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Evoking Blinks with Natural Stimulation and Detecting Them with a Noninvasive Optical Device: A Simple, Inexpensive Method for Use with Freely Moving Animals

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Abstract

Many laboratories studying eyeblinks in unanesthetized rodents use a periorbital shock to evoke the blink. The stimulus is typically delivered via a tether and usually obliterates detection of a full unconditioned response with electromyographic (EMG) recording. Here we describe the adapter we have used successfully for several years to deliver puffs of air to the cornea of freely moving rats during our studies of eyeblink conditioning. The stimulus evokes an unconditioned response that can be recorded without affecting the EMG signal. This allows a complete analysis of the unconditioned response which is important for studies examining reflex modification or the effect of drugs, genetic manipulations, or aging on the unconditioned blink reflex. We also describe an infrared reflective sensor that can be added to the tether to minimize the number of wires that need to be implanted around the eye, and which is relatively immune to electrical artifacts associated with a periorbital shock stimulus or other devices powered by alternating current. The responses recorded simultaneously by EMG wires and the optical sensor appear highly correlated and demonstrate that the optical sensor can measure responses that might otherwise be lost due to electrical interference from a shock stimulus.

INTRODUCTION

Eyeblink conditioning is used as a model paradigm to examine the neural basis of learning and memory. The rabbit (a lagomorph) was initially used after Gormezano demonstrated that these subjects are very tolerant of restraint and have large eyes with few spontaneous movements (Gormezano, 1962). However, rodents have become very popular since rats are less expensive and more readily available to study the effects of aging, and mice can be used to study the genetic basis of learning and memory. Rodents also have a more varied behavioral repertoire than do rabbits which enables comparisons across different learning tasks (Kuo, Lee & Disterhoft, 2006). Although eyeblink conditioning has been done in restrained rats using airpuff and a reflective sensor (Schmajuk & Christiansen, 1990), the freely moving rat preparation developed by Skelton (1988) has gained popularity over the years.

Most laboratories using rodents to study eyeblink conditioning use a tether to allow limited movement within an arena and to connect signals from the subject to the equipment (Bangasser

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& Shors, 2007; Stanton, Peloso, Brown & Rodier, 2007; Freeman et al., 2007; Takehara-Nishiuchi, 2006; Weiss et al., 2002). A periorbital shock is one such signal that is often used as an unconditioned stimulus (US) to evoke a blink. This stimulus is delivered via a pair of subdermal microwires that terminate in a connector that is surgically mounted to the skull. A second set of microwires is often used to record electromyographic (EMG) activity from the eyelid so that blinks can be recorded and measured.

A disadvantage of a periorbital shock US is that the stimulus activates a different set of sensory receptors than would be activated by a natural tactile stimulus such as a puff of air or foreign object approaching the cornea. The shock stimulus also often saturates the inputs of the amplifier used to record EMG signals from the eyelid when the stimulus is delivered as a train of pulses that is typically used during eyeblink conditioning experiments (e.g. Takatsuki, Kawahara, Takehara, Kishimoto & Kirino, 2001). This shock artifact masks detection of a complete unconditioned response (UR), even if the US is of short duration (e.g., Bangasser & Shors, 2007), applied as a single pulse (Servatius, 2000), or applied directly to the supraorbital nerve (Dauvergne & Evinger, 2007). Loss of the UR is a significant disadvantage, especially if one is interested in reflex modification (Evinger & Manning, 1988; Schreurs, Oh, Hirashima & Alkon, 1995; Weisz & LoTurco, 1988) or the effect of aging or different drugs on behavior (Moskal et al., 2005). Lastly, the shock US requires an isolated power source that can deliver regulated currents on the order of a milliamp. These devices can become unreliable if the impedance between the stimulating wires becomes too great.

In order to avoid the disadvantages of a shock US, and to record a complete UR, we have been using a puff of air directed to the cornea of freely moving rats. The supply line for the airpuff terminates with a barbed right angle fitting that forms a nozzle to aim the airpuff at the cornea. The nozzle is permanently attached directly to the tether in each training chamber (we train as many as four rats simultaneously) and the skull-mounted connector aligns the nozzle at the eye every time the tether is attached to the animal. A schematic of the tether is shown in Figure 1 and a picture of a typical tether is shown in Figure 2.

The first part of the Methods describes the “puff adapter” we use for delivering puffs of air to the cornea of freely moving animals. The second part of the Methods describes a light-weight infrared reflective sensor that can be attached to the tether to detect blinks. The circuitry for this sensor requires only a minor modification of that described by Thompson, Moyer, Akase and Disterhoft (1994). An advantage of the optical sensor is that trauma to the eye is minimized since fewer wires are used, and no subdermal wires are needed if an airpuff stimulus is used instead of a periorbital shock. The optical sensor is also relatively immune to the stimulus artifact if one does use periorbital shocks or AC electrical signals near the recording site.

MATERIALS AND METHODS

Puff-Adapter

The puff-adapter is based upon the 221 series nylon strip connectors, gold plated Relia-Tac pins (commonly referred to as Amphenol pins) manufactured by Cooper Interconnect (formerly Wire-Pro, Inc.), and brass strips approximately 0.25" wide and 0.020" thick. Table 1 lists the different parts for the tether. The brass strips are available at many hobby and home improvement shops. We order our connectors, pins and sockets from Electronic Connector Corp. (Chicago, IL), although several companies supply these parts. The nylon strips are offered with three different center to center spacing (.100", .075", .050"). The .075" spacing provides a good compromise between overall space savings on the animal's head and the ability to work with individual pins within the nylon strip. We use this size strip connector for eyeblink conditioning studies with both mice and rats. The “Plug Strip” accepts the Relia-Tac sockets and is permanently attached to the tether of each conditioning chamber (Figure 1&Figure 2).

The “Receptacle Strip” accepts the Relia-Tac pins and is permanently attached to the skull of each animal after a minor surgical operation. This is how most laboratories implant a connector with wires that terminate within or near the eyelids.

The basic connector for EMG recordings and shock stimulation requires seven complete holes (ground + 2 shock + 2 EMG + spacer hole + locking screw). The basic connector for EMG recordings and airpuff stimulation requires five complete holes. The locking screw is a 0–80 machine screw with a 0–80 nut glued (with cyanoacrylic) up against the head of the screw so that it can be turned easily by hand. The hole for the locking screw is tapped all the way through the plug strip so that the screw does not fall out of the tether (no drilling is needed prior to tapping). This requires a notch to be cut out of the top of the plug strip since the standard 0–80 tap is not long enough to reach the bottom of an intact plug strip. The tap to make the threads for the locking screw in the receptacle strip attached to the head is turned approximately ten times so that when the screw is turned it will bottom out and indicate that the lock is complete.

We made a puff adapter for each training chamber and one “surgical tether” for each stereotaxic device used during surgery (Figure 3). The adapters were all made at the same time using the same rat or mouse so that the adapters would be interchangeable among the different training chambers if needed. The adapter for the surgical tether has an extra piece of brass attached to the plug strip (on the side opposite to the side with the puff adapter) so that the strip can be held and positioned with a stereotaxic manipulator (Figure 3). This extra piece of brass is short and can be held with a standard electrode holder with a clamp (e.g. Kopf Model 1770 or 1771), or a hole in the piece of brass can be used to accept a screw that secures the adapter to a block attached to the bottom of the stereotaxic arm (similar to Kopf Model 1776). The hole also allows the pitch of the receptacle strip to be adjusted prior to cementing the strip in place on the skull. This provides one more degree of freedom for aiming the adapter at the cornea.

The brass strip for the puff adapter is cut to the proper length and a small hole is made at one end to accept the nozzle of a plastic barbed elbow fitting. Several barbed elbow fittings are available. We use a 1/16" × 1/16" nylon barbed elbow from Small Parts, Inc. The barb of one end is removed so that the straight tube will fit through the hole in the brass strip (Figure 3). It is then secured to the brass strip with two-part epoxy. The brass strip is bent to create two 90 degree turns approximately 15 mm apart for our adult rats. The short (proximal) section of approximately 6 mm is attached to the plug strip connector with cyanoacrylic glue and then with two part epoxy. The distal section (with the nozzle) is bent in the same direction (i.e. a flat U turn is made) at a point that will position the nozzle in front of the pupil. This step is done while the animal is in the stereotaxic so that the air nozzle can be aimed at the eye. The length of brass between the two bends is long enough to straddle the head from the midline to the side of the face. Final adjustment of the brass strip may require the use of two needle nose pliers to secure and twist/bend the nozzle into position. This often requires a slight rotation towards the rostral end of the animal in order to get the nozzle aligned in front of the pupil. The position of the strip connector could be placed more laterally if access to midline brain structures is desired. The surgical tether/puff adapter and receptacle strip on the head are separated after the cement on the skull has finished curing.

Air Delivery

The air nozzle is connected to the air source in several steps. The first step connects a 7 French (13 gauge) silicone tube from the puff adapter nozzle to a blunted 16 gauge needle (Fig. 1&2). The needle is attached to a 1 cc plastic syringe which has had the flange removed from it. The syringe is connected to a short piece of tubing (1/4" ID, 3/8" OD) then to a right angle plastic fitting (which is in the top of our training chamber) and then to another longer piece of tubing of the same size (we use Tygon R3603). The four long pieces of tubing (from our four chambers) are connected to two Y fittings, and the two Y fittings are connected to another Y

fitting with short lengths of tubing. Finally, this last Y fitting is connected to a single solenoid valve that releases the air puff from a source of compressed gas when the solenoid is signaled by a computer or other timing device. Each chamber has the same total length and size of tubing so that the distance and resistance between the solenoid and the eye is the same for each chamber. This allows the pressure and latency of air delivery to be the same among all four chambers, as long as all four tethers are connected, i.e., even if a chamber is not being used to test a subject.

Blink Detector

The optical blink detector (Fig. 4) is based upon the Optek OPR5005 surface mount infrared reflective sensor (Carrollton, TX, www.optekinc.com). The end of a length of wire-wrap wire is soldered to each of the four contacts and the other end is attached to a phone style cord and plug for the blink detector circuit described by Thompson, Moyer and Disterhoft (1992). The sensor is then glued to the end of the brass piece with or without a barbed elbow for the optional air delivery nozzle. The addition of the optical detector to the puff delivery adapter requires the use of a slightly wider piece of brass than if only EMG signals are being used.

The OPR5005 works with the same circuitry as for the OPB704 that we use with our experiments on rabbits, except that an offset control needs to be added for the rodents (resistor R7, Fig. 5). This extra control is needed since the sensor will be in a fixed position and not able to be adjusted as with our system for rabbits. The offset control is added by disconnecting pin 3 of the TL072 operational amplifier from ground, and connecting it instead to the wiper of a 10 kilohm potentiometer. The other two leads of the potentiometer should be connected to a +12V source and ground respectively. The potentiometer is used to adjust the baseline of the output signal to a value of about 0.25V. The baseline needs to be greater than 0V so that the response will remain positive if integrated responses are used for analysis. Other than the offset adjustment, the Thompson et al. circuit works well and provides a 0–5V output when the gain is set appropriately with resistor R5. The gain of the optical amplifier was set after verifying that a full blink would not saturate the system. The gain was not adjusted after that since the relative position of the sensor and the eye remained constant for each animal. Lastly, since the sensor is sensitive to wavelengths of approximately 900nm, it can still be used with visible light stimuli from LEDs since the spectral output is different enough from the sensitivity of the OPR5005 sensor. Fluorescent lights should be avoided however since they can emit IR energy that is detected by the sensor. The parts needed for the circuit are indicated in Table 2.

RESULTS

Figure 6 shows examples of EMG activity from the upper eyelid of a rat conditioned with tones and corneal airpuffs. An example is shown for a trial with a conditioned response (4A) and for a trial without a conditioned response (4B). Note that the entire unconditioned response was recorded. The raw EMG signal can be analyzed by thresholding the signal and counting spikes that cross the level (Weiss et al., 2002) or by integrating the activity and analyzing it as an analog signal (Weiss et al., 1999).

Figure 7 shows a different example of a response that was recorded simultaneously with both the infrared reflective sensor (7A) and with EMG recording wires (7B) from a conditioned mouse. Note that there appears to be a good correlation of EMG and optical signals, but that the optical signal occurs slightly later due to the time it takes the lid to start closing after the orbicularis oculi muscle is activated. An integrated version of the optical signal is not shown since the output is analog in nature.

DISCUSSION

We have been using airpuff stimulation with rats for our eyeblink conditioning studies since 1999 (Weiss, Boumeester, Power & Disterhoft) and have published six original peer-reviewed papers with this technique. We have also demonstrated our technique to the Bickford laboratory which has used a version of it successfully (Cartford, Gemma & Bickford, 2002; Cartford, Samec, Fister & Bickford, 2004). The air puff US produces good URs that are free of artifacts and which are readily conditioned in the rat when paired with a CS. We have also tried this system in the mouse (Weiss, Lindemuller, Pak & Disterhoft, 1997), but the conditioning was not robust until we switched the mice to a shock US (unpublished observations). This species-dependent difference will be verified and explored in the future.

We routinely use optical detection of blinks in rabbits, but the technique is relatively new for our rodents, especially since the EMG signal has been very reliable in the hands of experienced surgeons, and the EMG signal does provide a better indicator of response timing than does an optical signal. The different response times may be important to those developing computer models for the generation of CRs. However, the optical sensor does offer the advantage of less damage to the eyelid when implanting EMG electrodes or Hall effect devices (Koekkoek, Den Ouden, Perry, Highstein & De Zeeuw, 2002), and importantly, it is relatively immune to AC electrical signals which might have significant advantages in future studies with other electrically driven devices.

Note that our method does not use a commutator or slip ring as reported by some laboratories doing eyeblink conditioning with freely moving rodents. We have not found this component to be necessary, even when we use an airpuff US. Our animals tend to stay relatively still within the inner conditioning chambers (rats: 10" × 10" × 9.5", mice: 5" diameter × 4" tall) that are located within a larger sound and light attenuating chamber, so the tether and air tube only occasionally get tangled, and only in a few subjects (our tether is approximately 2" longer than the height of the chamber). A tangled air line becomes obvious by a decrease in the UR, and can be untangled quickly in between trials. We may also be avoiding any tangling due to our use of only 30 trials per session, which leads to good conditioning for us and others (Weiss, et al., 1999; Cartford et al., 2002)

Lastly, the tether adapter can be used for stimuli other than an airpuff. For example, we have used the bracket to support a green LED in order to use a light flash as a CS (Kronforst-Collins, Weiss & Disterhoft, 1996; Weiss, Kronforst-Collins & Disterhoft, 1996; Weiss, Lindemuller, Pak & Disterhoft, 1997). In fact, any lightweight device that needs to be aimed at the head or dorsal/lateral body could probably be attached to the tether with slight variations in the brass strip we describe in this report. The minimum requirement would be a nylon strip with three holes used to hold the locking screw, a spacer hole, and at least one pin, or a second locking screw to keep the tether adapter aligned with the connector attached to the skull.

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Figure 1.

A. A schematic of the assembled tether. a) shielded cable between connectors and amplifier or computer, b) female half of connector secured to top of chamber, c) top wall of chamber, d) male half of connector, e) 221 “plug” strip with female sockets, f) brass strip with two 90 degree angles, g) tygon tubing i.d.: 1/4”, o.d.: 3/8” h) nylon barbed elbow, i) distal end of a 1cc syringe, j) 16 gauge hypodermic needle with the point removed, k) 17 French silicone tubing, l) barbed elbow connector with one barb removed. The distances from the bottom and side of the plug strip to the nozzle is indicated for the rat (this can be adjusted for different size rats or if midline structures need to be accessed). The smaller numbers represent the distances for the optical sensor on a mouse tether (the sensor would be where the air nozzle is located, and there would be no air tubes). **B.** A magnified view of the terminal end of the tether. e) plug strip with a notch cut out of it so the front hole can be completely threaded with a tap, f) brass strip with a hole for the air nozzle, m) two part epoxy to secure cable to connector, n) 0–80 machine screw with a 0–80 nut glued to the head of the screw, o) female sockets with conductors soldered into the top of each socket. **C.** The strip connector that is secured to the head of each animal. Additional pins could be added if more connections are desired, e.g. if shock was to be added or if neural signals needed to be relayed to an amplifier or computer. p) “receptacle” strip for male pins. q) male pins with wires soldered into the bottom of each pins. r) the front hole is threaded part way through the depth of the receptacle strip to accept the locking screw (n).

Figure 2.

A photograph of an actual tether after removing it from the chamber. Our tether uses a round Amphenol connector to connect two EMG wires and a ground wire to the amplifier. The 16 gauge blunted needle is used to couple air from a syringe tip to the air hose and on to the barbed elbow connector within the brass strip that is secured to the strip connector with two part epoxy.

Figure 3. The surgical tether adapter is shown attached to the bottom of a stereotaxic manipulator. A strip connector with EMG wires would be attached to the adapter before each surgical procedure. The hole in the brass strip would be positioned in front of the pupil before cementing the strip connector in place on the skull.

Figure 4.

Pictures of the tether with the optical sensor attached to the strip connector with a short piece of brass. The brass piece has a hole in it to attach to the stereotaxic (see Fig 2 for the stereotaxic holder). The top sensor had a hole drilled in between the emitter and detector so that a narrow tube could be placed through it, although a larger diameter tube can be glued to the side of the device. Note the telephone style cable that is convenient to use for the four conductors needed by the optical sensor. The bottom picture shows another optical sensor while it was receiving power. The LED (right side of device) appears bright when photographed with a digital camera, but the infrared is not visible to the unaided eye.

Figure 5.

A modified version of the Thompson et al. (1994) circuit for detecting blinks with an infrared reflective sensor (OPR5005 instead of OPB704). The gain is set by potentiometer R5. The offset is adjusted by potentiometer R7. Capacitor C5 is used to smooth the output of the circuit; the value was selected to optimize smoothing and response time of the circuit. The two operational amplifiers are packaged into one dual integrated circuit (TL072).

Figure 6.

Example of a response from a rat during a CR (A) and nonCR (B) trial. The CS was a tone and the US was a puff of air to the cornea. The bottom of each panel shows the raw EMG signal. The top of each panel shows the EMG signal after it has been rectified and integrated.

Figure 7.

Example of a blink recorded simultaneously with both the optical sensor and EMG recording wires. Note that the signals from the two methods appear highly correlated

Table 1

Parts and materials needed to make the puff adapter.

Part Number	Description	Location/size	Company
221-2653	4" Receptacle Strip	Head	Electronic Connector Corp.
221-2553	4" Plug Strip	Tether	Electronic Connector Corp.
220-PO2	Relia-Tac Pin	Head Strip	Electronic Connector Corp.
220-SO2	Relia-Tac Socket	Tether Strip	Electronic Connector Corp.
	Brass strip	~.25" × .020"	Local hardware/hobby store
TFP-BE062	Barbed Elbow	1/16" × 1/16"	Small Parts
OPR5005	IR reflective sensor	tether	Optek Technology

Table 2

Parts needed for the blink detector circuit.

Capacitors		Comments
C1, C2	47 μ F, 25 VDC electrolytic	
C3	10 μ F tantalum	
C4	2.2 μ F tantalum	
C5	.001 μ F tantalum	Filters ripple in output
Resistors		
R1, R4	1.2 k Ω	
R2	4.7 K Ω	
R3	220 K Ω	
R5	4.7 K Ω , (nominally 2.4 K Ω)	Adjusts gain of output
R6	33 K Ω	
R7	10 K Ω	Sets baseline level
Diode		
D1	1N914	
Operational Amplifier	TL072	Dual amplifier chip
Infrared reflective sensor	OPR5005	Surface mount chip