Second-order conditioning: Different outcomes in fear and eyelid conditioning

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Second-order conditioning has been frequently observed with the fear response but not with the eyelid response. The present experiments manipulated the temporal relationship between the second-order and first-order stimulus on second-order conditioning trials. Our results indicated that a trace second-order procedure is not effective with either response system. Second-order fear conditioning was most prominent when the second-order CS terminated at the onset of the first-order CS. This arrangement, however, did not produce second-order eyelid CRs. In eyelid conditioning, the second-order CS appears to inhibit responding to the first-order CS which immediately follows it.

Pavlov (1927, p. 33) reported that a response originally conditioned to one stimulus could be conditioned to another when the two were paired in the absence of the US. Investigations employing the conditioned fear response have demonstrated reliable second-order conditioning. McAllister and McAllister (1964) trained rats to hurdle-jump to avoid shock. A group which received forward pairings of the second-order and first-order stimulus (CS_2 and CS_1 , respectively) showed consistent second-order responding, whereas a backward conditioning control group did not. Other control procedures, such as noncontingent presentations of either CS_1 and the US (Rizley & Rescorla, 1972, Experiment 1) or CS_2 and CS_1 (Kamil, 1968), have also been examined. These studies demonstrate that both CS_1 and the US, as well as CS_2 and CS_1 , must be paired in order for responding to CS_2 to develop. These outcomes clearly establish second-order fear conditioning as a bona fide associative phenomenon.

In contrast, investigations of second-order conditioning with the eyelid response have not been notably successful. Maisiak and Frey (1977, Experiment 2) established stable responding to CS_1 and then presented conditioning sessions containing both CS_1 -US and CS_2 - CS_1 trials. Very few second-order CRs were observed. Overall responding to CS_2 was less than 2%. A companion study (Maisiak & Frey, 1977, Experiment 1), however, demonstrated reliable

second-order fear conditioning with an analogous set of procedures.

The difficulty in obtaining second-order eyelid conditioning may be due to the use of an inappropriate time relationship between CS_2 and CS_1 . This hypothesis is based on Pavlov's (1927, p. 33) specification that for second-order conditioning to be effective, a temporal gap must separate CS_2 and CS_1 ; i.e., "the essential condition is that the new stimulus should be withdrawn some seconds before the primary stimulus is applied." Fear conditioning data have not supported this notion. For example, Maisiak and Frey (1977, Experiment 1) found that second-order fear conditioning was less probable when a gap separated CS_2 and CS_1 than when the onset of CS_1 coincided with the termination of CS_2 . Similarly, Rescorla (1973) found that a delay second-order fear conditioning procedure was superior to a trace procedure. The present experiments provide a more detailed analysis of Pavlov's hypothesis by systematically manipulating the temporal relationship of CS_2 and CS_1 in second-order conditioning.

EXPERIMENT 1

Method

Subjects. The subjects were 48 male albino rabbits ranging from 15 to 20 weeks in age and weighing approximately 1.8 kg. The animals were housed individually with free access to water and Purina Rabbit Chow. Four animals died of respiratory infections, and their data were eliminated. The colony room was maintained at 21°C ($\pm 1^\circ$) and 50% relative humidity ($\pm 15\%$). The overhead fluorescent lights were on from 7 a.m. to 8 p.m. daily.

Apparatus. The conditioning equipment used in the present study was similar to that described in Frey and Butler (1977, Experiment 2). The rabbits were conditioned in a large walk-in chamber (2.6 m long, 2.0 m wide, and 2.1 m high). Its temperature (20°C , $\pm 1^\circ$) and humidity (50% relative, $\pm 10\%$) were kept relatively constant. A Grason-Stadler (Model 901B) white-

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noise generator maintained the background sound level in the chamber at 74 dB SPL. The animals were individually restrained within Plexiglas and aluminum stocks. A photoelectric system (see VanDercar, Swadlow, Elster, & Schneiderman, 1969) and a six-channel oscillograph (Beckman Model 504D) graphically recorded eyelid movements.

The first-order CS was a flashing light consisting of two small light bulbs (Chicago Miniature No. 1820), each mounted behind a plastic translucent lens cap. The bulbs were 5 cm apart and positioned approximately 12.5 cm in front of the rabbit's head and 12.5 cm above its eye level. The lights flashed alternately, somewhat like a railroad crossing, except at a faster rate (5 Hz). The second-order CS was a 3,000-Hz tone presented at 10 dB over the background noise level in the conditioning chamber. The US was a 100-msec constant current (4-mA) electric shock delivered through two stainless steel electrodes.

Procedure. The rabbits were habituated to their cages for at least 2 days prior to surgery. After being anesthetized, they were surgically prepared for conditioning by the placement of two cheek electrodes (25-ga stainless steel wire) just below and to either side of the right eye. Two stainless steel bolts (2.56 × 13 mm) were embedded into the skull to the animal's left and right of bregma and resting on the dura. A third bolt (2.56 × 5 mm), placed to the left of bregma, was used for additional support in conjunction with a covering layer of dental cement. The animal's skin was sutured over the cement platform.

After a 2-day recovery period, each animal had a 45-min habituation session in the restraining stock with the recording equipment attached. Acquisition training involved six sessions (Days 1-6), each consisting of 30 CS₁-US trials at a 90-sec inter-trial interval (ITI). The duration of CS₁ was 600 msec, and the CS₁-US interval was 500 msec. This was followed by two 50% reinforcement sessions (Days 7-8) designed to ensure stable responding to CS₁ during the second-order conditioning phase which was to follow. The performance of the animals on the 6th day of acquisition was rank ordered. Nine animals failed to meet a criterion of 50% responding during that day and were eliminated from the experiment.

On Days 9-14, the animals received six sessions of second-order conditioning. Four different CS₂-CS₁ intervals were examined. The onset of the 500-msec CS₂ occurred 500 (*n* = 8), 700 (*n* = 9), 1,000 (*n* = 9), or 5,000 (*n* = 9) msec prior to the onset of CS₁. The duration of CS₁ was 600 msec, and the CS₁-US interval was 500 msec. Each session consisted of 30 trials of an alternating presentation of CS₁-US and CS₂-CS₁ trials. The temporal parameters for CS₁-US trials were the same as for acquisition. The ITI was maintained at 90 sec.

After completing second-order conditioning, each animal was given a threshold test (Day 15). Shock intensity was reduced to a level which just barely elicited an unconditional response (recorded measurement: approximately 5 mm in size). Four CS₂-US_{weak} pairings were alternated with four US_{weak}-alone trials. During the final phase of the experiment, the animals were given a transfer test which consisted of 30 CS₂-US pairings per session until each animal met a criterion of 10 CRs. The CS₂-US interval was 500 msec and the ITI was 90 sec.

Results and Discussion

The gain of the recording instruments was adjusted so that a complete eyelid closure produced a near-maximal pen deflection for our recording system (i.e., 20-25 mm). A CR was defined as a pen movement of at least 1 mm in the appropriate direction between the onset of the CS and the onset of the stimulus which followed.

As has been the case with previous eyelid condi-

tioning studies (Maisiak & Frey, 1977, Experiment 2; Stern & Frey, 1978, Experiment 2), there were very few overt second-order CRs. All groups showed less than 3% responding to CS₂.

For the threshold test, the dependent measure was a UR ratio that compared the mean amplitudes (millimeters) of the URs on CS₂-US_{weak} trials to the URs on US_{weak}-alone trials. There were no reliable differences among the groups' UR ratios; $\bar{X}$ = 1.20, 1.15, 1.20, and 1.34 for Groups 500, 700, 1,000, and 5,000, respectively. On the transfer test, subjects reached a criterion of 10 CRs to CS₂ at similar rates; $\bar{X}$ = 54, 64, 73, and 66 trials, for Groups 500, 700, 1,000, and 5,000, respectively. These results imply that CS₂ had gained neither excitatory nor inhibitory properties as a result of the second-order conditioning trials. The observed UR ratios on the threshold test and the rate of conditioning on the transfer test are similar for all groups. If CS₂ had gained excitatory or inhibitory properties, one or more of these groups should have differed from the implicit control subjects (Group 5,000).

An analysis of variance was used to examine performance to CS₁. There were no reliable differences among groups in the number of trials needed to achieve a criterion performance level of 10 CRs to CS₁ during acquisition ($\bar{X}$ = 91 trials). As shown in Table 1, performance to CS₁ at the end of acquisition (Days 7 and 8) was similar for the four groups (*F* < 1.0). During the 50% reinforcement sessions (Days 7 and 8), responding was higher to CS₁ on the CS₁-alone trials than on the CS₁-US trials [*F*(1,31) = 91.31, *p* < .001]. Since the two trial types were alternated, this result indicates that US presentation increased the likelihood of making a CR on the subsequent trial. This effect has been reported previously in rabbit eyelid conditioning (Frey & Ross, 1967).

Responding to CS₁ during the second-order conditioning phase was examined after performance had

Table 1
Frequency of Conditional Responses (CRs) to the First-Order Stimulus (CS₁) in Experiment 1 as a Function of the Duration of the Interstimulus Interval (ISI) on Second-Order Trials

ISI	Percent CRs to CS ₁			
	Acquisition		Second-Order Conditioning	
	CS ₁ -US	CS ₁ Alone	CS ₁ -US	CS ₂ -CS ₁
500	55.2	77.3	69.7	44.2
700	54.8	77.8	59.0	55.3
1,000	55.3	76.5	61.2	74.3
5,000	51.5	65.5	50.6	78.3

Note—ISIs are given in milliseconds. Acquisition trials took place on Days 7-8, second-order conditioning trials on Days 12-14.

stabilized (Days 12-14). The main effects of ISI and CS₁-US vs. CS₂-CS₁ trial type were not significant [$F(3,31) = 1.06$ and $F < 1.0$, respectively]; there was a significant interaction between the two variables [$F(3,31) = 11.58$, $p < .001$]. As shown in Table 1, responding to CS₁ on CS₂-CS₁ trials showed a large increase as the gap separating CS₂ and CS₁ was lengthened; however, responding to CS₁ on CS₁-US trials decreased slightly as the gap was increased. This systematic effect of the CS₂-CS₁ temporal manipulation on responding to CS₁ on second-order trials indicates that CS₂ can act as an inhibitor in respect to a stimulus which follows immediately after it even though the animal's response tendency to CS₂ itself does not appear to be altered. One interpretation of this inhibitory effect is that CS₂ becomes a discriminative stimulus (i.e., a safety signal), indicating a period when no US will be presented. An equally likely nonassociative interpretation is that CS₂ simply masks the onset of CS₁. Both of these explanations are consistent with the observation that this inhibitory effect was less prominent when CS₂ terminated 500 msec or more prior to the onset of CS₁.

EXPERIMENT 2

There was no evidence of second-order conditioning with any of the ISI durations used in Experiment 1. It is not clear whether Pavlov's expectations are incorrect or whether the anticipated results are simply not present in eyelid conditioning. Since the conditioned suppression paradigm has provided consistent nonartifactual second-order conditioning (e.g., Davenport, 1966; Kamil, 1968; Rizley & Rescorla, 1972), Experiment 2 examined the ISI manipulation in fear conditioning.

Method

Subjects. The subjects were 96 male and female gerbils, ranging from 4 to 12 months in age. The animals were born and raised in our own colony and were individually caged. They were maintained on a 20-h food-deprivation schedule, with Purina Rat Chow being available for a 4-h feeding period immediately after each session. Water was constantly available. The gerbil colony was maintained at 23°C ($\pm 1^\circ$) and 50% relative humidity ($\pm 15\%$). Overhead fluorescent lights were on from 7 a.m. to 7 p.m. daily.

Apparatus. The gerbils were runway trained and tested for second-order conditioning in an automated runway which was enclosed in a sound-attenuating metal box. A detailed description of the runway appears elsewhere (Sears, 1978).

For second-order conditioning, the gerbils were individually placed in aluminum and Plexiglas boxes which were 61.0 cm long, 20.3 cm high, and 5.1 cm wide. A speaker was mounted in the center of the back wall of each box. The boxes were located within a walk-in, sound-attenuating chamber identical to the rabbit conditioning chamber described in Experiment 1. A Grason-Stadler (Model 901B) white-noise generator maintained the background noise level in the chamber at 76 dB SPL. A 10-flash/sec strobe light, which served as CS₁, was attached to the ceiling, centered above the four conditioning boxes. The

second-order CS was a 2,000-Hz tone presented at 10 dB above the background white-noise level. This stimulus, in both the runway and the conditioning boxes, was generated by a Hewlett Packard audio oscillator. The US was a .5-sec 4-mA footshock which was delivered across the grid floor of the conditioning box.

Procedure. The food-deprivation schedule began 5 days prior to the onset of runway training. During each day of this period, several Noyes food pellets were placed in the gerbil's home cage immediately before its 4-h feeding. Acquisition of the runway response occurred during nine daily sessions (Days 1-9). The subjects received 10 trials per session with a 5-sec delay between trials.

On Day 4 of runway training, off-the-baseline second-order conditioning began. The mean cumulative approach time (i.e., time to traverse the distance between the startbox and goalbox) was calculated for each animal for Days 3 and 4 of runway training. The running speeds were rank ordered, and this information was used to assign the animals to six similar groups of 16 animals each. Five of the groups received two trials per session—a first-order trial followed by a second-order trial, at a 12-min ITI. On the CS₁-US trial, a 5-sec strobe light presentation was followed immediately by the US. The groups differed in the duration of the ISI on CS₂-CS₁ trials. Its duration was 5, 10, 15, 25, or 125 sec, as measured from the onset of CS₂ to the onset of CS₁. The duration of CS₂ was constant at 5 sec and no US was presented on those trials. The time relationship for the 125-sec group is similar to that commonly used for an unpaired control group. The animals in the sixth group provided a baseline measure of performance. That group received a single trial per day in which CS₂ was paired with the US—that is, it received first-order excitatory conditioning with the tone CS (Group FO). This trial was presented at the same time in the session as the CS₁-US trial was for the other groups (12 min). There were six daily sessions of second-order conditioning (Days 4-9).

Upon completion of the runway training and the off-the-baseline second-order conditioning phases on Day 9, there were two test sessions in the automated runways (Days 10 and 11). All animals were treated the same as during the runway training sessions, with the exception that, on Trials 4 and 7, CS₂ was presented for 5 sec simultaneously with the opening of the startbox door.

Results and Discussion

The dependent measure used for examining the excitation that developed to CS₂ was the amount of suppression of the ongoing runway response. A suppression ratio was defined as $\alpha/(\alpha + \beta)$, where α was the mean approach speed (centimeters per second) on the two test trials in which the tone was presented and β was the mean approach speed on the trials immediately preceding and following the test trials (i.e., Trials 3, 5, 6, and 8). A suppression ratio of 0 indicates that the stimulus had acquired complete suppressive properties, whereas a ratio of .5 indicates that it had no suppressive properties.

A trend analysis for unequal intervals (Myers, 1972, p. 386) was conducted on the mean suppression ratios across the 2 days of testing as a function of log seconds ISI. This analysis was done for the five groups in which a temporal gap was introduced between CS₂ and CS₁. As may be seen in Figure 1, the amount of suppression increased as the duration of the ISI was shortened [linear component $F(1,60)$

Figure 1. Amount of suppression to the second-order stimulus (CS₂) during the test sessions following second-order conditioning. Groups 5, 10, 15, 25, and 125 differed in the time interval between the onset of CS₂ and the onset of CS₁ on second-order trials. Training for the first-order group consisted of pairing CS₂ with the US, i.e., regular first-order conditioning.

= 10.98, $p < .01$]. As also shown in Figure 1, Group FO evidenced reliably more suppression of the runway response than Group 5 [$t(30) = 4.67$, $p < .001$]. This suggested that the degree of excitation established as a result of second-order conditioning was not as great as that found with a normal first-order conditioning procedure.

Experiment 2, using the conditioned suppression paradigm, demonstrated reliable second-order conditioning. Group 5 reliably showed more suppression than Group 125 [$t(30) = 2.68$, $p < .02$]. In addition, the results indicated that the temporal gap that was interposed between CS₂ and CS₁ for Groups 10, 15, and 25 did not facilitate second-order conditioning. This finding clarifies the interpretation of Experiment 1. The temporal relationship between CS₂ and CS₁ is important in second-order fear conditioning, with the optimal arrangement being either a short gap or simultaneous offset and onset. The temporal arrangements employed in Experiment 1, therefore, should have been successful if our second-order eyelid conditioning procedure was viable in other respects.

GENERAL DISCUSSION

The present experiments examined whether second-order conditioning has not been observed with the eyelid response because the first- and second-order stimuli have been presented in an inappropriate temporal relationship. In Experiment 1, there was an absence of second-order eyelid conditioning with all of the interstimulus intervals examined. With the fear response in Experiment 2, reliable second-order con-

ditioning was observed when the onset of the first-order stimulus coincided with the termination of the second-order stimulus. Extending the interval between the first- and second-order stimulus produced a decrease in the amount of second-order conditioning. This implies that the negative results observed with second-order eyelid conditioning in Experiment 1 would not have been changed if additional CS₂-CS₁ separations had been examined.

The virtual absence of second-order eyelid conditioning in Experiment 1 is consistent with other eyelid research (Maisiak & Frey, 1977, Experiment 2; Stern & Frey, 1978, Experiment 2). In each of these studies, as well as in the present investigation, attempts were made to maximize the possibility of observing second-order CRs. These included continued reinforcement of the first-order CS during second-order conditioning to insure that CS₁ would not extinguish, the introduction of a partial reinforcement schedule toward the end of the first-order conditioning phase to minimize generalization decrement at the beginning of second-order conditioning, and an examination of several time relationships between the first- and second-order stimulus. In addition, threshold and transfer tests were given, both of which are relatively sensitive follow-up procedures. Despite these efforts, little evidence for second-order eyelid conditioning was observed.

Pavlov (1927) was concerned about striking a balance between a complete overlap of CS₂ and CS₁, producing conditioned inhibition and a large separation of CS₂ and CS₁, producing no conditioning at all. He believed that second-order conditioning was probable when an intermediate temporal relationship was employed and that inhibition was probable when CS₂ and CS₁ overlapped. This idea is consistent with the data from several studies in which extended conditioning was given with CS₂ preceding and overlapping CS₁ (e.g., Herendeen & Anderson, 1968; Rescorla, 1972, 1973). In these studies, an initial excitatory phase of responding was followed by a subsequent inhibitory phase.

An interesting observation in Experiment 1 was that CS₂ had an inhibiting influence on CS₁ when the two stimuli occurred close together in time on second-order trials (Groups 500 and 700). This inhibitory effect, however, is not equivalent to that produced by a true conditioned inhibitor. Hearst (1972) and Rescorla (1969) have operationally defined conditioned inhibition in terms of the joint demonstration of reduced responding on a summation test and slower acquisition on a transfer test. The threshold test we employed provides essentially the same information as a summation test. Therefore, the negative results we observed with the threshold and transfer tests provide evidence that CS₂ was not a conditioned inhibitor. Apparently CS₂ has a

prominent inhibitory influence on stimuli which follow it, but little or no inhibitory effect during its own presentation. This pattern of results implies that CS₂ acts in a secondary inhibitory role either as a discriminative stimulus which modulates responding to a subsequent stimulus or as a distracting stimulus which overshadows or masks the response-eliciting properties of the stimulus which follows.

The present experiments offer no explanation for the prominent differences between second-order fear conditioning and eyelid conditioning. There is an established literature which demonstrates that the conditional eyelid response is carefully timed to occur just prior to US onset (e.g., Boneau, 1958; Frey, 1970; Frey & Ross, 1968; Leonard & Theios, 1967; Martin & Levey, 1969; Smith, 1968). This response system characteristic may be incompatible with the elicitation of overt responding to a second-order CS. Even if the second-order CS becomes a signal for US occurrence, the time relationship between the two is inappropriate for eliciting an immediate response.

Sears, Baker, and Frey (1979) have recently demonstrated that the first element of a two-element serial CS develops response-eliciting properties in eyelid conditioning. However, when this stimulus is presented by itself on a test trial, the CR occurs some time after its termination. This "late" response occurs at the time the US would normally occur on a regular serial trial. If second-order conditioning leads to the development of a timed second-order eyelid CR, then this response would also be expected to occur after the termination of the second-order stimulus. The establishment of an appropriate time relationship between the second-order stimulus and US onset requires that a conceptual superimposition of first-order and second-order trials be made. A complex process such as this may not occur in eyelid conditioning or it may occur only to a limited extent. Recent observations (Sears, Baker, & Frey, 1979, Experiment 4) are consistent with the latter possibility.

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