Brief Communication

Rats can acquire conditional fear of faint light leaking through the acrylic resin used to mount fiber optic cannulas

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Rodents are exquisitely sensitive to light and optogenetic behavioral experiments routinely introduce light-delivery materials into experimental situations, which raises the possibility that light could leak and influence behavioral performance. We examined whether rats respond to a faint diffusion of light, termed *caplight*, which emanated through the translucent dental acrylic resin used to affix deep-brain optical cannulas in place. Although rats did not display significant changes in locomotion or rearing to caplight in a darkened open field, they did acquire conditional fear via caplight-footshock pairings. These findings highlight the potential confounding influence of extraneous light emanating from light-delivery materials during optogenetic analyses.

[Supplemental material is available for this article.]

Optogenetic-based technologies have assisted rapid progress in understanding the neural mechanisms that mediate behavior (Zhang et al. 2010; Herry and Johansen 2014). These methods take advantage of light-sensitive opsin proteins, which can be transduced into target cells so that cell activity may be manipulated with light (Miesenböck 2009). In studies involving freely behaving rodents, the activating light is routinely delivered to the brain via surgically implanted fiber optic cannulas held in place on the animal's skull with dental acrylic resin (Zhang et al. 2010). During a pilot study using this configuration, we noticed that while the laser pulsed light through the light-delivery patch cable, a faint glimmer could be seen emanating from the dental acrylic "cap" on the rat's skull (Fig. 1A). Although this "caplight" had limited salience and was not in the rat's direct visual field, we were concerned that the rats might possibly perceive caplight and that it might influence behavioral performance. Indeed, rats are exquisitely sensitive to light (Muntz 1967; Rosenberger and Ernest 1971; Garcia et al. 2005; Burn 2008; Barker et al. 2010). They avoid even dim light (Campbell and Messing 1969), and they can condition to relatively low-intensity light cues (Rosenberger and Ernest 1971; Kaitz 1976), including those presented as part of a compound stimulus (Feldman 1975). Extraneous cues present in a testing situation might also absorb attention and distract an animal, and if presented in conjunction with other stimuli, it is possible that they diminish learning of other cues (Mackintosh 1976; Odling-Smee 1978).

Owing to these concerns, we tested whether rats can perceive or condition to caplight in two experimental settings. Because rats typically display increases in locomotion in response to sudden changes in illumination in an open field (Godsil and Fanselow 2004), we first tested whether caplight presentation correlated with alterations in locomotion or rearing. Subsequently the same animals underwent a Pavlovian fear conditioning procedure in which caplight served as the conditional stimulus (CS) that was explicitly paired with a footshock unconditional stimulus (US), and in which behavioral freezing (Fanselow 1980) was used as a measure of fear-related conditional responding. If caplight is a perceptible and salient stimulus, then we expected the animals would respond to it on the open field, and that they would also acquire conditional fear of caplight.

Twenty-two adult male Long Evans rats were individually housed in standard transparent Plexiglas cages with free access to food and water. To implant the fiber optic cannulas, rats were anesthetized with ketamine/xylazine (90 and 15 mg/kg, respectively) before being mounted into a stereotaxic device. The scalp was opened, the skull tissue was scraped away, and the head was leveled with respect to lambda and bregma before three burr holes were drilled through the bone. A single mono-fiber optic cannula (MFC_200/245-0.37_3.4mm_SM3_FLT, Doric Lenses) was positioned above the left prefrontal cortex (+3.2 mm AP/0.8 mm ML of bregma) and the lower edge of the cannula receptacle was positioned flush with the skull surface. Two anchoring screws were installed at −6.7 mm AP/5.4 mm ML to bregma, and −4.0 mm AP/3.0 mm ML to bregma. Next, bonding adhesive (Superbond L-type polymer, Sun Medical) was swabbed over the skull surface before a series of layers of self-curing composite dental resin (Dentalon Plus, Farbe L) was built up over the skull until a

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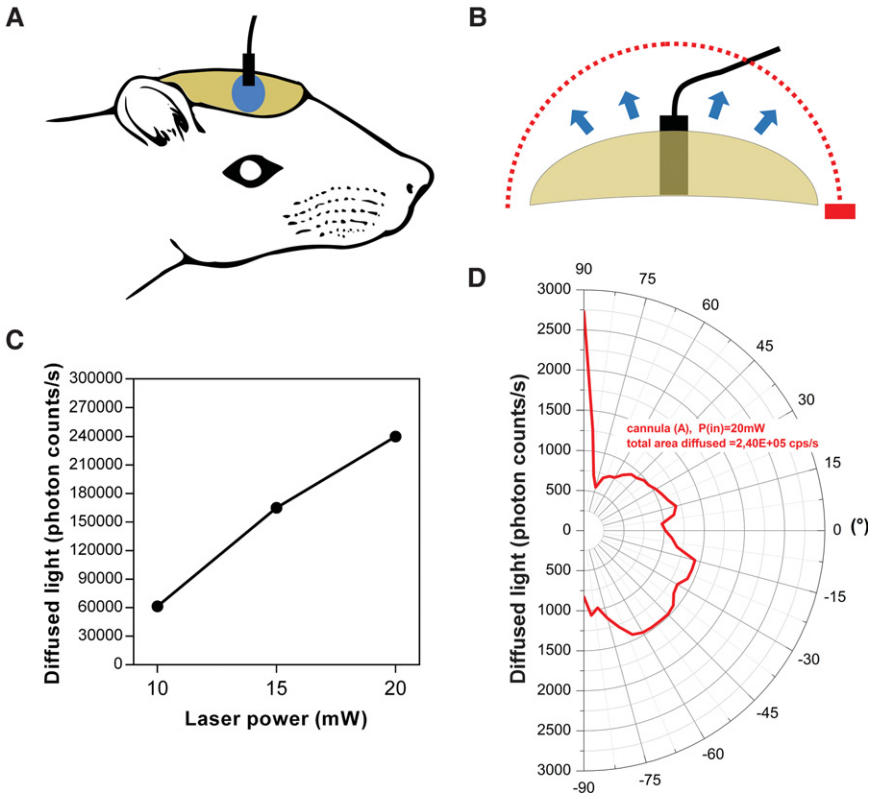

Figure 1. Characterization of simulated caplight stimulus. (*A*) Schematic representation of the caplight phenomenon. Caplight refers to a faint diffusion of light (blue oval) that can be seen emanating from the translucent dental acrylic resin cap (beige region) atop the head of a rat when light is pulsed through a fiber optic cannula (black rectangle). (*B*) Schematic showing the orientation of the detection arc (red arc) of the optical collection fiber (red rectangle) used to measure diffused blue light (blue arrow) from above a simulated cap (beige region). Methodology: To measure the power spectrum of light diffusing from the caps, three simulated caps were fashioned that had a similar size and shape as those used in the behavioral experiments. The 473 nm laser delivered light into the cannula via a single-mode fiber, with 10, 15, or 20 mW emanating from the fiber tip. During measurements, a collection fiber that was fixed to a goniometer (1 mm^2 surface area; positioned 9 cm from the cap) arced above the 180° anterior–posterior axis of the simulated cap (in reference to the rat's skull). 0° was aligned with the cannula axis, and +90° and −90° corresponded to the most "anterior" and "posterior" positions of the cap, respectively. In this way, the number of photons per second (which is proportional to the power of the light collected by the collection fiber) was quantified by a spectrometer (HR-460, Jobin Yvon), and these measurements were used to evaluate the power of the diffused light. (*C*) Sample input–output relationship between laser power and the diffused light from a representative cap. Values were estimated by calculating the area under the curve of the measurements collected across the sampling arc. (*D*) Sample diffusion emission diagram of one of the caps, which represents the number of photons per second measured at different positions along the sampling arc, with a laser input of 20 mW. The inhomogenuous shape in the emission diagram along the 180° arc corresponds to granularities present on the surface of the cap.

cap sufficient to hold the cannula in place was formed (approximate total mass: 1.2 g; approximate volume: 1.1 cm^2).

Behavioral testing began 2–3 wk after surgery. Rats were handled four times per day for 3 d prior to an open field Habituation session. Handling involved briefly removing the rat from its cage while manipulating its cannula's dust cap. For the Habituation session, rats were placed individually in a 50 × 50 cm darkened open field (Med Associates) for a 960-sec session while tethered by a patch cable connected to a rotary joint (FRJ_1×1, Doric Lenses) positioned above the arena, which allowed rats to move freely in the environment. Twenty-four hours later each rat was returned to the arena for a Test session that used an identical procedure as Habituation except that a 30 s caplight was presented beginning at 720 sec. Caplight was generated by delivering a 30 sec train of 20-Hz blue light (473 nm) from a laser (BL473T3-100FC, Shanghai Laser and Optics Century) through the patch cable/rotary joint assembly, and into the fiber optic cannula. The laser was controlled by a stimulus generator (33210A, Agilent Technologies) and was set to a 20% duty cycle with an intensity of ~20 mW at the tip of the patch cable. Activity data were collected with an automatic system (Activity Monitor, SOF-811, Med Associates), which estimated locomotor distance and rearing counts via infrared beam breaks. Statistical tests were computed with the R v3.2.3 statistical programming language software.

During the open field Test, locomotor activity gradually decreased across the session, yet the rats did not show a clear alteration in locomotion to caplight onset (Fig. 2A). Changes in behavior to the caplight onset were estimated by calculating a difference score for each rat [(distance traveled during the caplight) − (distance traveled during the 30 sec preceding the caplight)]. The mean difference score was 14.9 ± 12.1 cm, yet statistical analysis with a one-sample, one-tailed paired *t*-test indicated that this change in locomotion was not statistically significant [$t(21) = 1.23$, $P = 0.116$]. Rearing counts also did not appear to show significant changes to caplight onset (1.3 ± 1.2) [$t(21) = 1.03$, $P = 0.158$] (Fig. 2B). Considering that rats typically show robust increased locomotion and rearing in response to changes in illumination involving brighter stimuli (Godsil and Fanselow 2004; Godsil et al. 2005), the null effect suggests that the caplight cue lacked the degree of unconditional effectiveness to induce an activity response compared with brighter, or more salient, light cues.

Next, the animals were divided into two groups that would have different treatments during a subsequent Pavlovian Fear Conditioning session. Groups were matched such that they had similar performance with respect to locomotion and rearing in response to the caplight onset during the open field Test. Initially, we assigned 12 rats to the paired group and 10 rats to the unpaired group. Three rats were subsequently excluded from the fear conditioning analysis, however, because they displayed substantial generalized freezing to contextual cues prior to caplight presentation during the Fear Test (>80%). Thus, for the fear conditioning analysis, the paired and unpaired groups retained 11 and 8 rats, respectively. Re-analysis of the open field data suggested that these groups displayed similar locomotion and rearing during the open field test (Table 1). Thus, the groups did not exhibit preexisting differences with respect to their reactivity to caplight prior to fear conditioning.

Three days following open field testing, each rat was fear conditioned in a standard conditioning chamber (Context A) (VCF-007, Med Associates) kept inside a sound-attenuating

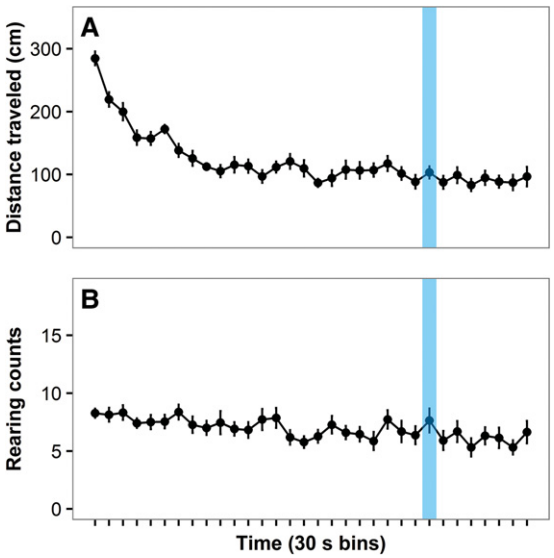

Figure 2. Behavioral response to caplight presentation in a darkened open field arena. The caplight stimulus was presented for 30 sec beginning 720 sec after the rat was placed in the arena. The interval shaded in blue corresponds to bin 25, during which the caplight was presented. (*A*) Mean locomotion during the open field Test. Data are represented as the distance travelled in centimeters during each of the thirty-two 30 sec time bins. (*B*) Mean rearing counts during the open field test. Data are represented as the number of counts during each of the thirty-two 30 sec time bins. All 22 animals were included in this experiment. Error bars denote the standard error of the means. No statistically significant changes in locomotion or rearing were detected in response to caplight presentation.

cubicle. This chamber had the internal dimensions of 30 × 24 × 33 cm, with aluminum sidewalls, an opaque polycarbonate rear wall, and a transparent Plexiglas door. The grid floor was connected to a shock scrambler and shock generator and ~0.2 mL of a scented cleaning solution (Simple Green, Sunshine Makers) was put in the collection pan. The chamber was dark, except for two infrared light sources located above the training box (NIR200, Med Associates). The rats were placed in the chamber individually and were connected to a patch-cable/rotary-joint/laser assembly that was controlled by Med Associates hardware and software. During Fear Conditioning, each rat received three 30 sec presentations of the caplight CS at 300, 512, and 724 sec after the initiation of the control program, which was activated immediately following placement in the chamber. Rats of the paired group received a footshock (2 sec, 0.6 mA) at each CS termination, whereas unpaired rats received a footshock 91 s after the termination of each caplight. The caplight cue was programed to have the same characteristics as those of the open field experiment.

During Fear Conditioning, both groups displayed limited freezing before and during the first caplight presentation, and freezing increased substantially during subsequent CS presentations (Fig. 3). A repeated-measures ANOVA indicated that there were no significant differences in freezing between the two groups during the caplights (Group: $F_{(1,17)} < 1$, $P = 0.76$; Group × Session interaction: $F_{(2,34)} = 1.39$, $P = 0.26$). This pattern suggests both groups acquired fear during the conditioning session. The fact that the unpaired group displayed comparable freezing as the paired group is consistent with their being high levels of conditioning to contextual cues, or that they developed nonassociative fear as a consequence of shock exposure.

To diminish generalized freezing evoked by contextual cues before testing, all of the rats were exposed to the eventual testing chamber (Context B) during three daily 25-min Context Exposure sessions. Context B was the same darkened chamber as Context A, but was scented with 1% acetic acid in the collection pan, and had white plastic floor and curved wall inserts. No caplight cues or shocks were presented, and freezing was scored during the first 5 min of each Context Exposure session. Both groups showed high levels of freezing during the first Context Exposure, but these levels decreased substantially across the sessions (Session: $F_{(2,34)} = 50.1$, $P < 0.00001$) and no statistical differences between the groups were detected (Group: $F_{(1,17)} < 1$, $P = 0.72$; Group × Session interaction: $F_{(2,34)} < 1$, $P = 0.56$). These results indicate that the rats in both groups initially showed a similar high degree of generalization between the training and testing contexts, and this generalized fear extinguished with increased exposure to Context B.

On the next day, each rat was tested individually in Context B for fear responses evoked by the presentation of three caplight cues (following the identical schedule of conditioning). Both groups displayed similar levels of freezing during the precaplight period of this Fear Test (two-sample, two-tailed *t*-test: $t(17) = 0.34$, $P = 0.71$), yet the paired group showed elevated freezing to the caplights afterward. A repeated-measures ANOVA of freezing during the three caplights confirmed that the paired group had significantly elevated freezing (Group: $F_{(1,17)} = 8.73$, $P < 0.01$; Group × Trial interaction: $F_{(2,34)} = 1.91$, $P = 0.16$). These results indicate that paired rats displayed significantly more fear responding to the caplight presentations during the Fear Test, which demonstrates they conditioned to the cue.

Considering these results, we examined the light that diffuses out of the upper surface of the caps, which we assume models the caplight that can be detected by the rats. To do so, we sampled a 180° arc above three simulated caps with a spectrometer equipped with moving detection fibers (Fig. 1B; Supplemental Materials). From these measurements, we estimate that, in total, ~20% of the light input into the cannula was diffused back into the environment. Notably, the caps exhibited linear input–output properties (Fig. 1C), and the light diffusion along the sampled arc was not uniform (Fig. 1D), which was likely due to inhomogeneity on the cap surfaces.

Table 1. Locomotion and rearing data of rats included in the fear conditioning analysis

Behavioral category	Group	*n*	Test interval: Before the caplight (sum of bins 1–24)	Test interval: Caplight (bin 25)	Test interval: After the caplight (sum of bins 26–32)	Response to caplight onset: Difference score (bin 25–bin 24)
Locomotion (cm)	Paired	11	3051 ± 156	101.5 ± 13.4	626.6 ± 72.7	7.1 ± 12.7
	Unpaired	8	3312 ± 183	114.6 ± 18.8	668.4 ± 89.0	15.3 ± 26.5
Rearing (counts)	Paired	11	166 ± 11	7.6 ± 1.8	38.7 ± 4.6	2.0 ± 2.0
	Unpaired	8	177 ± 9	8.4 ± 1.3	42.5 ± 6.1	1.4 ± 1.7

Data represented as group means ± SEM.

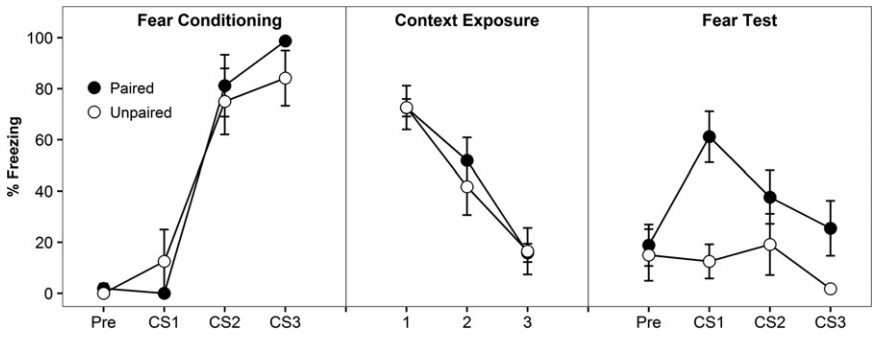

Figure 3. Behavioral freezing estimates for the paired and unpaired groups during the Fear Conditioning experiment. Rats were fear conditioned with three caplight-footshock pairings in Context A, before undergoing three 25-min exposures to Context B. During the Fear Test, rats were presented with three caplights without footshock in Context B. (*Left*) Mean percent freezing during the initial pretrial interval and during each of the three caplight cue presentations of the Fear Conditioning session. (*Middle*) Mean percent freezing during the first 5 min of each of the three Context Exposure sessions. (*Right*) Mean percent freezing during the initial pretrial interval and during each of the three caplight cue presentations of the Fear Test session. There was a main effect of Group during the Fear Test ($F_{(1,17)} = 8.73$, $P < 0.01$), indicating that the paired ($n = 11$) rats performed more freezing than rats in the unpaired ($n = 8$) group. Error bars denote the standard error of the means. (CS) conditional stimulus, (Pre) pre-CS interval.

Our findings demonstrate that, while the caplight may not be bright enough to arouse obvious unconditional changes in locomotor activity, rats can detect and condition to the low-intensity stimulus. Thus, although caplight may seem faint to the human eye, the fact that rats can acquire conditional fear of it demonstrates that such extraneous light can have a meaningful influence on behavioral performance.

We tested only one type of dental resin with procedures designed to maximize the detection of a caplight effect, and therefore the generalizability of these findings is presently unknown. One consideration is that dental composite resins are a diverse class of materials whose light transmittance depends on numerous factors, including properties inherent to the resin, the smoothness of the resin's surface, the delay in time since curing, and the thickness of the cap (Ferracane 1995; Inokoshi et al. 1996; Arikawa et al. 1998, 2007; Kim and Park 2013; Beltrami et al. 2014). Consequently, while we cannot assume that our finding is applicable to all the different resin materials used in optogenetic research, it is important to recognize that these resins are designed to be translucent in order to mimic the color of natural teeth (Ferracane 1995; Villarroel et al. 2011). Thus, since the degree of caplight transmittance likely varies between different resins, it is advisable to identify and utilize those formulations with less transmittance.

In addition to composite resin materials, we speculate that extraneous light could also leak into the experimental situation from other materials during optogenetic experiments. For example, some varieties of ceramic ferrules (which are used to connect patch cords to fiber optic cannulas) are translucent and can leak light. Also, bare optical fibers typically have transparent clad and it is conceivable that patch cables shielded with opaque outer shells can become frayed from the chewing or scratching of the animals. All told, our results imply that light leaking from other sources may also influence behavioral performance.

Our results demonstrate that rats can acquire fear of caplight, but we did not detect changes in the open field. An important question then is under which conditions might caplight be of concern? Space constraints do not allow full treatment of this issue, yet previous research demonstrates that rodents can respond to low-intensity stimuli in numerous ways. Similar to our study, rodents condition to weak visual cues when they are paired with a US alone (Mackintosh 1976), and it is possible that caplight could overshadow conditioning to background cues (Odling-Smee 1978), or to other low-intensity stimuli (Mackintosh 1976). The presence of extraneous light might also simply distract animals, which could lead to behavioral changes not mediated by the activity of opsins in the brain. Considering the diverse array of behaviors used in optogenetic experiments, these effects have the potential to confound many activity-related variables, which are often of greatest interest to behavioral neuroscientists. Also, comprehensive behavioral inventories are rarely conducted, and in some experimental settings automated methods are used to monitor behavioral performance. Such circumstances provide conditions where the presence and influence of caplight may be entirely overlooked. Indeed, in ambient light our acrylic caps appeared fairly opaque, and the extent of caplight noticeability was neglected until it was observed in a dark room.

A useful control against caplight effects is the use of light-stimulated animals that lack active opsins in the brain. Such groups are typically regarded as controls against light-induced heating effects of brain tissue, but they also are useful against caplight. Considering that caplight likely varies with respect to the dimensions of the cap, and perhaps also with regard the animal's position in a chamber (owing to differences in light reflection), caplight may be a rather uneven phenomenon for both experimental and control animals, especially in procedures involving differential ambient lighting across experimental contexts, and this phenomenon may be especially problematic for experiments that examine CS pathways. These confounding effects might also be curtailed by counterbalancing experimental contexts, or by preexposing animals to laser stimulation, both to habituate them to caplight and to reduce potential state-dependent effects of laser stimulation.

The vast majority of photoreceptors in the rat retina are rods (Szél and Röhlich 1992) which makes rats well suited to low light levels. Indeed, rats can discriminate between a cue of 0.107 lux and darkness, which suggests that they are more sensitive to dim light than are humans (Campbell and Messing 1969; Burn 2008). Rats are also capable of perceiving light as pulsing up to ~40-Hz (Sauve et al. 2006; Gilmour et al. 2008). With such sensory capacities, it is worth noting that optogenetic tools vary with respect to the photostimulation regimes. "Excitatory" opsins (channelrhodopsin) are stimulated with pulsed light, whereas "inhibitory" opsins (ArchT and halorhodpin) require continuous light (Cardin et al. 2010; Mattis et al. 2012). Because pulsed and continuous stimuli appear to function differently as cues for rodent behavioral experiments (D'Amato 1961; Fox et al. 2013), it may be that the potential for caplight effects varies across excitatory and inhibitory opsin experiments.

Optogenetic techniques are powerful tools that have been widely integrated into neuroscience research. Here, we call attention to the potential confounding influence of caplight and other forms of extraneous light in the experimental setting. Our findings provide evidence that caplight can alter behavioral performance, and therefore the possibility that such extraneous light might be leaking into the experimental setting should be carefully scrutinized.

Acknowledgments

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