Patterned light stimulation, does it affect neuronal activity?

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Abstract

Neuronal sensitivity to light stimulation can be a significant confounding factor for assays that use light to study neuronal processes, such as optogenetics and fluorescent imaging. While continuous light stimulation has been shown to be responsible for a decrease in firing activity in several neuronal subtypes, discontinuous light stimulation commonly used in optogenetic experiments is supposed to have a negligible action. In the present report, we experimentally test this theoretical prediction by evaluating the effect produced by ten of the most commonly used patterns of discontinuous light stimulation under several electrophysiological parameters.

Introduction

The continuous light stimulation of brain tissue has been shown to affect the electrophysiological properties of several neuronal subtypes,*in vitro* and *in vivo* , by producing a modification of firing rate associated with a hyperpolarizing outward current (Stujenske*et al.* , 2015; Ait Ouares *et al.* , 2019; Owen *et al.* , 2019) . The sensitivity of neurons to light can therefore represent a confounding factor for those assays that use light as a tool to investigate neuronal processes such as optogenetics and fluorescent imaging. Because the effect of light is primarily mediated by the increase in tissue temperature and discontinuous light has a weaker heating action than continuous light, it can be assumed that the use of specific patterns of discontinuous light stimulation is preferable to avoid non-specific effects. In particular, theoretical models (Stujenske*et al.* , 2015) and experimental measurements (Senova *et al.* , 2017) have demonstrated a decrease in light-induced heating concurrent with decreasing power, frequency and duty cycle of light stimulation. For example, 10 mW of discontinuous light stimulation at 10 Hz with a duty cycle of 10 % is responsible for a temperature increase equivalent to that produced by 1 mW of continuous light stimulation (Stujenske *et al.* , 2015). As we previously showed that the latter protocol had no impact on neuronal activity (Ait Ouares *et al.* , 2019), 10 mW of discontinuous light stimulation at 10 Hz should also be free of artifactual effect assuming that light effects are exclusively due to temperature increase. However, light has also been shown to be responsible for temperature independent effects (Ait Ouares *et al.* , 2019; Tyssowski & Gray, 2019), including the activation of encephalopsins (Friedmann *et al.* , 2015; Wang *et al.* , 2019) or cytotoxic processes (Duke *et al.* , 2020). It is therefore important that theoretical predictions about the "safety" of a specific pattern of optical stimulation be confirmed by empirical evidence. Based on a literature review using the PubMed query "Optogenetics", we selected 10 different patterns of discontinuous light stimulation commonly used in optogenetic research and we tested their impact on several electrophysiological properties of mitral cells (MCs). More specifically, we analyzed the effects of light on the generation of outward hyperpolarizing current as well as on the amplitude and latency of action potentials (APs) induced by short steps of positive current,

as well as on neuronal firing produced by continuous depolarization. All these parameters were consistently modified by continuous light stimulation (Ait Ouares *et al.* , 2019). We therefore tested whether patterned light stimulation would be responsible for: 1) the generation of an outward current, 2) a decrease in AP amplitude, 3) an increase in AP latency and 4) a reduction in firing frequency. Finally, the pattern producing the most pronounced effects in MCs was also applied to cortical pyramidal neurons (PYRs) and fast-spiking interneurons (FSIs), in striatal medium-sized spiny neurons (MSNs) and in hippocampal granular cells of the dentate gyrus (GCs).

Methods

Animals.

Male C57Bl6/J mice (Charles River Laboratories, France) aged between 60 and 90 days were used. To identify FSIs, mice expressing the fluorescent tomato protein in parvalbumin neurons were generated by crossing B6;129P2-Pvalbtm1(cre)Arbr/J strain with B6.Cg-Gt(ROSA)26Sortm9(CAG-tdTomato)Hze/J strain from Jackson Laboratory11The initial proposal planned to use wild type mice. The use of transgenic mice expressing tomato protein in parvalbumin neurons was introduced to identify the fast spiking interneurons in cortical slices. This changes in the protocol received editorial approval prior to data collection, on 9 Feb 2022.. All procedures were in accordance with European Union recommendations for animal experimentation (2010/63/UE). Mice were housed in groups of up to five in standard laboratory cages and were kept on a 12-hour light/dark cycle (at a constant temperature of 22°C) with food and water ad libitum.

Electrophysiology.

Protocols for slice preparation and recordings of olfactory bulb, hippocampal and cortical neurons.

Mice were anaesthetized with an intra-peritoneal injection of ketamine (50 mg/ml) and decapitated. The head was quickly immersed in ice-cold (2-4°C) artificial cerebrospinal fluid (CutACSF) of the following composition: 125 mM NaCl, 4 mM KCl, 25 mM NaHCO3, 0.5 mM CaCl2, 1.25 mM NaH2PO4, 7 mM MgCl2 and 5.5 mM glucose (pH = 7.4 oxygenated with 95 % O2/5 % CO2). The osmolarity was adjusted to 320 mOsm with sucrose. Horizontal olfactory bulb (OB) slices (400 µm thick), cortical coronal slices (400µm thick) and hippocampus coronal slices (350 µm thick) were prepared with a vibratome (Leica). Slices were then incubated in a recovery chamber at 30 ± 1°C using an ACSF solution with a composition similar to the Cut ACSF, except for changes to $CaCl_2$ and $MgCl_2$ concentrations (2 mM and 1 mM, respectively). Slices were transferred to a recording chamber mounted on an upright microscope and continuously perfused with oxygenated ACSF (4 ml/min) at 30 ± 1°C. Neurons were visualized using a microscope (Zeiss axioscope) with a 40X objective (Zeiss Plan-APOCHROMAT). Data were acquired with the amplifier RK 400 BioLogic at full sampling frequency of 25 kHz using a 12-bit A/D-D/A converter (Digidata 1440A, Axon Instruments) and PClamp software (PClamp10, Axon Instruments). Patch-clamp whole-cell recordings were achieved with borosilicate pipettes having a resistance 4-8 M and filled with: 126 mM K-gluconate, 5 mM KCl, 10 mM HEPES, 1 mM EGTA, 1 mM MgCl2, 2 mM ATP-Na2, 0.3 mM GTP-Na3, and 10 mM phosphocreatine (pH = 7.3, 290 mOsm). The membrane potential was corrected for the junction potential (-15 mV). All experiments were performed in the presence of ionotropic receptor antagonists (NBQX 10 µM, APV 40 µM and Gabazine 10µM).

Protocol for slice preparation and recordings of striatal neurons . Mice were deeply anesthetized with ketamine/xylazine (100/10 mg/kg, i.p.) and transcardially perfused with an ice-cold N-methyl D-glucamine (NMDG)-based solution containing (in mM): 93 NMDG, 2.5 KCl, 1.2 NaH_2PO_4, 30 $NaHCO_3$, 20 HEPES, 20 glucose, 10 $MgCl_2$, 93 HCl, 2 Thiourea, 3 sodium pyruvate, 12 N-acetyl cysteine and 0.5 $CaCl_2$(saturated with 95% O_2 and 5% CO_2, pH 7.2-7.4). The brain was then removed from the skull and glued to the stage of a vibratome (Leica, VT1000S) where it remained submerged in ice-cold oxygenated NMDG-based solution. Coronal slices (250 µm thick) containing the striatum were collected. Slices were immediately transferred to recover in NMDG-based solution at 35°C for 5 min and then stored for at least 1h at room temperature in normal artificial cerebrospinal fluid (ACSF) containing (in mM): 126 NaCl, 2.5

KCl, 1.2 MgCl2, 1.2 NaH2PO4, 2.4 CaCl2, 25 NaHCO3 and 11 glucose, to which 250 µM kynurenic acid and 1 mM sodium pyruvate had been added. For the recordings, slices were transferred one at a time to a submersion-type chamber and perfused continuously with warm ACSF (32-34°C) at a rate of 3 ml/min. Solutions were continuously equilibrated with 95% O2 / 5% CO2. All experiments were performed in the presence of ionotropic receptor antagonists (NBQX 10 µM, APV 40 µM and Gabazine 10µM). MSNs were visualized on an upright microscope (Nikon Eclipse FN1) equipped with DIC optic using an IR 40x water-immersion objective (Nikon). Patch-clamp electrodes (4-6 MΩ) were prepared from filamented borosilicate glass capillaries (PG150T-7.5, Harvard Apparatus) using a micropipette puller (PC-10, Narishige) and were filled with an intracellular solution containing (in mM): 126 KMeSO4, 14 KCl, 3 MgCl2, 0.5 CaCl2, 5 EGTA, 10 HEPES, 2 NaATP and 0.5mM NaGTP, 10 Na-Phosphocreatine, pH adjusted to 7.25 with NaOH and osmolarity adjusted to 270-280 mOsm/L. Recordings were obtained using motorized micromanipulators (MP-85, Sutter Instrument), a Multiclamp 700B amplifier, a Digidata 1550B digitizer, and pClamp 10.7 acquisition software (Molecular Devices, San Jose, CA, USA). Signals were low-pass filtered at 10 kHz online and sampled at 10 kHz. Electrode capacitances were compensated electronically during recording. In current-clamp mode, the bridge was continuously balanced and input resistances were monitored. Cells showing more than 20% input resistance variation were excluded from the analysis.

Optical stimulation and fluorescence detection.

Olfactory bulb, hippocampal and cortical experiments. Blue light stimulation at 470 nm (430-495 nm) was performed using a Dual Port OptoLED (CAIRN, UK) dichroic mirror 495 nm at a power of 13, 5 or 1 mW measured at the output of the x40 objective. Power loss at the working distance (2.5 mm from the objective), due to the presence of ACSF, was empirically estimated at 13%. The average power density in the tissue was estimated by dividing the power at the working distance by the empirically measured illumination area (˜ 7 mm^2) which gives 1.61 mW/mm^2 for a output power of 13 mW , 0.62 mW/mm^2 for 5 mW and 0.12 mW/mm2 for 1 mW. The excitation light for the detection of tomato expressed in cortical parvalbumin interneurons was produced by a white light LED (Dual Port OptoLED, CAIRN, UK) filtered at 545/25 nm and reflected on the sample by a dichroic mirror of 570 mn (Zeiss). The emission light was filtered at 605/70 nm (Zeiss filter). To avoid an effect of the light for tomato detection on neuronal activity, only short light pulses (<100 ms) at power < 0.2 mW were used. The illumination frequency was lower than 0.5 Hz, the total time of green led illumination was lower than 2s and a minimum of 3 min was applied between green and blue light stimulation.

Striatal experiments. Light was delivered under the control of the acquisition software via the x40 microscope objective lens using wide-field 475 nm LED illumination (Spectra Light Engine, Lumencor, Optoprim).

Temperature measurement.

Bath temperature in the recording chamber was measured and controlled by a ThermoClamp-1 device (Atomate Scientific).

Experimental procedure.

For MC recordings, light stimuli consisted of fifty pulses delivered according to one of the ten different patterns showed in table 1 (N=24 MCs for each pattern). For experiments in MSNs, GCs, PYRs and FSIs, only the pattern producing the maximal effect on MCs was tested (pattern 4, N= 15).

Procedure 1: determining the profile of temperature modification produced by light patterns

The thermal modifications produced by the different stimulation patterns was determined by placing the thermal probe on the surface of OB slices at the center of the objective field. Each pattern was repeated 10 times (interstimulus time: 10 s) and the median normalized temperature trace was used to calculate the temporal profile of temperature variation for each pattern in each slice. This procedure was repeated on 10 different slices and the comparison between patterns was performed on the average trace (± 95% confidence interval).

The following experimental procedures 2, 3 and 4 were applied to each recorded neuron.

Procedure 2: Calculation of the membrane resistance (Rm)

Cells were held at -60 mV in voltage-clamp mode. Ten steps to -65 mV (1s duration @ 1 Hz) were generated and recorded on fifteen separate electrophysiological traces and a median trace was calculated. The Rm was calculated on the median trace according to ohm's low $R=\Delta V/\Delta I$ where ΔV=-5mV and ΔI was the difference between the median current in the last 100 ms of the hyperpolarization and the median current in the 200 ms pre-step period. Access resistance A_R was calculated as $A_R=\Delta V/\Delta I_A$ where ΔI_A is the difference between the peak of transient current and the median current in the 200 ms pre-step period.

Procedure 3: Determination of light effect on membrane currents

Cells were held at -60 mV in voltage-clamp mode. The stimulation pattern was repeated three time at 10 s intervals for the three powers (1mW, 5mW, and 13mW). The discontinuous light patterns were followed, after 10 s, by 1 s of continuous light stimulation. Since 1 s stimulation was expected to produce a consistent outward current (Ait Ouares *et al.* , 2019), the effect of continuous stimulation was used as positive control. In the protocols for which the total illumination time (T.I) during patterned stimulation was different than 1s, a continuous T.I. light step was also applied. Fifteen electrophysiological traces were recorded for each neuron, these were aliened on the pre-light control period and a median trace was calculated. The light effect was quantified on the median trace by subtracting, for each of the 50 light steps, the average membrane current in the 1 s preceding the application of light (the CTR period) from the average membrane current during light period. Only one of the ten stimulation patterns was applied on each MC. Procedures 2 and 3 were performed on the same electrophysiological acquisition.

Procedure 4: Determination of the light effect on AP amplitude and latency.

Cells were recorded in current-clamp mode at their resting membrane potential. In cases where spontaneous firing was present, a hyperpolarization of the membrane potential was produced by injecting negative current. Two consecutive trains of 50 APs, separated by 10 s, were generated with 5 ms depolarizing steps applied at the frequency of the light stimulation pattern chosen for that specific neuron. In half of the recorded neurons, the first train was generated in the absence of light and the second train generated during light stimulation, with the depolarizing steps concomitant to the last 5 ms of the illumination. In the other half of the neurons, light stimulation train preceded the no light train. After 10 s, a single depolarizing step (test step) was generated at the end of a 1 s continuous light stimulation. Since the latter procedure is expected to produce a consistent modification of AP amplitude and latency (Ait Ouares *et al.* , 2019), the AP generated by the test step was used as positive control. In the protocols for which the total illumination time (T.I) during patterned stimulation was different from 1 s, one AP was also generated at the end of the continuous T.I. step. This protocol was repeated 10 times for each neuron. For each of the ten acquired traces, the effect of light was assessed trough a step-by-step comparisons of AP in the absence and in the presence of light (i.e., 1st AP no LED vs 1st AP LED, 2^{th} AP no LED vs 2^{th} AP LED$\cdots\cdots$ 50^{th} AP no LED vs 50^{th} AP LED). The AP of the test step and T.I. step was compared to the 1st AP generated in the no LED condition. For each AP, the latency was quantified as the time of the AP peak relative to the beginning of the depolarizing step (Ait Ouares *et al.* , 2019). AP amplitude was calculated as the difference between AP peak and the median membrane potential calculated in the 200 ms preceding the first depolarizing step. Steps presenting AP failures, or AP for which the time of AP peak exceeds the step duration, were excluded from the analysis. In half of the recorded neurons, the steps of current in the no-light condition preceded the steps of current in the light condition and vice versa for the other half of the neurons. The effect of light on each step was expressed as the average difference in AP amplitude and latency, between the light and no light conditions for that specific step. Only one out of the ten stimulation patterns was applied on each MC.

Procedure 5: Determination of the light effect on average firing frequency on MCs.

The protocol was the same as that described in procedure 4 except that the current steps for AP induction were replaced by a long-lasting depolarization. The average firing frequency under light and no light condition

was calculated during a 2.5-s current-induced depolarization (N=15 MCs).

Statistics:

Since the normality of the data was not known and normality tests have low statistical power for a sample size of 24 (Öztuna *et al.* , 2006), non-parametric one-sample Wilcoxon signed rank test and one way Kruskal-Wallis test were used for the analysis. Type I error probability was kept at 5% by using Bonferroni-Holm method for correction of multiple comparisons (stats.multitest.multipletests function from Statsmodels module, Python, Holm method).

Data analysis and statistics:

For each of the ten patterns, the hypotheses tested were:

Hypothesis 1 : patterned light stimulation is responsible for a decrease in AP amplitude.

Hypothesis 2 : patterned light stimulation is responsible for an increase in AP latency.

Hypothesis 3 : patterned light stimulation is responsible for the generation of an outward current.

Hypothesis 4: patterned light stimulation is responsible for a decrease in the average firing frequency.

The sample sizes required to have a statistical power > 90 % were estimated based on the Cohen'd effect size observed in Ait Ouares et al. (2019) (ES = mean of light effect /standard deviation of light effect).

Hypothesis 1 was tested as follows:

For each light pulse, the effect of light on AP amplitude was calculated as the difference in AP amplitude between the LED condition and the no LED condition (i.e., 1st AP amplitude LED – 1st AP amplitude no LED; .....; 50^{th} AP amplitude LED – 50^{th} AP amplitude no LED). For each MC the median effect was calculated. This resulted in the median light effect for each of the 50 pulses (i.e., effect of light on the amplitude of the 1st AP; .....; effect of light on the amplitude of the 50^{th} AP). The values calculated for all recorded cells were used for descriptive statistics by calculating the average population effect ($\pm$ 95% CI) as well as the Cohen's d ES (average of population effect /STD of population effect). Similar analyses were performed for the positive control (test LED) and the total continuous illumination exposure (T.I. LED), for which the effect was evaluated as: LED AP amplitude - 1st AP amplitude no LED. In order to reduce the probability of Type I error produced by multiple comparisons correction, statistical tests were made only for three light pulses (the 1st, 25^{th}, and 50^{th}) by using a unilateral one-sample Wilcoxon signed rank test (tested hypothesis, median effect < 0). The σ risk level was set at 0.05 and Bonferroni-Holm correction was applied. Experiments were conducted on 24 neurons for each pattern of light stimulation and cell type (336 neurons in total). This sample size was determined based on a previously published experiment on MCs where continuous light stimulation reduced AP amplitude with ES = -1.2 (Ait Ouares *et al.* , 2019) and takes into account the Bonferroni-Holm correction for 30 statistical comparisons. As a consequence, the statistical power to observe an effect as small as that produced by 1 s continuous light stimulation is estimated between 98% et 99% depending on the level of correction (calculated with G*Power 3.1.9.2, (Faul *et al.* , 2007). The 24 MCs were recorded from 57 mice. The homogeneity of the effect produced by the "Test LED" between the groups was assessed by one-way Kruskal-Wallis test. Exploratory analysis was performed to evaluate a possible difference between the average AP amplitude (over the 50 depolarizing steps) in the presence and in the absence of patterned light stimulation.

Hypothesis 2 was tested as follows:

The general procedure was the same as for testing the hypothesis one except that AP latency was measured instead of AP amplitude

Exploratory analysis was performed to evaluate whether light stimulation modifies other parameters of the AP such as AHP amplitude and action potential full width at half maximum (FWHM).

Hypothesis 3 was tested as follows:

Light effect on membrane current was calculated on the median trace of the 15 recorded traces. For each of the 50 light pulses, the effect of light on membrane current was calculated as the difference between the average membrane current in the 1 s preceding the beginning of patterned light (the CTR period) and the average membrane current during the light period. For each of the three powers used, this resulted in the median light effect for each of the 50 pulses (i.e., effect of the 1^{st} light pulse; .....; effect of the 50^{th} light pulse). The values calculated for all recorded neurons were used for descriptive statistics by calculating the average population effect ± 95% CI as well as the Cohen's d ES (average of population effect /STD of population effect). Similar analyses were performed for the positive control (Test LED) and the total continuous illumination (T.I. LED), for which the effect was evaluated as the difference between the average membrane current during light stimulation and the average membrane current in the respective CTR period. Statistical test was performed only for three light pulses (the 1^{st}, 25^{th}, and 50^{th}) by using a unilateral one-sample Wilcoxon signed rank test (tested hypothesis, median effect >0). The σ risk was set at 0.05 and a Bonferroni-Holm correction was applied. The experiment weas conducted on 24 neurons for each pattern of light stimulation and cell type (240 neurons in total). This sample size was determined based on a previously published experiment showing an effect size (ES) of continuous light stimulation on MC membrane current equal to 1 (Ait Ouares *et al.* , 2019) and takes in account the Bonferroni-Holm correction for 30 statistical comparisons. Therefore, the statistical power to observe an effect as small as that produced by 1 s continuous light stimulation is between 91% et 99%, depending on the level of correction (calculated with G*Power 3.1.9.2). The 24 MCs were recorded from 51 different mice (i.e., different patterns were applied on slices coming from the same animal). The homogeneity between the groups produced by the "Test LED" was assessed by a one-way Kruskal-Wallis test.

Hypothesis 4 was tested as follows:

For each of the 10 repetitions on each recorded neuron, the effect of light on AP frequency was calculated as the difference between the firing frequency in the LED condition and the firing frequency in the no LED condition. For each neuron, the median effect of light was calculated. The values calculated for all recorded neurons were used for descriptive statistics by calculating the average population effect ± 95% CI as well as the Cohen's d ES (average of population effect /STD of population effect). Statistical tests were performed using unilateral one-sample Wilcoxon signed rank test (tested hypothesis, median effect <0). The σ risk level was set at 0.05. The experiment was conducted on 15 neurons. This sample size was determined based on a previously published experiment on MCs and MSNs, in which continuous light stimulation reduced firing frequency with an ES=-1 at -1.6, respectively (Ait Ouares *et al.* , 2019). Therefore, the statistical power to observe an effect as small as that produced by 1-s continuous light stimulation was between 97% and 99% (calculated with G*Power 3.1.9.2).

See supplementary table 1 for a summary of statistical analysis.

The results obtained from procedure 1 were used for exclusion criteria based on Rm (see later) as well as for exploratory analysis aimed at correlating the eventual effect of light stimulation with Rm.

Analyses were performed with custom scripts (Python 3.7).

Data sharing.

All raw electrophysiological traces, scripts for the analysis and raw data are accessible via the Open Science Framework website (https://osf.io/fe6t3/)

Exclusion criteria.

Cells were excluded from experiments when i) their resting membrane potential (Vrest) was above -50 mV, ii) the membrane resistance was higher than 1000 M, and iii) no AP could be elicited in the no light condition. Traces were excluded from the analysis when: i) the starting Vm (calculated in the 100 ms preceding the depolarizing current step) in the no light condition differed by more than 5 mV from the starting Vm in the light condition, ii) there was an instability in membrane potential, and iii) a degradation of neuronal whole-cell recording (depolarization of resting potential > 10 mV, AP overshot below 0mV, or access resistance >

60 m 11Revised from initial proposal of access resistance > 40 m, after editorial approval on 13 Sept 2021. Analysis using 40 m criteria lead to qualitatively similar results that are available at https://osf.io/amvkq).

Competing Interests: the authors declare no competing financial interests.

Results

3.1 Tissue temperature increases with light duty cycle and pulse duration during patterned light stimulation (procedure 1)

We first evaluated the modification of tissue temperature when fifty blue light pulses were delivered to OB slices following the stimulation patterns described in table 1. The temperature of the tissue gradually increased over the fifty pulses for all patterns (Figure 1, inset). The higher the power, the duty cycle, and pulse duration, the more pronounced the temperature increase (Figure 1). Interestingly, temperature change did not appear to be correlated with the frequency of the stimulation within each duty cycle. At 13mW, most of the patterns induced a temperature increase that overcame 0.1 °C, a thermal condition that has been previously associated with modifications in neuronal activity (Ait Ouares *et al.* , 2019; Owen *et al.* , 2019).

3.2 Patterned light stimulation produces an outward current in MCs (Procedure 3)

The effect produced by the ten different patterns on MC membrane current was studied using the protocol depicted in figure 2A. Whatever the pattern, light intensities of 1mW had no effect, while for 5mW, the light tended to produce an outward current that did not reach statistical significance (Figure 2B and supplementary Figures 1-2). This lack of effect appears mainly to result from the low statistical power produced by the correction for multiple comparisons (see p-values before correction https://osf.io/e6brs). Moreover, the exploratory analysis performed by calculating the average effect produced over all 50 light pulses produced a small but significant outward current for all patterns (supplementary table 2). When the light power was set at 13mW, the light induced an outward current that tended to increase with pulse repetition (Figure 2B, C and supplementary Figure 1). A significant effect was consistently observed at the 50th pulse for most of the patterns (Figure 2C). Similar to the effects of light on tissue temperature, the amplitude of the light-induced outward current increased with duty cycle. The effect of the pulse duration was less pronounced (figure 2C). The difference between patterns cannot be attributed to sample variability since the effect produced by the test LED was the same for all patterns (Supplementary figure 1, Kruskal-Wallis test, H=0.79, p= 0.612). For patterns with a high duty cycle, the effect produced by the continuous stimulation (TI LED) was not different from the effect produced by patterned light. However, for lower duty cycles, discontinuous stimulation produced a lower effect than continuous ones (Figure 2C). Interestingly, exploratory analysis showed a covariation between the effect produced by each pattern on tissue temperature and the generation of outward current (Figure 4A). These results suggest a causal link between the light effect on MC electrophysiological properties and the light-induced modification of tissue temperature, supporting previous findings (Ait Ouares *et al.* , 2019; Owen*et al.* , 2019).

3.2 Patterned light stimulation reduces AP amplitude without affecting AP latency in MCs (Procedure 4)

The effect produced by the different patterns on AP properties was studied using the protocol depicted figure 3A. Most of the recorded neurons were previously submitted to the voltage-clamp protocol shown in figure 2A. Light stimulation induced a reduction in AP amplitude that reached a significant effect only for patterns 3, 4, 6, 7 and 8 (Figure 3B-C and supplementary figure 3). The lack of effect of the other patterns is likely due to the low statistical power that follows the Bonferroni-Holm correction. Indeed, with the exception of pattern 1, all patterns had a p-value lower than the α risk (0.05) before the correction (see *https://osf.io/h7g8p/*). Moreover, exploratory analyses for which the effect was assessed on all 50 APs showed a significant decrease in AP amplitude for all tested patterns (supplementary table 3). Again, the light effect was higher for patterns that have a longer duty cycle. However, the covariation between the duty cycle and the AP amplitude reduction was less pronounced than the covariation between the duty cycle and the light-induced outward current shown in figure 2. In addition, no clear relationship emerged between step

duration and AP amplitude reduction. The effect of light on AP amplitude had a moderate covariation with the tissue temperature increase produced by the different patterns (Figure 4B). Patterned light did not alter AP latency (Figure 3 C, bottom, and supplementary figure 4). We further performed exploratory analysis to look for other eventual modifications produced by light on other AP parameters. In particular, we looked at the full width at half maximum (FWHM) and at the AHP amplitude. Both of these parameters were reduced by patterned stimulation (supplementary table 3). To test whether light also alters APs during more physiological stimuli, we induced neuronal firing with longer pulses of current (2.5 s). We used only the pattern and power that produced the largest overall effect in previous tests (pattern 4 at 13mW). As shown in figure 5, light stimulation had a heterogeneous action on MC population. The firing activity was mildly reduced or unaffected in around 80% of the recorded neurons and strongly reduced in the remaining cells. The average frequency modification was -16 ± 30 Hz (N= 15, p=0.013).

Effect of patterned light stimulation on other neuronal types (procedures 3 and 4)

We next determined whether discontinuous light stimulation also affects the activity of GCs, PYRs, FSIs and MSNs by using the pattern that produced the largest effect on MCs (pattern 4 at 13mW power). While continuous light stimulation induced an outward current in all cell types (Ait Ouares *et al.* , 2019), discontinuous stimulation had this effect only in PYRs, GCs and MC (figure 6A). Interestingly, in one of the recorded GC, we observed a clear inward current upon light stimulation (supplementary figure 6) suggesting that light can have an excitatory action on a small fraction of neurons. Patterned light reduced AP amplitude in all neuronal types, but this effect was less pronounced than that produced by continuous stimulation (Figure 6B). Similar to our observation for MCs, we did not find any effect of patterned light on AP latency (supplementary figure 7).

Discussion

The main conclusion of this study is that a significant number of discontinuous light stimulation patterns, commonly used in optogenetic experiments, can affect neuronal electrophysiological activity. However, their impact is, in general, smaller than that produced by continuous light stimulation. It also highlights how the different parameters of the stimulation impact the light action, providing several guidelines to minimize the artefactual effects associated with the use of light in neuroscientific investigations. Indeed, a bibliographic analysis done on the first 50 discontinuous patterns retrieved when using the query "Optogenetics" on PubMed showed that the light parameters investigated here are among the most commonly used in optogenetic experiments (https://osf.io/anvsj). We used the change in AP amplitude and latency and the generation of an outward current as a read-out for light action, all of which were modified by continuous light stimulation (Ait Ouares*et al.* , 2019). We found that patterned light consistently reduced AP amplitude without significantly altering AP latency in all cell types and produced an outward current only in MCs, PYRs and GCs. The lack of effect on AP latency is likely related to the low statistical power of the pre-registered analytical method, since exploratory analysis shown in supplementary table 3 suggest that AP latency does indeed slightly increase during patterned light stimulation. The effects of light on membrane current and AP amplitude are small, with a change of only a few pA for membrane current and a ˜0.5 mV reduction in AP amplitude for the pattern with the highest effect. However, even small variations in AP amplitude can consistently modify synaptic transmission (Rama *et al.* , 2015). Moreover, discontinuous light produced a strong reduction in firing frequency for a subpopulation of MCs when neurons where depolarized to a membrane potential that allow spontaneous activity. A possible reason for the heterogeneity of this effect might be explained by a diversity of biophysical properties among different MCs (Padmanabhan & Urban, 2010; Fourcaud-Trocmé *et al.* , 2022). However, the effects of discontinuous light where in general lower than those produced by continuous stimulation showing that patterned stimulation can reduce the artifactual effect of light. Both the induction of the outward current and the reduction in AP amplitude induced by discontinuous light occur rapidly after few pulses and quickly reach a plateau. Therefore, reducing the number of stimuli does not appear to be an optimal strategy to reduce the artefactual effect of light. On the other hand, the light effect can be attenuated by reducing the stimulation power, the duty cycle and, in some cases, the pulse duration. Note that varying the frequency of the light stimulation does not change the artefactual effects as

long as the duty cycle is the same. This is particularly important for experiments whose goal is to induce high frequency firing by optogenetic stimulation. As a rule, we recommend adapting the pulse duration to the stimulation frequency in order to keep the duty cycle at or below 5%. Using a light power of 1 mW or less also appears to be an effective means of preventing unspecific effects during discontinuous stimulation compared to the higher powers, which induce an outward current (see supplementary table 1). The reduction in AP amplitude induced by pattern 4 is observed in all cell types, whereas an outward current is induced only in FSIs and MSNs, suggesting a cell-specific effect of light. The greater reduction in the discharge frequency of FSIs induced by light compared to pyramidal neurons (Owen *et al.* , 2019) also suggests a differential effect of light across cell types. However, we did not observe such a difference here where all experiments were done in the presence of ionotropic glutamatergic receptor antagonists. Since light appears to reduce glutamatergic synaptic transmission (Ait-Ouares et al., 2019), the pronounced decrease in FSI firing observed by Owen et al., (2019) may be related to a reduction in their excitatory inputs. However, we cannot rule out the possibility that the weaker effect observed here is due to the presence of the tomato fluorescent protein in the recorded FSI. The covariation between the change in tissue temperature and the effect of light suggests a causal relationship between the two factors, as previously reported (Stujenske *et al.* , 2015; Senova *et al.* , 2017; Ait Ouares *et al.* , 2019; Owen *et al.* , 2019). The light-induced temperature changes observed in this study (between 0.1 and 0.8 °C) remain below the threshold for tissue damage but may modulate a set of voltage-dependent channels involved in neuronal discharge (Podgorski & Ranganathan, 2016; Ait Ouares *et al.* , 2019). Our results suggest that light is able to modulate different type of K^+ channels beside the inwardly rectifying potassium channels (Kir) (Owen *et al.* , 2019). For instance, the reduction in AP duration suggests an increase in Kv conductance's responsible for AP repolarization (Bean et al., 2007) while the reduction in AHP amplitude suggests an impact of light on the calcium- and voltage-dependent potassium channels (Sah & Faber, 2002). The light-induced reduction in AHP amplitude after a single AP contrasts with the increase in AHP measured at the end of a long depolarization that we observed previously (Ait Ouares *et al.* , 2019). This result suggests that light may oppositely modulate the K^+ channels involved in the fast-medium and slow AHP. However, because the AHP in MCs is modulated by recurrent glutamatergic transmission (Duménieu *et al.* , 2015), we cannot rule out that the light-induced reduction in AHP amplitude is partially due to the presence of ionotropic glutamatergic receptor antagonists. Finally, because AP initiation in MCs is promoted by hyperpolarizing events (Fourcaud-Trocmé *et al.* , 2022), the light-induced decrease in AHP could also contribute to the reduction of the firing frequency of these cells.

Conclusions:

Prevention of off-target effects during optogenetic stimulation is fundamental for reducing misinterpretation of experimental results. The use of discontinuous light at low power levels and/or duty cycle can achieve this goal. However, such conditions could reduce the efficiency of optogenetic protocols in neurons expressing first-generation opsins, a limitation that can be overcome by using new-generation opsins such as ChRmine or ChroME (Marshel *et al.* , 2019; Sridharan *et al.* , 2022).

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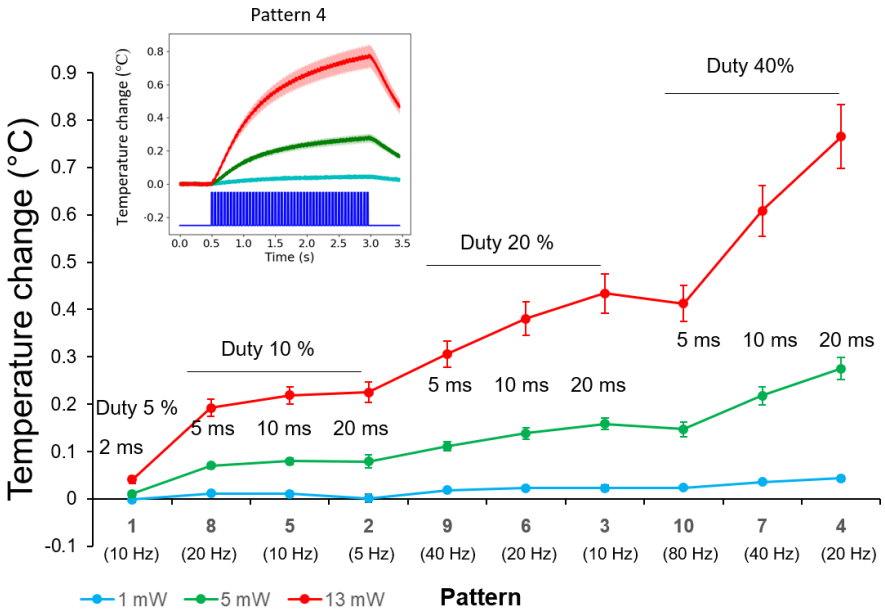

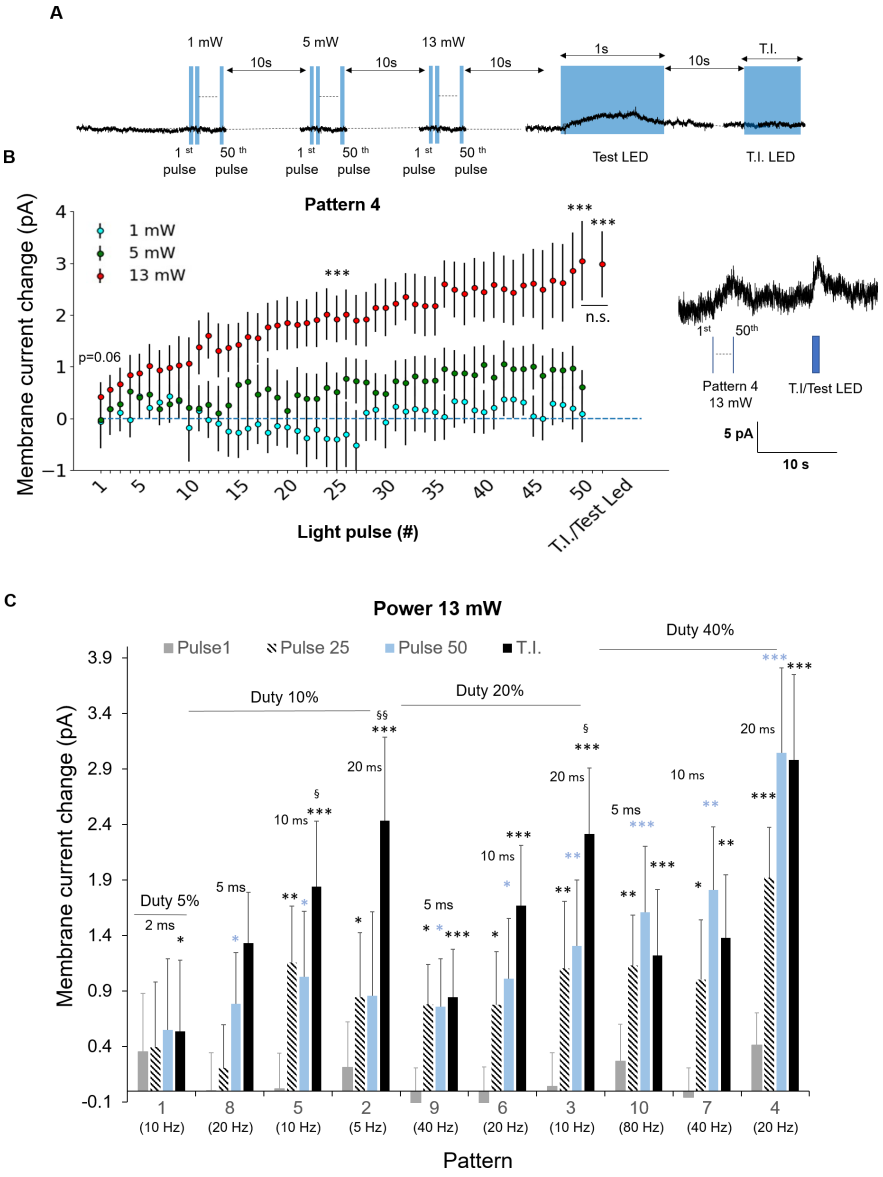

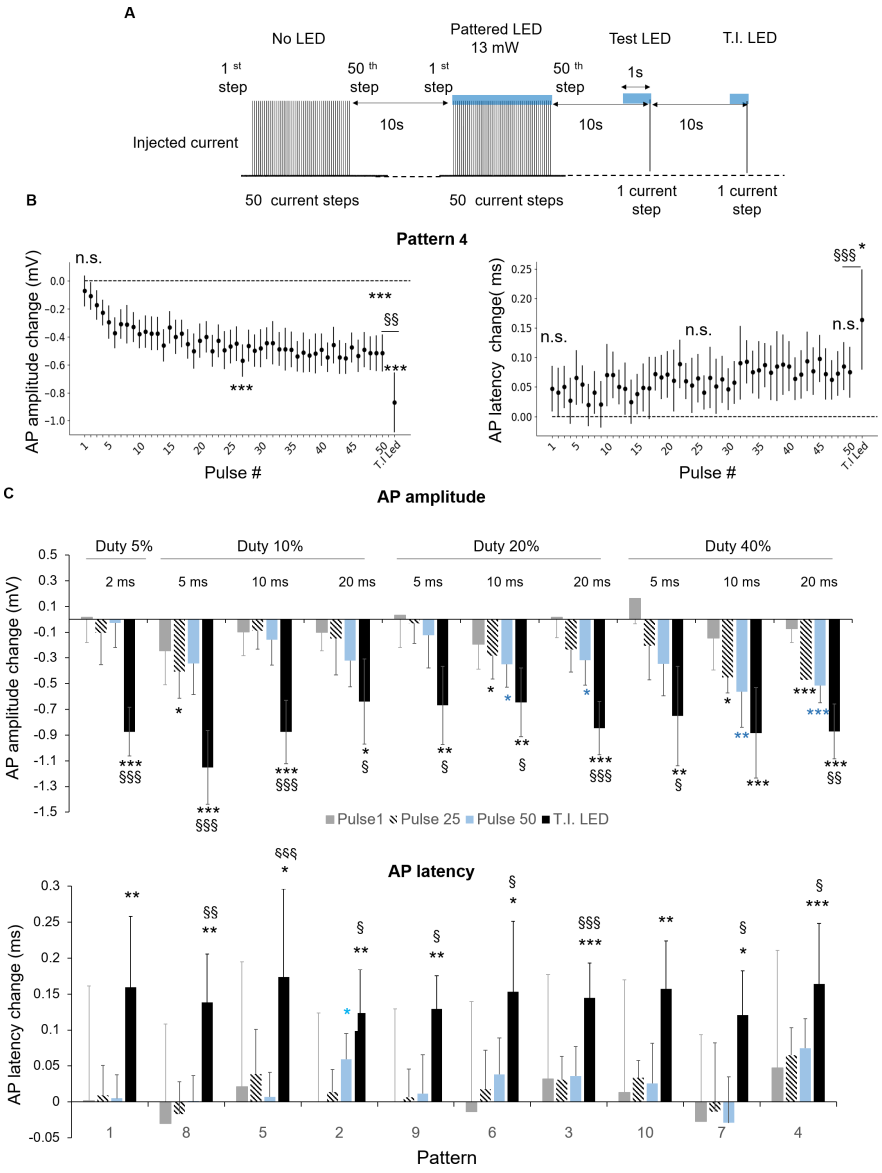

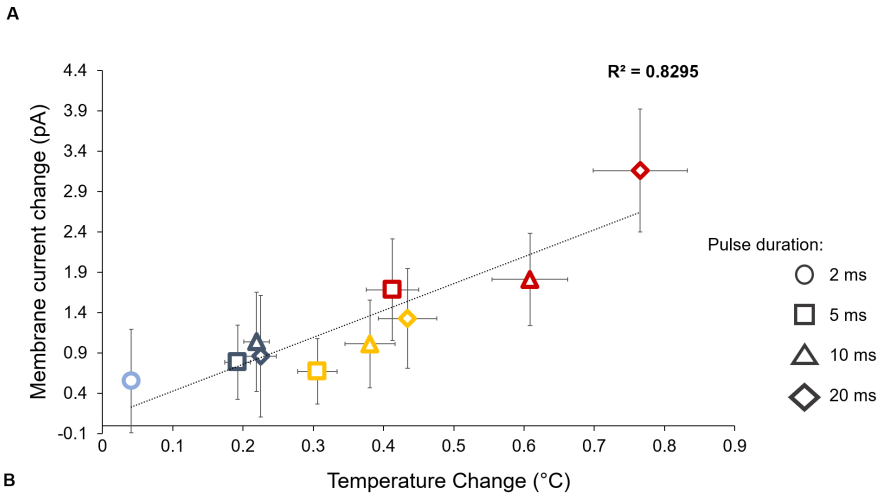

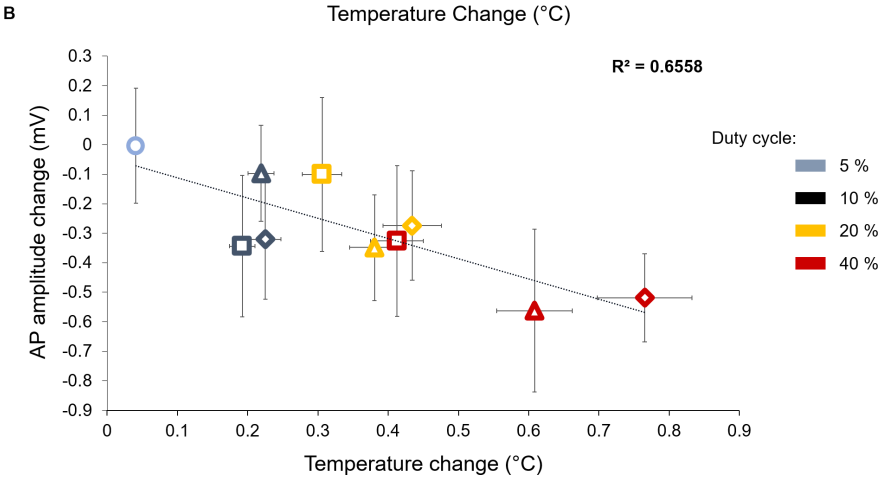

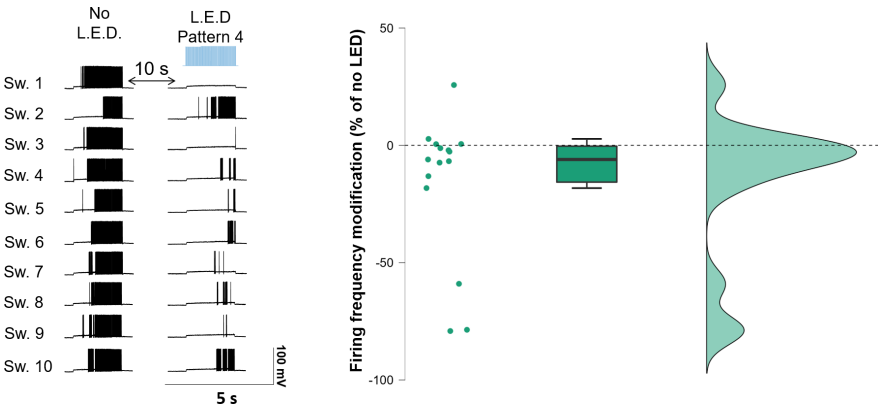

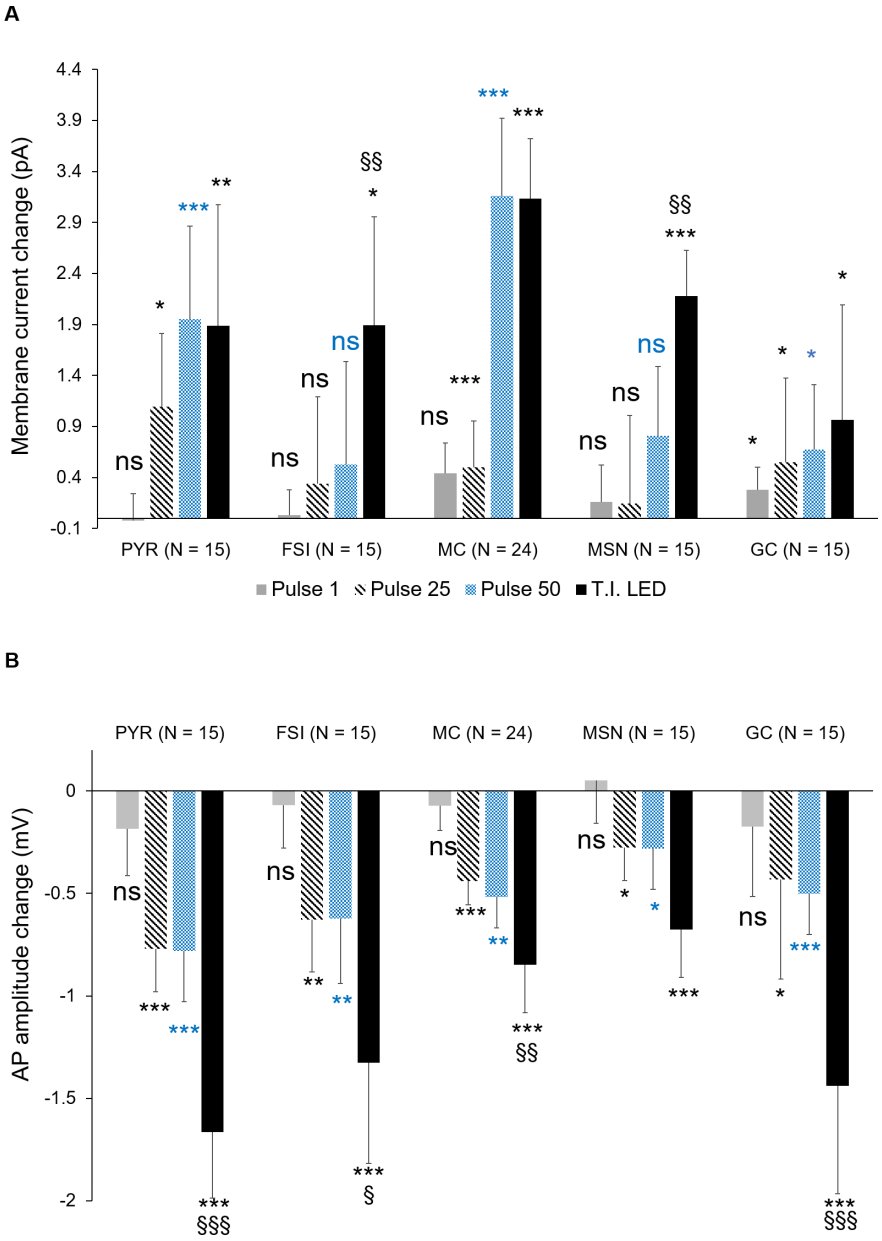

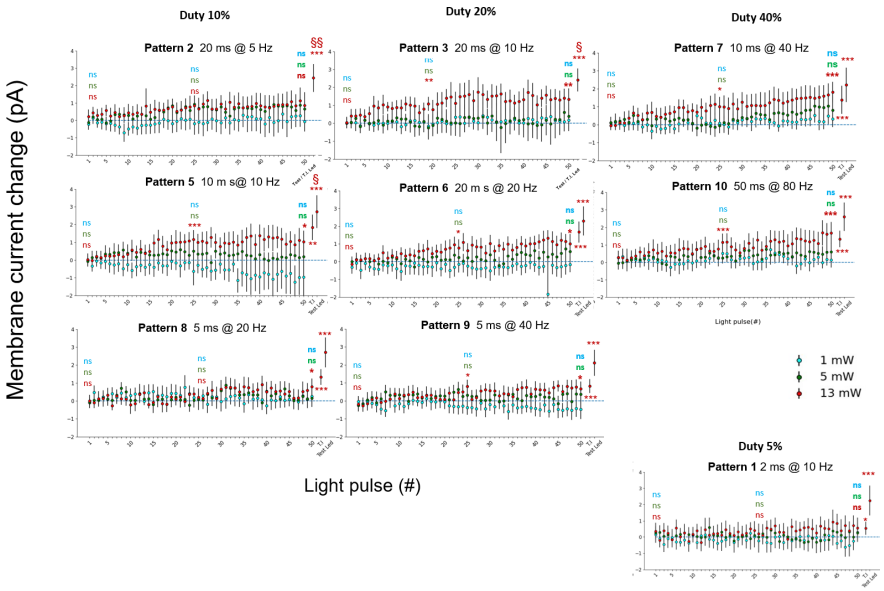

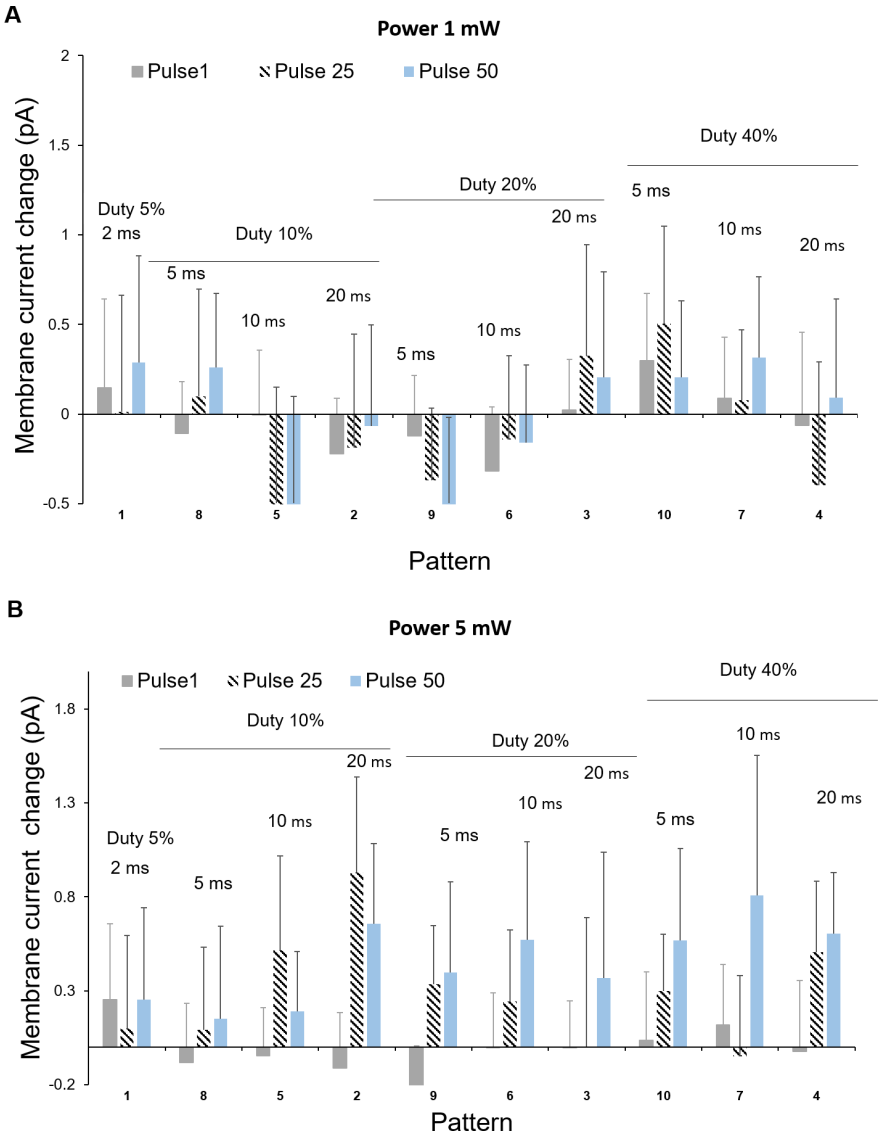

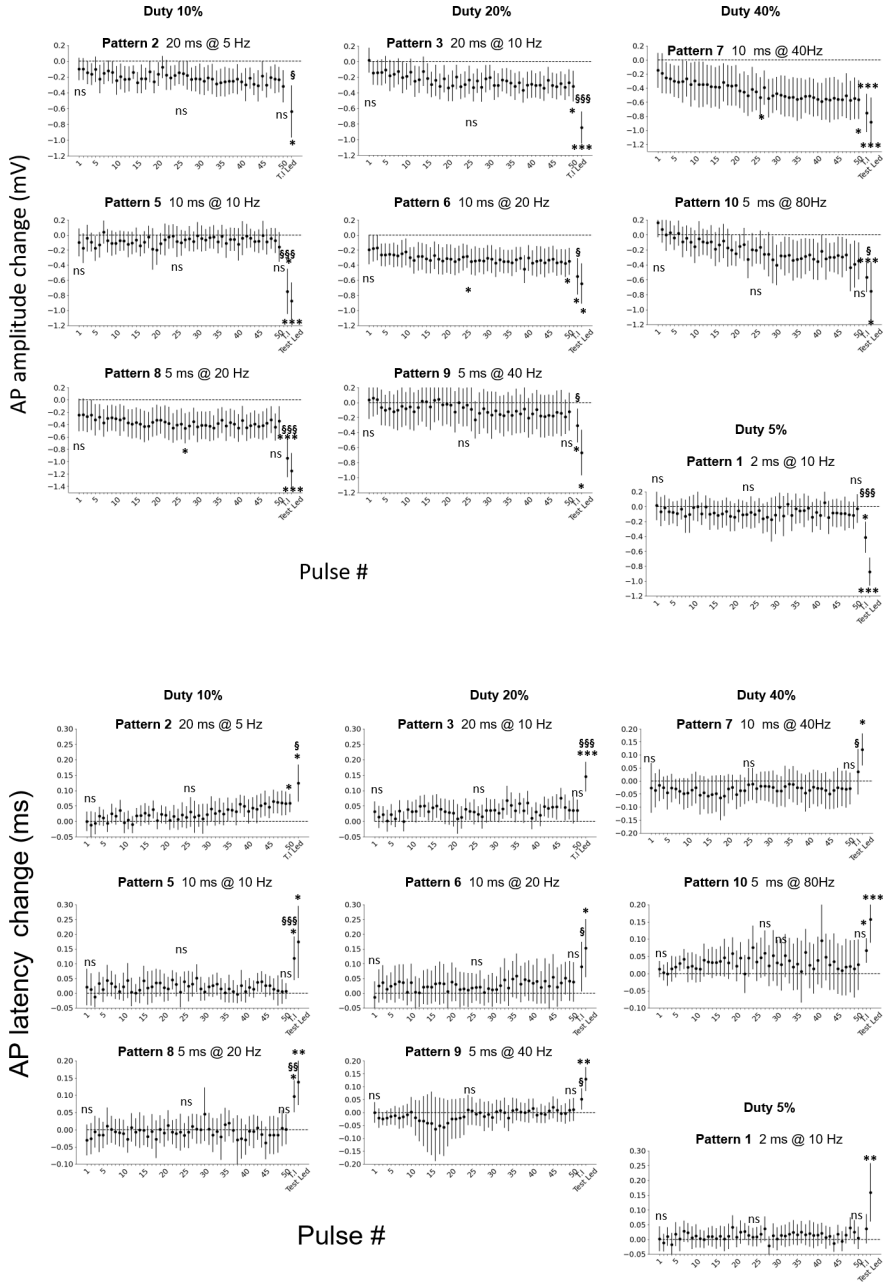

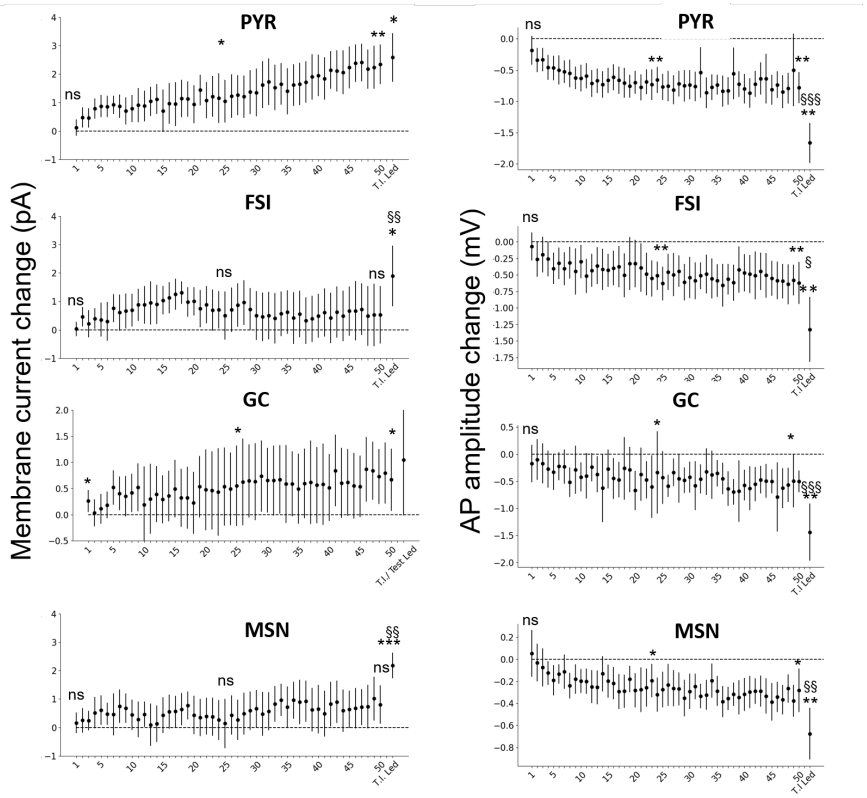

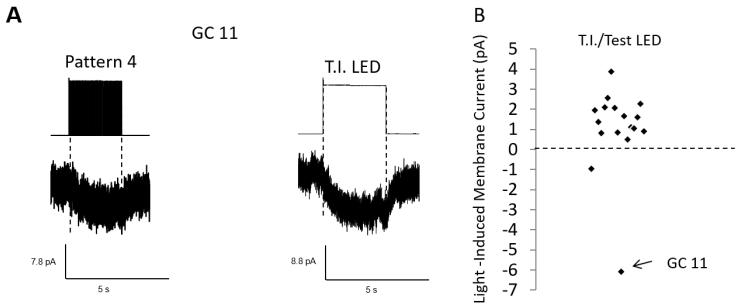

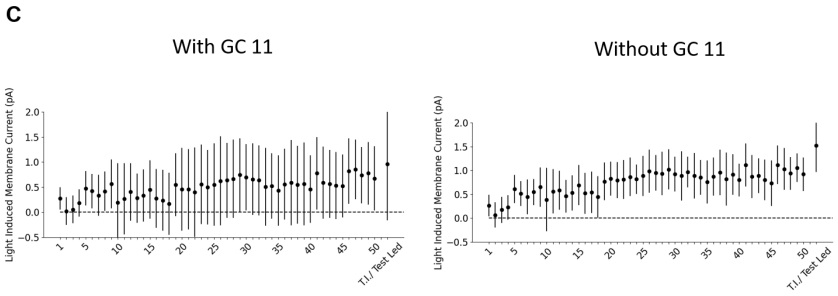

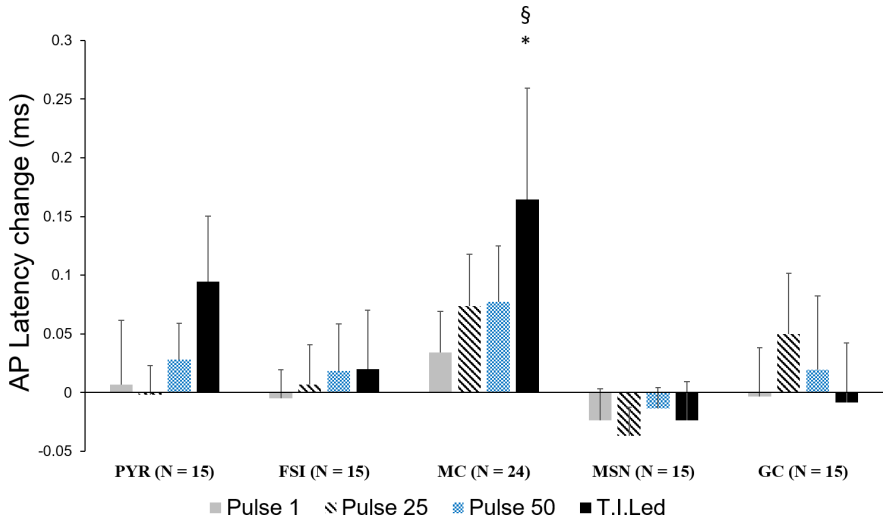

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