Selective Impairment of Long-Term but Not Short-Term Conditional Fear by the N-Methyl-D-Aspartate Antagonist APV

Jeansok J. Kim, Michael S. Fanselow, Joseph P. DeCola, and Jesus Landeira-Fernandez
University of California, Los Angeles

Previous research has indicated that the competitive N-methyl-D-aspartate (NMDA) antagonist APV (DL-2-amino-5-phosphonovalerate) prevents the Pavlovian conditioning of fear to contextual stimuli when tested 24 hr, but not immediately, after training. The present study investigated this differential time-dependent effect of APV on fear conditioning. Rats were given either APV or saline and presented with 3 footshocks in a distinctive chamber. Promptly after the shock, rats that had received APV exhibited a species-typical fear response—freezing. However, the freezing lasted for only a short period of time (< 3 min) compared with that of controls. An immediate-shock procedure showed that freezing was entirely a conditional response to the chamber. In addition, the results of a savings test suggest that APV impairs storage rather than retrieval processes. These results indicate that there are two temporally distinct associative fear processes, a short-term NMDA-independent conditional fear and a long-term NMDA-dependent conditional fear.

Pavlovian fear conditioning occurs when arbitrary stimuli such as tones, lights, or distinctive environments are paired with aversive unconditional stimuli (US) such as footshock. Through association formation, neutral stimuli can become conditional stimuli (CS) that are capable of eliciting fear responses (e.g., Fanselow, 1984). Recently, N-methyl-D-aspartate (NMDA) receptors have been implicated in fear conditioning (Kim, DeCola, Landeira-Fernandez, & Fanselow, 1991; Miserendino, Sananes, Melia, & Davis, 1990). Intraventricular infusion of the selective NMDA receptor antagonist APV (DL-2-amino-5-phosphonovalerate) was found to prevent the learning without affecting the performance of conditional fear as assessed by the freezing response (Kim et al., 1991). This suggests that NMDA receptors mediate the acquisition but not the expression of conditional fear. The site of drug action appears to be in the amygdala because local application of APV into the amygdala also blocks fear learning (Fanselow, Kim, & Landeira-Fernandez, 1991; Miserendino et al., 1990).

APV is also well known to affect a form of synaptic plasticity known as long-term potentiation (LTP). LTP generally refers to an experimentally induced stable enhancement in synaptic transmission that has several properties desirable of an information storage system (e.g., see Bliss & M. Lynch, 1988; G. Lynch & Baudry, 1984). Antagonism of NMDA receptors in the hippocampal areas with APV has been demonstrated to block the induction but not the expression of LTP (Collingridge, Kehl, & McLennan, 1983; Harris, Ganong, & Cotman, 1984; Kauer, Malenka, & Nicoll, 1988; Muller, Joly, & G. Lynch, 1988). LTP involving glutamatergic pathways has also been demonstrated at the amygdala (Clugnet & LeDoux, 1990). Because APV affects LTP and fear conditioning in a similar manner, LTP has been proposed as a potential neural mechanism underlying fear conditioning (Kim et al., 1991; Miserendino et al., 1990). Kim et al. (1991) reported that although APV blocked fear conditioning when the test was given 24 hr after training, it did not affect the fear response that occurred immediately after training. This suggests that there may be two temporally distinct fear processes—short-term conditional fear and long-term conditional fear. Moreover, long-term, but not short-term, conditional fear is NMDA dependent. The present experiments attempted to further document the existence and clarify the nature of these two temporally distinct fear processes.

**Experiment 1**

We previously examined rats during the 20-s period immediately after shock or 24 hr after shock (Kim et al., 1991). APV completely abolished fear responding in the latter test but had no effect in the former test. Thus, NMDA-dependent fear seems to occur sometime between 20 s and 24 hr after shock. The purpose of this first experiment was to pinpoint the time when the NMDA-dependent memory emerges by tracking continuously after training the APV effect on the fear response. As in previous studies, the freezing response was used as a behavioral measure of fear. Freezing is a frequently used, well-documented, and highly reliable index of fear (e.g., R. J. Blanchard & D. C. Blanchard, 1969; Bolles & Collier, 1976; Fanselow, 1980; LeDoux, Iwata, Cicchetti, & Reis, 1988).

**Method**

**Subjects and Surgery**

The subjects were 23 naive adult female Long-Evans rats that weighed between 195 and 276 g at the time of surgery. They were individually housed in a colony room on a 12:12-hr light-dark cycle.
Test procedures were conducted during the light phase of the cycle. Animals had ad lib access to food and water except during the testing.

While under pentobarbital anesthesia (45 mg/kg ip), rats had a 22-gauge stainless steel guide cannula (Plastic Products, Roanoke, VA) implanted into the right lateral ventricle. With the skull level, the stereotaxic coordinates were 0.5 mm posterior to bregma, 1.5 mm lateral to midline, and 3.2 mm ventral to the surface of the cortex. All rats were allowed a minimum of 7 days to recover from the surgery. During this time, subjects were adapted to transportation and handled daily. Each dummy cannula was removed and replaced with a clean one.

**Drugs and Injection**

APV, which was purchased from Sigma Chemical Company (St. Louis, MO), was dissolved in sterile isotonic saline (SAL). An injection volume of 3 μl was delivered using a syringe infusion pump (Harvard Apparatus, Inc., South Natick, MA) at a rate of 3 μl/min. A 28-gauge internal cannula, which extended 0.5 mm beyond the guide cannula, remained in place for at least 1 min after the infusion before being pulled out. A day before the experiment, subjects were infused with SAL (3 μl) to habituate them to the infusion procedure.

**Apparatus**

Behavioral testing took place in four identical observation chambers (28 × 21 × 10.5 cm; Lafayette Instrs., Co., Lafayette, IN) that were placed inside sound-attenuating chests. The chambers were illuminated with a white light bulb (1280 bayonet bulb, 28 V) that allowed the experimenter to observe and videotape the subjects' behavior. Ventilation fans provided background noise at 78 dB (A scale).

The floor of each chamber was made up of 18 stainless steel rods (4 mm in diameter) that were spaced 1.5 cm center to center and wired to a shock generator and scrambler (Lafayette Instrs.). Before testing, each chamber was cleaned with a 5% solution of ammonium hydroxide and tap water.

**Procedure**

Animals were randomly assigned to either SAL (n = 11) or APV (n = 12) groups. On the experimental day, subjects were infused intracerebroventricularly with either APV (5 μg/rat) or SAL and placed into the observation chambers. After 3 min had elapsed, three successive footshocks (1 mA, 1 s in duration, 20 s apart) were presented. After the last shock, animals remained in the chamber for 8 min in the absence of additional shock. Behavior was videotaped throughout the session.

During the 3-min preshock (baseline) and the 8-min postshock periods, the amount of freezing was measured with a time sampling method. An observer who was uninformed of the subjects’ treatment scored 1 of 4 animals sequentially every 2 s. Thus, each rat was scored once every 8 s. Behavior was judged to be freezing or not at the moment the sample was taken. Freezing was defined as the absence of all visible movement of the body and vibrissae aside from movement necessitated by respiration. All other behaviors were scored as general activity.

**Histology**

At the conclusion of the experiment, the animals were overdosed with sodium pentobarbital and infused intracerebroventricularly with 3 μl of India ink. Approximately 5–15 min later, they were perfused intracardially with 10% formalin. The brains were removed, and coronal sections were made along the cannula tract. Positive cannula placement was verified by the presence of ink throughout the cerebral ventricles. The cannula placements were verified by an experimenter who was uninformed of the subjects' treatment. Only those animals for which positive placement was verified were included in the analyses. In this first experiment, 5 rats were excluded before the analyses because of negative cannula placement.

**Results and Discussion**

Figure 1 shows the mean percentage of time spent freezing for the SAL and APV groups 3 min before the first shock and 8 min after the last shock. As can be seen from the figure, APV per se did not produce defensive freezing in naive animals before the shock. After the shock, however, both APV and SAL groups started to freeze; maximal freezing (reflecting fear level) occurred approximately 2 min after the shock. To get a

![Figure 1](image-url)
metric of time at which APV exerted its effect, the latency to the last freeze was analyzed by an analysis of variance (ANOVA). Freezing behavior terminated significantly earlier in the APV group ($M = 35 \, \text{s}$) compared with the SAL group ($M = 167 \, \text{s}$), $F(1, 17) = 25.09, p < .001$. In fact, freezing completely disappeared within 3 min after the shock in the APV animals, whereas the SAL group showed some freezing for the entire 8-min testing period. Hence, postshock freezing seems to be composed of an APV-insensitive component that lasts for only a short duration and an APV-sensitive component that lasts for a longer duration.

One concern is that the emergence of APV’s effect 3 min after the shock reflects the interval from APV administration to testing rather than the shock-to-test interval as just argued. However, we have previously demonstrated that the presence or absence of APV during testing has no effect on this preparation (Kim et al., 1991). For example, in this previous study, APV did not affect freezing that was tested 3 min after the drug treatment, but it did eliminate the freezing 24 hr after the drug treatment. In addition, even if APV is administered up to 20 min before shock, rats will still show normal immediate-postshock freezing, but they do not freeze when returned to the chamber 24 hr later (DeCola, Kim, & Fanselow, 1991). What is clear from the available data is that the critical determinant of APV’s effect on freezing is the drug administration-to-shock interval not the drug administration-to-test interval. That is, APV must be present during the shock to have an effect on freezing even though that effect is not seen immediately after the shock. The second experiment in this study provided an additional demonstration of this fact.

**Experiment 2**

Experiment 1 demonstrated that the freezing response after shock had two components (i.e., a short-term NMDA-independent component and a long-term NMDA-dependent component). Experiment 2 attempted to clarify certain aspects of each of these components. The question raised about the short-term freezing was whether it was mediated by an associative process or a nonassociative process. The question raised about the long-term freezing was whether APV was acting on a storage process or a retrieval process.

One obvious possibility about the short-term NMDA-independent freezing is that it is an unconditional reaction to the shock and is not associatively based. However, considerable evidence on postshock freezing indicates that it is entirely associative (Blanchard, Fukunaga, & Blanchard, 1976; Fanselow, 1986). One line of evidence for this is the immediate-shock freezing deficit. When a footshock is presented immediately after placing a rat in the chamber, the animal fails to freeze (Blanchard et al., 1976; Fanselow, 1986). This occurs because the animal fails to associate the cues of the chamber with the shock. Therefore, the chamber cues do not elicit a fear response. This procedure was used to determine whether the NMDA-independent freezing was a conditional response or an unconditional response.

Previous studies on the long-term NMDA-dependent component of fear indicated that APV blocks the acquisition of fear conditioning (Kim et al., 1991; Miserendino et al., 1990), which suggests that the drug affects the learning at the level of storage process. However, the effect of the drug can be interpreted differently. For instance, APV-treated animals may have learned, but the available memory may have become inaccessible somehow. If this is the case, then reminder treatments, such as retraining in the absence of the drug, should produce a savings effect (Riccio & Richardson, 1984). Therefore, we tested for such savings by giving rats that received shock under APV a second shock while drug free. In this case, the immediate-shock animals served as “no-learning” controls against which a savings effect could be assessed (Fanselow, 1986).

**Method**

**Subjects and Surgery**

Thirty-six naïve adult female hooded rats (223–292 g) of Long-Evans descent served as subjects. They were implanted intracerebroventricularly with cannulae and housed in manners identical to those in Experiment 1.

**Drug and Injection**

Animals received either APV (5 μg/rat) or SAL in the manner previously described.

**Apparatus and Procedure**

The apparatus used in this experiment was the same as that described for Experiment 1. Subjects were randomly assigned to each cell of a $2 \times 2$ factorial design. The factors were APV or SAL administration and immediate-shock (IS) or delayed-shock (DS) presentation; group designations were SAL-IS ($n = 9$), SAL-DS ($n = 9$), APV-IS ($n = 9$), and APV-DS ($n = 9$; drug/shock treatments).

**Immediate test.** The purpose of the immediate test was to determine whether the short-term NMDA-independent freezing response was associative or not. This was accomplished by comparing rats with delayed and immediate shock.

On Day 1, animals received either APV or SAL and were placed in the observation chamber. Half of the animals in each drug condition received a single footshock (1 mA, 3 s in duration) immediately after placement in the chamber (IS), whereas the other half received the same shock after 3 min in the chamber (DS). All animals stayed in the chamber for 90 s after the shock and were then returned to their home cage. This short postshock interval was used because it corresponded to the NMDA-independent period and prevented substantial extinction of fear. An observer who was uninformed of the experimental conditions scored a single rat every 2 s to increase the sampling size.

**Delayed test.** The purpose of this test was to confirm that the long-term freezing response was totally NMDA dependent.

On Day 2, animals were placed back into the chamber in which they received a shock the day before. This was to assess long-term retention of the conditioning that occurred the day before. For 3 min, the amount of fear conditioned to the chamber was measured in the manner described in Experiment 1. Afterward, animals received a shock that was identical in intensity (1 mA) and duration (3 s) to that of the previous day. Hence, on this day, all animals received a delayed shock. This treatment was done to serve as a reminder for the previous shock that had occurred under either APV or IS conditions (or both). Immediately after the shock, animals were returned to their home cage.
Savings test. The purpose of this test was to estimate the amount of fear retained in memory after the reminder treatment.

On Day 3, each rat was placed back in the chamber for 5 min in the absence of shock. Again, freezing was assessed by a time-sampling procedure (Experiment 1).

Histology

The procedure was the same as that of Experiment 1. In this experiment, 3 rats were excluded from the analyses due to negative cannula placement.

Results and Discussion

Immediate Test

Figure 2 depicts the mean percentage of freezing for the SAL-DS, SAL-IS, APV-DS, and APV-IS groups during the 90-s postshock period on Day 1. As can be seen in the figure, animals that received an immediate shock, regardless of drug treatment, failed to show any postshock freezing behavior. In contrast, rats that received a delayed shock, regardless of drug treatment, showed substantial postshock freezing. This was supported by a two-way ANOVA that showed a significant main effect of shock treatment, $F(1, 30) = 18.18, p < .001$, but no effect of drug treatment, $F(1, 30) < 1$. The Drug × Shock interaction did not approach statistical reliability, $F(1, 30) < 1$. Because only the delayed shock, but not the immediate shock, produced postshock freezing, the freezing behavior is not an unconditional response to the shock. This indicates that for 90 s after shock there is an associatively based freezing response that is NMDA independent.

Delayed Test

Figure 3 presents the mean percentage of freezing for the four groups during the 3-min testing period that occurred 24 hr after the shock (Day 2). Inspection of the figure indicates that the SAL-DS animals showed freezing, whereas the other three groups (SAL-IS, APV-IS, and APV-DS) did not. This was confirmed by a two-way ANOVA that indicated a statisti-

cally reliable Drug × Shock interaction, $F(1, 30) = 4.08$, $p < .05$. Although APV had no effect on immediate-postshock freezing to a delayed shock (Day 1), it completely blocked freezing when tested 24 hr after the shock. This indicates that the associative memory tested remotely is NMDA dependent. Note again that this test was made 24 hr after APV administration.

Savings Test

Figure 4 illustrates the mean percentage of freezing for the four groups during the 5-min testing period that occurred 24 hr after the second shock. According to the figure, SAL-DS animals froze more than any other group. A one-way ANOVA yielded a significant group difference, $F(3, 30) = 16.60, p < .001$, and post hoc analyses (Newman-Keuls) indicated that the SAL-DS group differed reliably from the other three groups (all $p < .01$). If the freezing deficit associated with APV is due to some sort of retrieval failure, then the delayed shock (a reminder treatment) given in the absence of the drug (Day 2)
should have resulted in a savings effect. Fanselow (1986) showed that animals that received an immediate shock when subsequently presented with a delayed shock froze equally to those animals that received only a single delayed shock, which indicates that immediate shock does not result in savings and thus provides an ideal control with which to assess savings of a context–shock association because it equates any nonassociative effects of exposure to shock and handling. In this second experiment, the APV-DS group, if anything, exhibited less freezing than did the SAL-IS group. Therefore, there was no savings after receiving shock under APV. This suggests that APV blocks fear conditioning exclusively at the encoding or storage level. SAL-IS animals froze reliably more than did either the APV-DS or APV-IS groups (both ps < .01, Newman–Keuls). The reasons for this result cannot be ascertained from the present procedure.

General Discussion

There seem to be two forms of associative fear memory that activate the freezing response that emerges shortly after footshock. The first is a short-term conditional fear that is independent of NMDA receptors. This memory, which is not affected by APV, is associative because it results in a freezing response to delayed shock but not to immediate shock. The second is a long-term conditional fear that is dependent on NMDA receptors. This memory too is associative, and it is responsible for freezing that starts at about 3 min after shock. Unlike the short-term fear, this is blocked by APV.

There are other possible explanations for these findings that should be entertained. For example, APV may somehow accelerate extinction of acquired fear. Extinction occurs when a CS is presented repeatedly without the US (Pavlov, 1927). In Experiment 1, animals remained in the chamber immediately after receiving the footshock. It is possible that the difference in freezing observed during this time may reflect a difference in extinction between SAL and APV animals. This is unlikely, however, because when SAL and APV animals were removed from the chamber before extinction occurred, only the SAL animals exhibited freezing the next day (Experiment 2; Kim et al., 1991). In addition, APV appears to arrest rather than facilitate extinction of conditional fear (Falls, Miserendino, & Davis, 1990). Another possibility is that APV may produce performance (motor or motivational) deficits and therefore shorten the freezing response. This is also unlikely in view of prior studies that showed that APV does not affect performance of previously acquired fear (Kim et al., 1991; Miserendino et al., 1990).

LTP has been suggested to be a synaptic mechanism that underlies long-term NMDA-dependent fear memory (Kim et al., 1991; Miserendino et al., 1990). This hypothesis was largely based on the behavioral evidence that the effect of APV on fear conditioning closely parallels its effect on LTP. For instance, central administration of APV was found to block the acquisition but not the expression of long-term conditional fear (Kim et al., 1991). APV also blocks the acquisition, without affecting the expression, of LTP (Collingridge et al., 1983; Harris et al., 1984).

In addition to LTP, brief repetitive activation of excitatory fibers results in NMDA-independent synaptic changes that are of short duration (Collingridge et al., 1983). Posttetanic potentiation (PTP) is a form of short-term (2–3 min) synaptic plasticity (for a review see Zucker, 1989). The mechanism underlying PTP is different from that of LTP in that NMDA antagonists do not block PTP (Collingridge et al., 1983; Griffith, 1990). In brief, PTP develops from a transient increase in availability of intracellular Ca ++ that subsequently leads to an increase in transmitter release (Zucker, 1989). It has been suggested that PTP may be one of the simplest forms of memory for which a neuron remembers its recent history of activity (Eccles, 1964). There is an intriguing possibility that processes such as associative PTP (Sastry, Goh, & Auyeung, 1986) may participate in short-term conditional fear. This speculation obviously requires additional testing.

The reminder–treatment experiment indicates that APV-treated animals do not show a savings effect when subsequently trained without the drug (Experiment 2). Savings is a means to estimate the amount of information retained in memory by determining how difficult it is to relearn the information (Baddeley, 1974). It is a very liberal test of weak memories compared with other reminder treatments. The finding that APV animals do not show any savings when later trained in the absence of the drug suggests that APV affects long-term conditional fear at the level of the encoding (or storage) process.

In summary, there seems to be two temporally distinct associative fear memories, an NMDA-independent conditional fear and an NMDA-dependent conditional fear. The former appears to last for only a short period of time (about 3 min), whereas the latter lasts considerably longer.

References


The Publications and Communications Board of the American Psychological Association announces the appointment of Thomas H. Carr, PhD, Michigan State University, as editor of the Journal of Experimental Psychology: Human Perception and Performance for a 6-year term beginning in 1994. As of January 1, 1993, manuscripts should be directed to

Thomas H. Carr, PhD  
Department of Psychology  
Michigan State University  
East Lansing, Michigan 48824

Manuscript submission patterns for JEP: Human Perception and Performance make the precise date of completion of the 1993 volume uncertain. The current editor, James E. Cutting, PhD, will receive and consider manuscripts until December 31, 1992. Should the 1993 volume be completed before that date, manuscripts will be redirected to Dr. Carr for consideration in the 1994 volume.