Behavioral Expression of Learned Fear: Updating of Early Memories

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The expression of learned fear emerges in a response-specific sequence where freezing occurs before fear potentiated startle (FPS) to an odor conditioned stimulus (CS; Postnatal Day [PN] 16 vs. PN 23; e.g., Hunt, 1997; Richardson, Paxinos, & Lee, 2000). Studies have shown that learned fear is expressed in a manner appropriate to the animal’s age at training and not its age at test (Richardson & Fan, 2002; Richardson et al., 2000). Specifically, animals trained with an odor CS at PN 16 exhibit avoidance but not FPS when tested at PN 23. The present study shows that subsequent training with a different CS can “update” an early memory, allowing it to be expressed in a manner appropriate to the animal’s age at test. This updating effect appears to be modality specific, whereby the subsequent training must involve a CS of the same sensory modality as the original training.

Keywords: Fear potentiated startle, odor, development, learned fear, freezing

In a Pavlovian fear conditioning preparation, a conditioned stimulus (CS) such as a tone, light, or odor is paired with an unconditional stimulus (US) such as an aversive shock. A consequence of these pairings is that an association is formed between the CS and US, and subsequent presentations of the CS elicit fear. There are several potential measures of learned fear elicited by the CS including changes in heart rate, freezing, and potentiation of the acoustic startle response (e.g., LeDoux, 1993; McAllister & McAllister, 1971). Studies with adult rats indicate “equivalence” of these conditioned responses (CRs); in other words, these measures of fear acquisition tend to covary consistently across parametric variables and experimental manipulations in adult rats (Hunt & Campbell, 1997; Leaton & Cranney, 1990; Stanton, 2000). However, tests of Pavlovian conditioning in the developing rat suggest that learning emerges in an asynchronous manner that is both sensory specific and response specific. Specifically, studies have shown that conditioned fear responses emerge at different points in development depending on the modality of the CS used in the conditioning procedure (Hunt & Campbell, 1997). For example, whereas freezing to an auditory CS occurs at Postnatal Day [PN] 15, freezing to a visual CS does not emerge until about PN 17 (Moye & Rudy, 1985). Hunt and Campbell pointed out that the asynchrony in the expression of learning observed in young rats cannot be due to independent maturational changes of the motoric or sensory pathways. Rats have the motoric ability to exhibit freezing to an auditory stimulus at 15 days, but they do not express this particular index of fear when the CS is a visual cue. Therefore, the failure to observe conditioning to a visual CS is not merely due to the rat’s inability to freeze. In addition, the asynchrony in the expression of learning cannot be attributed to sensory immaturity, as infant rats readily detect an auditory stimulus and exhibit freezing to it at 15 days of age (Moye & Rudy, 1985) but do not exhibit conditioning to that stimulus when change in heart rate is used as the index of learning until 21 days of age (Campbell & Ampuero, 1985). Thus, it appears that the CS-US binding process is functional at quite an early age. However, the neural pathways connecting the CS sensory inputs and the efferent structures mediating the various behavioral expressions of fear appear to emerge at different points in time across development (Hunt & Campbell, 1997).

A common measure of learned fear in the rat is fear potentiated startle (FPS; e.g., Brown, Kalish, & Farber, 1951; Falls & Davis, 1993; Hunt, 1999; Richardson, Vishney, & Lee, 1999). The startle reflex consists of a rapid sequential contraction of muscles, predominantly around the face, neck, and shoulders. When an aversive CS precedes the startle stimulus, such as a sudden acoustic, visual, or tactile stimulus (Fendt & Fanselow, 1999), the amplitude of the elicited startle reflex is greater than when the startle stimulus is presented alone (e.g., Davis, Falls, Campeau, & Kim, 1993). The potentiation of startle has been demonstrated using a variety of conditioned stimuli (e.g., Campeau & Davis, 1995; Richardson et al., 1999) and is highly correlated with other behavioral measures of fear (e.g., Leaton & Cranney, 1990). An interesting aspect of FPS is that there is considerable evidence that it emerges relatively late in ontogeny (e.g., Hunt, 1999; Hunt & Campbell, 1997; Hunt, Richardson, & Campbell, 1994;
Richardson et al., 1999). Moreover, the emergence of FPS does not appear to depend on CS modality, as is the case with freezing and heart rate. FPS emerges at PN 23 for visual (Hunt, 1999), auditory (Hunt et al., 1994), and olfactory stimuli (Richardson, Paxinos, & Lee, 2000).

Given that FPS emerges relatively late in development, Richardson and colleagues have used this index of learned fear to examine whether the expression of early learning is appropriate to the rat’s age at training or to its age at test (Richardson & Fan, 2002; Richardson et al., 2000). Specifically, Richardson et al. (2000) demonstrated that an odor paired with shock potentiated the startle response for rats that were trained at PN 23. However, this same CR was absent for rats trained at 16 days of age. They also reported that FPS was absent for rats trained at 16 days and tested at 23 days of age. In other words although these rats possessed the ability to exhibit potentiated startle at the age of test (~PN 23), they did not express this particular conditioned response as they did not exhibit it at the age of training (PN 16). The failure to detect odor potentiated startle at test in these rats could not be attributed to retention or acquisition deficits because they exhibited a pronounced avoidance of the odor CS. This finding was also reported using a within-subject design (Richardson & Fan, 2002). In addition, Hunt and Barnet (2002) reported that PN 18 rats given light–shock pairings did not exhibit FPS to the light CS when tested at PN 25 even though they displayed light-elicited freezing comparable to that of rats that were trained at PN 24. In summary, these studies suggest that the expression of learning is appropriate to the rat’s age at training and not its age at test.

In the present series of experiments, we addressed whether the behavioral expression of a CS-US association acquired at PN 16 can be “updated” or “activated” by subsequent training at an older age. Specifically, we examined whether rats would exhibit FPS to an odor trained at PN 16 if they were conditioned to a second CS at PN 23. We predicted that rats should exhibit FPS to the second CS, as it was trained at an age when FPS has emerged (PN 23). However, of particular interest was whether FPS would also be observed, or activated, to the odor conditioned at PN 16 as a consequence of training to a second CS at PN 23.

Experiment 1

Experiment 1 examined whether rats were able to update the expression of early learning if they were conditioned to a second stimulus at a more mature age. Specifically, Experiment 1 explored whether rats given Odor 1–shock pairings at PN 16 and Odor 2–shock pairings at PN 23 (Group 16(O1)–23(O2)) would exhibit both freezing and FPS to Odor 1 when tested at PN 23. There were two control groups: (a) rats trained only to Odor 1 at 16 days of age (Group 16(O1)) and (b) rats trained only to Odor 2 at 23 days of age (Group 23(O2)). The former group was included to demonstrate that rats perform in a manner appropriate to their age at training rather than their age at test (i.e., they should exhibit freezing but not FPS to Odor 1). The second control group, given Odor 2–shock pairings at PN 23, was included to control for the possibility of generalization across different odors. All rats were tested at 24 and 25 days of age to each odor, and the order of test was counterbalanced (see Table 1 for the experimental design).

We predicted that rats given Odor 1–shock pairings at 16 days of age would exhibit freezing to that odor when tested at 24 to 25 days of age regardless of whether they exhibit FPS to that odor (Group 16(O1)–23(O2) and Group 16(O1)). We also predicted that rats given Odor 2–shock pairings at 23 days of age would demonstrate both freezing and FPS to Odor 2 (Group 16(O1)–23(O2) and Group 23(O2)). Moreover, we expected rats to be able to discriminate between the two odors based on pilot data obtained in our laboratory. That is, rats in Group 23(O2) would exhibit CRs only to an odor that had been previously paired with a shock (Odor 2) and not to a novel odor (Odor 1). Similarly, we expected rats given Odor 1–shock pairings at PN 16 (Group 16(O1)) to freeze to Odor 1 and not Odor 2. Finally, if conditioning to a second odor at PN 23 updates or activates the expression of learning acquired at PN 16, then rats in this condition should exhibit FPS as well as freezing to Odor 1 at test (Group 16(O1)–23(O2)).

Method

Subjects

Thirty experimentally naive Sprague-Dawley rats obtained from the breeding colony maintained by the School of Psychology at the University of New South Wales were used. All rats were 16 (±1) days of age at the commencement of the experiment. Rats were housed in groups of 8, with their mother, in plastic boxes (37 × 24.5 × 27 cm; length × width × height), and kept in a room with a 12-hr light–dark cycle (lights on at 6 am). No more than one rat per litter was used in any experimental group. Food and water were continuously available. All rats were treated according to the principles of animal use outlined in The Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th ed., 2004), and the Animal Care and Ethics Committee at the University of New South Wales approved all procedures.

Apparatus

Conditioning and startle tests occurred in two identical startle chambers (13 × 9 × 9 cm; length × width × height). The startle chambers were

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Note. In group designations, numerals represent age in days. PN = postnatal day; O = odor; FPS = fear potentiated startle.
constructed of Plexiglas and stainless steel bars. The front wall, rear wall, and ceiling of each chamber were constructed of clear Plexiglas and stainless steel bars. The two sidewalls were made of 3-mm stainless steel rods. The rods were vertically positioned relative to the floor of the chamber. The floor was composed of 3-mm stainless steel rods spaced 13 mm apart, center to center. Electric shock (0.6 mA, 1 s in duration) could be delivered to the floor of each chamber via a custom-built constant shock generator. The chamber was attached to a piece of Plexiglas onto which a sheet of piezoelectric film had been laminated. Movement in the startle chamber caused the piece of Plexiglas to flex, which consequently produced voltage in the piezoelectric film. This voltage is proportional to the amount of movement within the chamber, with larger movements producing larger voltages. These voltages were amplified and digitized (at a 1-kHz rate) in order to measure startle amplitude. The peak voltage (converted into arbitrary units ranging from 0–32,000; data rounded to 0–320 prior to analysis) in the 250-ms period after stimulus onset was taken as the index of the startle response.

The acoustic startle stimulus was delivered through two high-frequency speakers mounted 8 cm on either side of the startle chamber. The startle stimulus was a 100-ms, 100-dB burst of white noise, with a 1-ms rise–fall time. The intensity of the startle stimulus and the background noise was measured with a Bruel & Kjaer (Naerum, Denmark) precision sound level meter (Type 2235) placed in the center of the startle cage. A computer controlled the stimulus presentations and recording of data. The software and hardware were custom-developed at the University of New South Wales.

The chambers were housed in wood cabinets in order to reduce external noise and visual stimulation. Ventilation fans located on the sidewall of the wood cabinets provided a low-level background noise (~60 dB) at all times, and illumination was provided by a 15-W red light on the front door of each cabinet. A removable tray that contained rat bedding was located beneath each cage and was cleaned and replaced with the removal of each rat.

Alternative chambers were used to measure freezing. The two freezing chambers (19 cm long × 16 cm wide × 10 cm high) were constructed entirely of clear Plexiglas, with 30 holes 1 cm in diameter drilled into the floor to enable the odor to enter the chamber during testing. Three walls of each freezing chamber were lined with contact paper that had vertical black and white stripes. Each stripe was 1 cm wide. The front wall was made of clear Plexiglas. The ceiling of each chamber was lined with a black sheet. The freezing chambers were housed within a wood cabinet like the one used for the startle chambers. Located on the door of this wood cabinet was a 24-V red light that illuminated the chambers at all times. Testing sessions were recorded using a Panasonic BP-100 video camera.

**Odor**

The odors used in this study were 0.1 ml of grape flavor (Grape No. 182380019 from Wild Flavours, Heidelberg, Germany) and eucalyptus (Goanna Eucalyptus Oil; Herron Pharmaceuticals, Queensland, Australia). The odors were squirted onto a piece of paper towel inside a plastic specimen jar.

**Procedure**

**Training.** Rats in Group 16(O1)–23(O2) and Group 16(O1) received Odor 1–shock training at PN 16, whereas rats in Group 23(O2) received shock-only training at PN 16 (n = 10). The shock-only treatment was included to equate the number of shocks received across experimental groups. Fifteen Odor 1–shock pairings were given at PN 16. A two-stage training procedure was used for odor conditioning. The doors of the wood cabinets in which the startle cages were located were kept slightly ajar throughout both stages of training. In Stage 1 of training, rats were placed in the startle chamber and given a 5-min adaptation period, at the end of which the first of 15 shocks was given. Seven to 10 s prior to the delivery of shocks, a specimen jar containing an odor was placed approximately 10 cm beneath the startle chambers. The jar contained grape for half the rats and eucalyptus for the remaining rats. That is, Odor 1 was either grape or eucalyptus. Rats that received shock-only training experienced identical procedures with the exception that the specimen jar contained water instead of an odor. For all groups, immediately after the shock, the jar was removed and the lid replaced. The jar was then placed on a bench approximately 2 m from the startle chamber. The interval between the shock presentations was 1.5, 2, or 2.5 min (average = 2 min), and varied pseudorandomly. After the last shock, rats were removed from the startle chambers and placed in their home cages. In addition to producing an association between the odor and shock, the training procedure also produces an association between the context, that is, the startle chambers, and shock. In order to reduce the level of contextual fear, rats were returned to the startle chambers 50 to 80 min later for the second phase of training. In Stage 2 of training, for groups that had received Odor 1–shock pairings in Stage 1 (Group 16(O1)–23(O2) and Group 16(O1)), a specimen jar containing water was placed beneath the chamber for 7 to 10 s after a 2-min adaptation but no shock was delivered. For the group that had received shock-only presentations in Stage 1 (Group 23(O2)), rats were placed in the context for Stage 2 with no stimulus presentation. The specimen jar presentation was repeated 15 times in Stage 2 (i.e., the same number of trials as in Stage 1), and the interval between presentations was 1.5, 2, or 2.5 min (average = 2 min), and varied pseudorandomly (i.e., same as Stage 1). Both stages of training lasted approximately 33 min, and at the end of each stage, the doors and windows were opened for at least 10 min to allow ventilation of the experimental room.

Rats trained at 23 days of age received five Odor 2–shock pairings (Group 16(O1)–23(O2) and 23(O2)). The procedures were identical to those used for rats trained at PN 16 with the exception that training consisted of 5 trials instead of 15 and the training session lasted approximately 15 min instead of 33 min. Rats in 16(O1) received shock-only treatment at PN 23. Therefore, Group 16(O1) received identical procedures to those used for the other groups at PN 23 with the exception that the specimen jar contained water instead of an odor in Stage 1 of training. In Stage 2 of training, rats in Groups 16(O1)–23(O2) and 23(O2) received contextual extinction and five water presentations. The interval between water presentations was kept the same as Stage 1 odor presentations (1.5, 2, and 2.5 min, average = 2 min). Rats in Group 16(O1) were placed in the context in Stage 2 with no stimulus presentations.

**Test.** All rats were tested four times: (a) FPS to Odor 1, (b) FPS to Odor 2, (c) freezing to Odor 1, and (d) freezing to Odor 2. These tests were conducted over 2 days when the rats were 24 and 25 days of age. On the 1st day of testing, rats were tested for FPS and freezing to one stimulus (either Odor 1 or Odor 2), and on the 2nd day, they were tested for FPS and freezing to the other odor. The stimulus tested on the 1st day of testing was counterbalanced such that half the rats were tested to Odor 1 on the 1st day and the other half were tested to Odor 2. Furthermore, the order of test type on each day was also counterbalanced, such that half the rats were tested for FPS to each stimulus first, whereas the other half were tested for freezing first. On each day of testing, the two tests were separated by 2 to 4 hr.

**Test for FPS.** Rats were returned to the startle chamber in which they had been trained. After a 5-min adaptation period, 30 startle-eliciting noise bursts, separated by 30 s, were presented. No odor was present during these initial 30 bursts. The average startle response on these initial 30 trials was taken as an estimate of the rat’s baseline startle response. During a 60-s period following the 30th noise burst, a jar containing either the grape or eucalyptus odor was placed beneath the startle chamber floor and remained there for the rest of the test session (approximately 20 min). After introduction of the odor, an additional 30 noise bursts, each separated by 30 s, were presented. At the end of the testing session, subjects were removed from the startle chamber and returned to the home cage. Odor potentiation of startle (OPS) was examined by comparing the average startle amplitude
when the odor was present to the average startle amplitude during baseline
and was calculated using the following equation: \(\frac{(T - B)}{B} \times 100 = OPS\),
where \(T = \) mean startle amplitude over the 30 test trials, \(B = \) mean startle amplitude
over the 30 baseline trials, and \(OPS = \) percentage change in startle amplitude.

**Test for freezing.** In the first stage of the freezing test, subjects were
placed in the freezing chamber for 1 min in the absence of either odor to
establish baseline freezing. Rats were retested for baseline freezing if the
initial test exceeded 50% freezing. A maximum of three baseline trials
were given before the rat was excluded from the experiment. At the end of
the 1-min baseline test, rats were returned to their home cages. Prior to the
second stage of the freezing test, the specimen jar containing either the
grape or eucalyptus odor was placed approximately 1 cm below the
chamber floor. Rats were then returned to the chamber for the second stage
of testing where freezing was scored in the presence of the odor for 2 min.
The interval between baseline and CS test was approximately 10 min.

**Scoring of freezing.** Both stages of the freezing test were recorded. A
time sampling technique was then used in which each rat was scored as
freezing or moving every 3 s. Scoring commenced approximately 10 s after
the rats were placed in the chamber. Freezing was defined as the absence
of all movement except for those related to breathing (Fanselow, 1980). A
second scorer, who was unaware of the experimental condition of each rat,
scored a random sample of approximately 35% of the rats tested. Interrater
reliability was found to be greater than 90%. Because of individual differ-
cences in baseline freezing, CS elicited freezing was calculated as a differ-
ence score. The CS difference score was calculated using the following
formula: \(CF = (T - B)\) where \(CF = \) CS-elicited freezing, \(T = \) percentage
of time spent freezing in the presence of the odor CS, and \(B = \) percentage
of time spent freezing during baseline.

**Statistical Analysis**
All statistical analyses for both baseline and CS-elicited responses used
planned orthogonal ANOVAs (Hays, 1972), and the decision-wise error
rate was set at \(\alpha = .05\).

**Results and Discussion**

**Test for Odor 1-Elicited Freezing**

The analyses suggested that there were no significant differ-
ences between groups prior to Odor 1 presentation, largest \(F(1, 27) = 2.25, p = .15\). Figure 1A shows the CS freezing difference scores for the three groups in the presence of Odor 1 at test.

**Test for Odor 1-Elicited FPS**

One subject from Group 16(O1)–23(O2) and one subject from
Group 23(O2) froze significantly more than rats in Group 16(O1), \(F(1, 27) = 11.86, p < .01\), and (b) levels of freezing in Group 16(O1)–23(O2) and Group 23(O2)
were not significantly different \((F < 1)\).

**Test for Odor 2-Elicited Freezing**

Analyses of baseline freezing performance suggested that there
were no differences between groups prior to Odor 2 presentation
\((Fs < 1)\). Inspection of Figure 1A suggests that rats conditioned to
Odor 2 at PN 23 froze more to Odor 2 than did rats that had not
been conditioned to that odor. Moreover, rats in the two groups
trained to Odor 2 showed similar levels of freezing to Odor 2.
Analyses of the test data confirmed these observations where (a)
Figure 1B illustrates the mean percentage of change in startle amplitude in the presence of Odor 1. Inspection of Figure 1B suggests that Group 16(O1)–23(O2) demonstrated greater FPS to Odor 1 than did Group 16(O1) and Group 23(O2). Note that a decrease in startle amplitude of ~30% to 40% is a typical level for odor potentiated startle in rats trained at ~23 days of age. Rats that had been trained to Odor 2 at PN 23 after initial training with Odor 1 at PN 16 exhibited greater FPS to Odor 1 compared with (a) rats that experienced only Odor 1–shock pairings at PN 16 and then tested at PN 24–25 (Group 16(O1)), and (b) rats that had only experienced Odor 2–shock pairings at PN 23 (Group 23(O2)). Statistical analyses were consistent with these observations whereby the levels of FPS to Odor 1 in Group 16(O1) and Group 23(O2) were significantly different from those in Group 16(O1)–23(O2), F(1, 25) = 14.98, p < .01. Moreover, the low levels of FPS shown by Group 16(O1) and Group 23(O2) were comparable. That means that although Group 16(O1) was trained to Odor 1 at PN 16, the level of FPS to Odor 1 in this group was not significantly different to that seen in rats that had never been trained to Odor 1 (Group 23(O2); F < 1).

Test for Odor 2-Elicited FPS

Two subjects in Group 23(O2) were excluded from the analyses because of procedural errors in the startle test. Analyses of baseline startle amplitudes indicated that there were no significant group differences (Fs < 1). Figure 1B shows that rats that had been given Odor 2–shock pairings at PN 23 (Group 16(O1)–23(O2) and Group 23(O2)) exhibited more FPS to Odor 2 than rats that had never been conditioned to this odor (Group 16(O1)), F(1, 25) = 12.32, p < .01. In addition, the performance by rats in Group 16(O1)–23(O2) was not significantly different from that of rats in Group 23(O2) in their level of FPS to Odor 2, F(1, 25) = 1.1, p = .30.

There are three main findings in this experiment. First, the results show that rats trained to Odor 1 at PN 16 exhibit freezing but not FPS to Odor 1 when tested at PN 24–25, which is consistent with previous reports from this lab (Richardson & Fan, 2002; Richardson et al., 2000). This experiment therefore provides further support for the hypothesis that expression of conditioned fear is appropriate to the rat’s age at the time of training and not to its age at the time of test. The second finding of interest in this experiment is that training to a second odor at PN 23 after initial training to Odor 1 at PN 16 activated FPS to Odor 1. Finally, the third finding of interest is that control rats that received Odor 2–shock only at PN 23 exhibited FPS only to Odor 2. This latter result indicates that FPS to Odor 1 in Group 16(O1)–23(O2) was not a result of simple generalization from Odor 2 to Odor 1.

Experiment 2

In the previous experiment, the results suggested that training to a second odor at PN 23 enabled rats to exhibit FPS to an odor trained at PN 16. Experiment 2 examined whether this activation effect occurs when the second CS is of a different sensory modality than the first CS. Specifically, would conditioning to a light CS at PN 23 activate the expression of FPS to an odor trained at PN 16? To answer this question, we used three groups in the second experiment (see Table 2 for the experimental design). In Group PN 16 (O), rats were given odor–shock pairings at PN 16 and tested at PN 24. This group was identical to Group 16(O1) in Experiment 1, and, therefore, we predicted rats in this group would express freezing but not FPS to the odor at test. The second group in this experiment was identical to Group 16(O1)–23(O2) in Experiment 1 with the exception that instead of receiving Odor 2–shock pairings at PN 23, rats in Group 16(O)–23(L) received light–shock pairings at PN 23. We predicted that rats in this group would show freezing to both the light and odor as they were conditioned to both CSs at ages when they are capable of expressing freezing to both stimuli. Moreover, rats in this group should also exhibit FPS to the light CS as the neural processes mediating the expression of FPS to a visual stimulus would have matured by PN 23 (Hunt, 1999). It is unclear, however, whether rats would show FPS to the odor conditioned at PN 16 if they are subsequently given light–shock training at PN 23. Finally, a third group of rats received light–shock pairings at PN 23 (Group 23[L]). We expected rats in this group to show freezing and FPS to the light CS and no conditioned responses to the odor. The inclusion of this group was to ensure that any FPS in the presence of the odor in Group 16(O)–23(L) was due to the odor–shock association acquired at PN 16 and not due to generalization of fear from the light CS to the odor CS.

Method

Subjects

Forty-two Sprague–Dawley rats were used in this experiment (n = 14). They were obtained from the same source as in the previous experiment. All rats were 16 (±1) days of age at the commencement of the experiment.

Apparatus

The equipment used in this experiment was the same as that used in the previous experiment with the exception that an 18-W white light instead of a second odor was used as a CS for some rats in this experiment.

Table 2

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<td>Light-shock</td>
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Note. In group designations, numerals represent age in days. PN = postnatal day; O = odor; L = light; FPS = fear potentiated startle.
Odor

The odor used was 0.1 ml of grape.

Procedure

Training. Rats in Groups 16(O)–23(L) and 16(O) received odor–shock training at PN 16, whereas rats in Group 23(L) received shock-only training at PN 16 (see Table 2 for experimental design). The training that occurred at PN 16 for all groups was identical to that performed in Experiment 1. Rats in Group 16(O)–23(L) and Group 23(L) received 10 light–shock pairings at PN 23, whereas rats in Group 16(O) received shock-only treatment at PN 23. Light training consisted of two phases. In the first phase of training, rats were placed in the startle chambers with the cabinet doors closed and received a 5-min adaptation period. Following this, rats in Groups 16(O)–23(L) and 23(L) were exposed to an 18-W white light for 8 s. A shock (0.6 mA/1 s) was administered during the last second of this light CS. There were 10 light–shock pairings, with the intertrial interval varying pseudorandomly between 2 and 3 min. Rats receiving shock-only training sessions received an identical number of shocks but no exposure to the white light. Following administration of the final shock, rats were returned to their home cage. All rats received a second phase of training in which they were placed in the startle chamber for 30 min with no programmed stimuli (i.e., no lights or shocks). This second phase of training occurred at least 2 hr after the initial phase.

Test. Tests for freezing and FPS to the odor were identical to those used in Experiment 1. The order of test each day (freezing or FPS) and the sequence of the stimulus (light or odor) tested were counterbalanced.

Test for light-elicited freezing. Rats were placed in the freezing chamber, and their baseline level of freezing in the absence of the light was recorded for 1 min. Following this, the light CS was presented, and the rat’s level of freezing in the presence of the light was recorded for 2 min.

Test for light-elicited FPS. As light is a discrete CS, as opposed to the continuous odor CS we used, the test for FPS to the light CS was different from the test used for the odor CS. For the test with the light CS, rats were placed in the startle chamber and received a 5-min adaptation period followed by 30 startle-eliciting bursts, each separated by 30 s. The chamber was illuminated only by a red light during this time. Following these initial 30 startle bursts, rats received a further 18 test trials. The intertrial interval between these trials varied pseudorandomly between 1 and 2 min, with a mean interval of approximately 1.4 min. On 6 of these 18 trials, the startle stimulus was preceded by an 8-s exposure to the light CS. The mean amplitude response on these 6 trials was taken as the average startle response in the presence of the light CS. The mean amplitude over the 12 test trials on which the light was not presented was taken as the rat’s baseline startle response. Potentiation of startle was calculated as the percentage difference in responding on baseline and test trials using the following formula: 

\[
\text{FPS} = \left( \frac{L - B}{B} \right) \times 100, \text{ where } FPS = \text{percentage increase in startle, } L = \text{mean startle amplitude in the presence of the light CS, and } B = \text{mean baseline startle amplitude.}
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Results and Discussion

There were three main findings in this experiment: (a) Rats that had been trained to a light CS at PN 23 exhibited FPS and freezing to the light CS at test, (b) rats that had been trained to an odor CS at PN 16 froze to the odor when tested at PN 23, and (c) rats that had been trained to an odor CS at PN 16 and trained to a light CS froze to the odor and the light at test. However, training to a light CS at PN 23 did not activate FPS to the odor CS acquired at PN 16.

Test for Odor-Elicited Freezing

One subject from Group 16(O)–23(L) and one subject from Group 16(O) were excluded from this analysis because they failed to meet baseline criterion for freezing (less than 50% on baseline). In addition, one subject from Group 16(O)–23(L) and one subject from Group 16(O) were excluded because of experimenter error.

An initial analysis of baseline freezing showed that there were no significant group differences (Fs < 1). Inspection of the levels of odor-elicited freezing (see Figure 2A) showed that rats in Group 16(O)–23(L) and Group 16(O) froze more to the odor than rats in Group 23(L). This difference was confirmed by statistical analysis, F(1, 35) = 6.43, p < .05. Moreover, there were no differences in the level of freezing between Group 16(O)–23(L) and Group 16(O), F(1, 35) = 1.09, p = .30. This latter result suggests that subsequent light training for Group 16(O)–23(L) did not affect overall freezing performance to the odor.

Test for Light-Elicited Freezing

Three subjects from Group 16(O)–23(L) were excluded from this analysis because they failed to meet baseline freezing criterion

![Figure 2. Results of Experiment 2: mean (± SEM) freezing difference score to odor and light (A) and mean (± SEM) percent change in startle amplitude to odor and light (B). CS = conditioned stimulus. In group designations, numerals represent age in days; O = odor; L = light.](image-url)
(less than 50%). In addition, 1 subject from Group 16(O) was excluded because of experimenter error. An initial analysis of baseline freezing showed that there were no significant group differences in baseline freezing ($F_{s} < 1$).

Figure 2A shows the mean freezing difference scores in the presence of the light CS for each of the three groups. Inspection of the figure suggests that rats in Group 16(O)–23(L) and Group 23(L) exhibit greater levels of light-elicited freezing than rats in Group 16(O). Statistical analysis confirmed that this difference was significant, $F(1, 35) = 7.41$, $p < .05$. Moreover, the level of freezing exhibited by Group 16(O)–23(L) and Group 23(L) to the light CS was not significantly different ($F < 1$).

Test for Odor-Elicited FPS

One subject from Group 16(O) and 1 subject from Group 23(L) were excluded from the analysis because of experimenter error. Analyses of the baseline startle amplitudes showed that there were no significant group differences ($F_{s} < 1$).

Figure 2B shows the average percentage change in startle amplitude of the three groups in the presence of the odor CS. Inspection of the figure suggests that there were no differences in levels of FPS between the three groups. This observation was confirmed by statistical analysis where Group 16(O)–23(L) did not exhibit greater FPS than Group 16(O) and Group 23(L) ($F < 1$). Moreover, Group 16(O) did not differ significantly from Group 23(L) ($F < 1$). As is clear in the figure, none of these groups exhibited FPS in the presence of the odor CS (cf. Figures 1B and 2B).

Test for Light-Elicited FPS

One subject from Group 16(O) was excluded from this analysis because of experimenter error. Furthermore, 1 subject from Group 16(O)–23(L) was excluded from the analysis because the percentage change in startle amplitude (%% FPS) exhibited by this rat was more than 8 standard deviations away from the group mean. Analysis of the baseline startle amplitude revealed no significant group differences ($F_{s} < 1$).

Figure 2B illustrates the mean percentage change in startle amplitude in the three groups when in the presence of the light CS. The figure suggests that rats in Group 16(O)–23(L) and Group 23(L) exhibited greater FPS to the light CS than did rats in Group 16(O). This observation was confirmed by statistical analysis, $F(1, 37) = 16.20$, $p < .05$. In addition, the analysis indicated that the levels of FPS exhibited to the light by Group 16(O)–23(L) and Group 23(L) were not significantly different, $F(1, 37) = 2.08$, $p = .16$.

In this experiment, we showed that rats retained odor–shock associations acquired at PN 16 when they were tested for freezing to that odor at PN 23 (Group 16(O)–23[L] and Group 16(O)). Moreover, the results also indicate that rats conditioned to a light CS at PN 23 demonstrated both FPS and freezing to the light CS when tested the next day (Group 16(O)–23[L] and Group 23[L]). However, training with a light CS at PN 23 did not activate the expression of FPS to the odor CS trained at PN 16 (Group 16(O)–23[L]). That is, although rats in Group 16(O)–23(L) exhibited freezing to the odor, they did not exhibit FPS to the same CS at test.

General Discussion

There are three main findings in this study. First, the results confirmed that rats trained to an odor CS at PN 16 display odor-elicited freezing but not FPS when tested at PN 24–25. This result is consistent with previous findings that have shown that preweaning rats behave in a manner that is appropriate to their age at training and not their age at test (Hunt & Barnet, 2002; Richardson & Fan, 2002; Richardson, Fan, & Parnas, 2003; Richardson et al., 2000; Richardson, Tronson, Bailey, & Parnas, 2002). Second, the present study also shows that if rats were subsequently trained to a second odor at PN 23 after receiving Odor 1–shock pairings at PN 16, then they exhibit FPS as well as freezing to the first odor. This effect, the activation of FPS to Odor 1 as a consequence of conditioning to a second odor at PN 23, could not have been due to simple generalization between the two odors because rats were able to discriminate between the two odors.

Our current finding that PN 23 rats exhibit freezing but not FPS to an odor conditioned at PN 16 is consistent with previous studies from this laboratory (e.g., Richardson et al., 2002, 2003). In other words, rats behave in a manner appropriate to their age at training and not their age at test. These findings are inconsistent with current models of conditioned fear. For example, the central state of fear theory suggests that once an aversive CS elicits a central state of fear, the animal should be able to express this fear in any number of ways (e.g., Davis, 1992; Stanton, Fox, & Carter, 1998). Moreover, these behavioral indices should be expressed in a manner appropriate to the animal’s age at the time of testing. This model cannot account for the aforementioned developmental findings. The developmental evidence suggests that rats trained at ~PN 17 and tested at ~PN 23 have acquired a central state of fear as indexed by their avoidance of, or freezing to, the CS (e.g., Richardson et al., 2000). However, they appear to be able to only express this fear in a manner that is appropriate to their age at training (e.g., avoidance–freezing) but not in a manner appropriate to their age at test (e.g., FPS).

A separate study with human infants provides further support for the hypothesis that the expression of learning is appropriate to the subject’s age at training and not its age at test. More specifically, Simcock and Hayne (2002) had children participate in a unique activity and then assessed their memory for the event 6 months or 1 year later. At the time of the event and at the time of the memory test, the verbal skills of the children were assessed. They found that across the retention period of 6 to 12 months, the children’s verbal skills improved. At test, however, these children exhibited verbal memory that was appropriate to their vocabulary at the time of the event (encoding), and not their vocabulary level at test. It appears then that the children’s verbal recall for the events was “frozen in time” (Simcock & Hayne, 2002, p. 229), reflecting their verbal skills at the time of encoding.
Activation Effects of Early Learning

The first experiment of the present study has shown, however, that expression of early learning in preweaners can be updated with subsequent training. Specifically, rats given odor–shock pairings at PN 16 and tested at PN 23 exhibited freezing but not FPS to the odor. However, rats conditioned to a second odor at an age when FPS has emerged (PN 23) exhibited FPS as well as freezing to the first odor. The transformation of the expression of early learning via a second activation event is consistent with a recent human infant study by Hartshorn and Rovee-Collier (2003). These investigators exploited the finding that memory in infants is highly dependent on context at 6 months but not at 8–9 months of age. Therefore, they examined whether infants who acquired an operant response at 6 months of age in one context would exhibit context-dependent or context-independent memory when tested at 8 or 9 months of age. They concluded that infants behaved in a manner appropriate to their age at test as infants trained at 6 months of age demonstrated context-independent memory of the event when tested at 8 months of age. A notable procedural feature in that study, however, was that all infants underwent “reinstatement” 1 month prior to test in order to facilitate retention of the event. The reinstatement procedure involved providing the infants with a brief reminder episode in which they were given a chance to perform the operant response. The reinstatement procedure may be similar to our activation procedure, where the second similar event essentially updates the expression of early learning. Specifically, Hartshorn and Rovee-Collier may have found learning that was appropriate to the infants’ age at test because their method included a reinstatement procedure that allowed for the updating of the information learned at 6 months of age.

Modality-Specific Effects

An interesting feature of our results is that this activation effect appears to be modality specific. That is, if the second CS encountered at PN 23 was a light instead of an odor, then rats that were trained with an odor CS at PN 16 only exhibited freezing but not FPS to the odor CS. In other words, a light CS did not activate FPS to an odor CS even though these rats did exhibit FPS to the light CS. The modality-specific effect is particularly interesting given a recent experiment by Hunt and Barnet (2002). In that study, rats experienced light–shock pairings at PN 18 and were tested for freezing and FPS at PN 25. These rats demonstrated freezing but not FPS to the light CS, a result that is consistent with our findings that learning is appropriate to the rat’s age at the time of training. However, R. C. Barnet and P. Hunt (personal communication, August 4, 2005) also found that a tone CS conditioned at PN 24 activates FPS to a light CS conditioned at PN 18. In other words, Barnet and Hunt obtained a modality-independent activation effect when the two CSs used in the arrangement were visual and auditory. One possibility that might account for the apparent inconsistency in terms of the modality specificity of the activation effect is that nonolfactory and olfactory mediated conditioned fear use different neural processes (Krettrek & Price, 1978; Price, 1973). Consequently, there may be more commonality in those processes mediating learned fear to auditory and visual cues than there is for those processes mediating learned fear to olfactory and visual cues.

Savings Effect

One potential explanation for our activation results, which can also account for our modality-specific effects, is that the FPS activation to the odor trained at PN 16 is a consequence of a “savings” effect generated by training to the second odor at PN 23. To elaborate, initial training with Odor 1 at PN 16 may have produced a partial or weakly formed Odor 1–shock association. This association is sufficient for eliciting freezing but not FPS at test (PN 24–25), as freezing may be more sensitive to lower levels of fear below the threshold for producing FPS (McNish, Gewirtz, & Davis, 1997). Additional training at PN 23 to a second odor may then strengthen the original Odor 1–shock association. This hypothesis is perhaps best captured within McLaren and Mackintosh’s (2002) framework for perceptual learning (also see McLaren, Kaye, & Mackintosh, 1988). This model stipulates that any stimulus is constituted of a number of elements and that any two stimuli (e.g., AX and BX) may share commonalities (X elements, e.g., duration, place of occurrence) as well as unique features (A and B elements, e.g., AX is visual, BX is olfactory).

Therefore, conditioning trials with AX and BX result in a sampling of various elements. If we consider that Odor 1 (AX) and Odor 2 (BX) share a number of common elements (X), conditioning to Odor 2 at PN 23 will also result in conditioning to common elements shared with Odor 1 (Experiment 1). This in turn should strengthen the original Odor 1–shock association and may cause that association to cross the response threshold for FPS at test. Moreover, this account may also explain why FPS was not observed to Odor 1 when the second conditioning episode involved a light CS at PN 23. Presumably, a light CS would share less common elements (X) with Odor 1 compared with a CS of the same modality (Odor 2), therefore ensuring that there is little or no further conditioning of the original Odor 1–shock association during light–shock pairings at PN 23.

Although theoretically plausible, this savings hypothesis is not supported by the available, albeit limited, empirical evidence. If the level of FPS were positively correlated with the strength of the odor–shock association, then retraining PN 23 rats to an odor initially conditioned at PN 16 would result in an accelerated emergence of FPS compared with that in naive rats first conditioned at PN 23. However, Richardson and Fan (2002) showed that rats that had acquired an odor–shock association at PN 16 took as many trials to express FPS to that same odor CS at PN 23 as did naive PN 23 rats that were trained to that odor CS for the first time. Given this finding, the activation of FPS in our study as a consequence of a second odor training is unlikely to be due to a savings effect because previous studies have shown that no savings occur when rats are trained at PN 16, retrained at PN 23, and tested using FPS as a behavioral measure (Richardson & Fan, 2002; see also Weber & Richardson, 2004).

Neural Plasticity in the Caudal Pontine Reticular Nucleus (PnC)

Another way of looking at these results is to consider the neurobiological bases of aversive Pavlovian conditioning. There is considerable agreement that the structure mediating the acquisition, retention, and expression of conditioned fear is the basolateral complex of the amygdala (BLC; Campeau & Davis, 1995;
Cousens & Otto, 1998; Kilpatrick & Cahill, 2003; Maren, 2001; Maren, Aharonov, & Fanselow, 1996). The BLC projects to the central nucleus of the amygdala (CeA), which has both direct and indirect projections to various brainstem structures that mediate specific behavioral expressions of learned fear. For example, the periaqueductal gray (PAG) and PnC mediate freezing and FPS, respectively. A lesion to the PAG impairs the freezing response but leaves intact other fear expressions such as conditioned arterial blood pressure (LeDoux, Iwata, Cicchetti, & Reis, 1988) or conditioned suppression (Amorapanth, Nader, & LeDoux, 1999). These efferent structures are conventionally thought only to contribute to the expression of a specific conditioned response but not to be involved in the learning and memory of classical conditioning (e.g., LeDoux, 1995).

For that reason, a prominent neurobiological view for Pavlovian learning is that neural plasticity in the amygdala is necessary and sufficient for the expression of conditioned fear (Davis, 1992). The BLC is thought to elicit a “central state of fear” in the presence of a CS previously paired with an aversive US, and this central state can be expressed in any manner available to the subject at the time of test. This theory stands in contrast to the current results, which suggests that neural plasticity in an efferent structure, the PnC, may also be necessary for the acquisition and expression of FPS to an odor CS. To elaborate, a potential explanation of the delayed emergence of FPS that has been reported in earlier studies is that the pathway between the CeA and PnC is not functional (Hunt & Campbell, 1997; Weber & Richardson, 2001). A further consequence of this pathway’s not being functional prior to about 23 days of age is that the proposed neural plasticity involving the PnC would not occur in rats trained at 16 days of age. However, subsequent training at a later age could lead to the activation of FPS to an earlier-acquired CS because this subsequent training causes the requisite neural plasticity in the PnC. More specifically, Odor 2–shock pairings at PN 23 produces the neural changes in the PnC necessary for the expression of FPS, and this therefore enables the expression of FPS to Odor 1 that was conditioned at PN 16 (i.e., Group 16[O]–23[O2], Experiment 1). Notably, it is unclear why a light CS trained at PN 23 does not activate FPS to the Odor CS trained at PN 16 (Group 16[O]–23[L], Experiment 2) as any proposed neural changes in the PnC must have occurred when the rat was trained to a light CS at PN 23 (i.e., rats did exhibit a pronounced FPS effect to the light CS). Further research is needed to examine this issue.

There are several other studies that support the hypothesis that efferent structures to the amygdala may be essential for acquisition, and not simply expression, of learned fear. For example, De Oca, De Cola, Maren, and Fanselow (1998) showed that the dorsal area of the PAG is involved in the acquisition of defensive responses. More recently, Weber and Richardson (2004) reported that temporary inactivation of the PnC with bupivacaine during conditioning in adult rats prevented the expression of FPS but not freezing to an odor CS. This result suggests that the PnC is critical for the acquisition of FPS. In addition, Weber and Richardson (2004) failed to observe the same effect if the CS was a light instead of an odor. This latter result further supports the hypothesis that different neural processes may mediate FPS to an olfactory CS and a visual CS and potentially accounts for our failure to observe activation of FPS when an olfactory CS is used at PN 16 and a light CS is used at PN 23 (Group 16[O]–23[L], Experiment 2).

Whatever the final interpretation of the present findings may be, our results show that (a) the expression of learning is appropriate to the rat’s age at encoding and not its age at test; however, (b) the expression of early learning can be updated and activated by a second event at an older age such that the expression of that early learning is appropriate to the rat’s age at test; and (c) this latter effect appears to be modality-specific, at least in our laboratory, such that the activation of FPS only occurs if the CSs used at PN 16 and PN 23 are of the same modality (olfactory). This effect is absent when the first CS is an odor and the second CS is a light.

References


Richardson, R., & Fan, M. (2002). Behavioral expression of learned fear in rats is appropriate to their age at training, not their age at testing. Animal Learning & Behavior, 30, 394–404.


