of light (Zhang et al., 2008) for bet-

ter reproducibility in responses has been proposed by Grosenick et al. (2015) and Dawydow et al. (2014).

Maintaining consistent responses or levels of activation over time of the optically sensitive channels is important for developmental studies if one wishes to manipulate activity of neural circuits to address neuronal plasticity and factors involved in neuronal circuit formation or control (see review by Giachello and Baines, 2017). This is not only the case for neurons, but also other tissues in which optically activated proteins are being used. It is of interest to understand the mechanisms behind the habituation of the responses as well as the biophysical properties underlying the light induced refractory period. A possibility to be tested is that over depolarization of the nerve by the high frequency of neural activity resulted in voltage gated sodium channels inactivating thus producing a refractory period. This phenomenon was reported by Lin et al. (2009) but without presenting data (data not shown, Lin et al., 2009). The possibility of the PMCA and other pumps, such as the Na^+-K^+ pump, over compensating and lowering the resting membrane potential could reduce the basal voltage-gated sodium channel inactivation and result in a lower threshold (Nadim et al., 1995). Another possibility could be activation of calcium activated potassium channels keeping the cell hyperpolarized; thus, inhibiting the cell from firing when electrically evoked. Future investigations need to be conducted to determine the cellular mechanisms for the electrical refractory during channel rhodopsin activation. It is very likely that the long lasting sodium-dependent afterhyperpolarization, driven by the electrogenic activity of Na^+/K^+ ATPase, may be a contributing factor. The long lasting effect was shown in larval motor neurons and it was determined that number of spikes and not the short burst frequency is the contributing factor (Pulver and Griffith, 2010). This may well be the underlying mechanism to explain our observations with using this highly sensitive channel rhodopsin.

The possibility of the expressed proteins being targeted in other membranes besides the cell, or being taken up in membrane recycling, brings to light other phenomenon to consider. Could the synaptic vesicles themselves incorporate the ChR proteins due to their bilipid nature, and thus influence glutamate loading and even fusion events? As far as we are aware this possibility has not been directly addressed. It is known that mast cells use calcium release activated Ca^{2+} (CRAC) channels that are mediated by IgE dependent activation through an internal inositol 1,4,5-triphosphate (IP3) system (Ashmole and Bradding, 2013), which would be similar to the ER within the nerve terminal during Ca^{2+} dumping. Thus, if ChR proteins were loaded into the ER membranes and activated in addition to the plasma membrane of the cell there would be various means of loading Ca^{2+} within the synaptic terminal resulting in vesicle fusion. A similar scenario of Ca^{2+} dumping could occur for mitochondria present within the nerve terminals. Even if the mitochondria were to be loaded with Ca^{2+}, due to the Ca^{2+} ions coming across the cell membrane and into the mitochondria, when light is present it could result in transit mitochondria damage which would result in Ca^{2+} dumping (Kislin et al., 2016). Likewise, if the lysosomes happen to incorporate ChR proteins and then are activated by light the result would be a Ca^{2+} surge within the terminal (Brailoiu and Brailoiu, 2016). There is precedence for proteins to be expressed and targeted to the ER which appear to have no functional use in the membrane of the ER. There is evidence that nicotinic acetylcholine receptors (nAChRs) can occur on Endoplasmic Reticulum (ER) derived microsomes (Moonschi et al., 2015), which suggest cell trafficking of proteins can result in ion channels being directed to other localizations besides the cellular plasma membrane and be functional (Colombo et al., 2013). Possibly using a pharmacological approach with Brefeldin A would prevent the vesicle formation from the endosome (Park et al., 2016). Thus, vesicles would not contain the channel rhodopsin from this means but blocking this pathway would also alter recycling of vesicle for synaptic transmission and would not necessarily inhibit channel rhodopsin from being in the ER or even to the vesicles by other means such as lipophilic attraction.

Another issue that could probably have an impact on cellular function is high intensity LED lights may cause organelle damage (Chamorro et al., 2013). The expression systems induced a high level of protein syntheses, such as channel rhodopsin, in cells which can interfere with native cellular processes (Palomares et al., 2004). In addition, the turnover rate of the channel rhodopsin may also be dependent on the frequency of activation and overall cellular activity (Ullrich et al., 2013). The amount of ATR in the diet or the endogenous production of ATR, depending on the animal model, is an aspect to consider in working out the effects on the channels sensitivity over longer period of time. These matters produce a changing target in order to deliver consistent optical responses in a cell. Despite some of the experimental problems with use of channel rhodopsins that we and others have encountered, there is promise that the use of light activated channels will provide technical breakthroughs for experimentation and potential use in therapeutics as well as long term manipulation during development of tissues, particularly of defined neural circuits (Iaccarino et al., 2016).

Author contributions

The contributions of the authors are that all authors participated in experimental design, implementation of the experiments and analysis as well as construction of the manuscript.

Conflict of interest

The authors declare no conflicts of interest.

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