

## **Introduction**

The process of learning has sparked a great deal of research in the field of biopsychology. The mechanism by which an animal acquires a memory, stores it, and later retrieves it is a procedure that has yet to be fully explained. Throughout history, paradigms such as operant conditioning and classical conditioning have provided possible models to more effectively study learning. The topic of how one learns has been narrowed even further; specifically, interest has been generated in the connection between stimuli and their fear-induced responses (Maren, Aharonov, Stote, and Fanselow, 1996). The fear response produced by an animal can both hinder and improve the survival and quality of life experienced (LAB LECTURE, 2006). In humans, fear arising from phobias or post-traumatic stress disorder (PTSD) may impede his/her ability to function on a daily basis. For example, if a patient has a phobia of heights, and he or she is hired by a company with an office on the hundredth floor of a building, a conflict is inevitable. Conversely, the ability to produce a fear response may actually be vital to one's survival; therefore, fear is not completely maladaptive. For example, the same patient's phobia of heights will prevent him or her from doing a potentially life-threatening activity like sky-diving. Understanding the mechanism by which a fear response is acquired and expressed will be invaluable to the advancement of pharmacology and psychology.

More specifically, scientists have become extremely interested in the cellular mechanisms involving the formation and storage of new memories. Recently, research has indicated the importance of the amygdala in fear conditioned learning (Maren et al, 1996). Studies have shown that both lesions in the amygdala and pharmacological inactivation produce insufficient responses in both the formation and demonstration of fear conditioned learning (Maren et al, 1996). Also, synaptic plasticity has been established as a critical feature of the

learning process in the brain, specifically, long-term potentiation (LTP) or the “receptor-dependent” strengthening of synapses (**Maren et al, 1996; LAB lecture, 2006**).

In this experiment, the connection between stimuli and the fear-induced response was studied using a Pavlovian fear conditioning model (Maren et al, 1996). In this model, a stimulus that does not normally elicit a response (conditioned stimulus, CS) was paired with an unconditioned stimulus (US) that normally elicits an aversive response. By the pairing of these two stimuli, the CS began to predict the US, and when the CS was presented alone it produced a fear response. The amygdala is the loci of the association of the US and CS; therefore, the significance of NMDA receptors in the amygdala during aversive learning conditioning was tested (Maren et al, 1996). The research intended to show that by the administration of D, L-2-amino-5-phosphonovalerate (“APV; a selective NMDA receptor antagonist”), LTP, and therefore, expression of the fear response, was disrupted (Maren et al, 1996). The glutamate receptor, *N*-Methyl-D-Aspartate (NMDA), was hypothesized to be vital to the generation of LTP in the amygdala for the expression of fear conditioned learning (**LAB LECTURE, 2006**).

## **Methods**

### Subjects:

Forty-Eight (two subjects per group) male Long- Evans (hooded) rats were used. The rats were supplied by Harlan and weighed from 175-199 grams when received. The rats were handled daily two weeks prior to surgery, and were maintained on a 12 hour light/dark cycle (light: 7:00 am; dark 7:00 pm). The dummy cannulae were changed daily after the surgery until the time of the drug infusion.

### Stimuli:

The unconditioned stimulus (US) was a mild (0.6 mA) one second footshock in the chamber which was given during the training session. The conditioned stimulus (CS) consisted of the environmental context; i.e. the chamber in which the rats were placed. The chamber was a small box with a transparent door. The floor of the chamber consisted of a metal grating of rods through which the shock was transmitted, and under the grating was a metal pan to collect any droppings. On one side of the chamber there was also a dim light. Rats were also subject to the odor of a vinegar solution which was used to clean the chamber in between trials. The unconditioned response (UR) was the unlearned reaction to the shock. This reaction entailed a startled reaction and specifically jumping in response to the US. The conditioned response (CR) involved the learned response of freezing. Freezing behavior in rats specifically consists of somatic immobility. Freezing is also associated with an increase in heart rate, and respiration. The independent variable for this experiment was the difference of drug administration between groups (APV treated rats v. control rats). Whereas the dependent variable was the rat behavior which was recorded (specifically freezing) for analyzing fear conditioning. Rearing, grooming and locomotion were also recorded and therefore additional dependent variables.

#### *Surgical Procedure:*

After anesthetization with sodium pentobarbital, rats were mounted on a stereotaxic surgical device. This device allows for the precise location of various brain regions to be mapped and for accurate implantation of guide cannulae. An incision of the skin was made in the scalp and the skin was held back by hemostats. A drill was used to make holes in the skull in order to implant the cannulae. Using coordinates found in a stereotaxic atlas, guide cannulae were implanted bilaterally into the basolateral amygdala. Screws were placed in the skull for support, and the screws and cannulae were held in place by a coating of commonly used dental cement which

covers the open surface of the skull. Dummy cannulae were inserted into the guides to avert any type of obstruction. The dummies were changed daily, as the rat was given a minimum of one week to recover from surgery.

#### Drug Infusion:

Drug or saline was infused using a 10  $\mu$ L Hamilton syringe which was attached to an infusion pump cannula which extended 1 mm further than the guide cannula. After removal of the dummy cannulae, the infusion cannulae were inserted into the guide cannulae. This ensured that the drug was locally administered to the amygdala, the brain region which is of primary concern. In the control rats, 0.5  $\mu$ L 0.9% sterile saline was infused at a rate of 0.1  $\mu$ L/min for five minutes. In the experimental group, the same volume of aminophosphonovalerate (APV) in 0.9% sterile saline was infused at the same rate. The infusion cannulae were removed one minute after the completion of the drug administration and replaced by clean dummy cannulae. One minute was given in order for the drug to diffuse, after which the rats were taken to the chambers for the training session.

#### Procedure:

##### **Week 1:**

**Pre-training:** Each rat was placed in a chamber, and three behaviors (freezing, grooming, and rearing) were independently scored, one by each team member. The session lasted four minutes, and recordings were made based on a time-sampling procedure. No footshocks or other stimuli were administered for this session. Recordings were determined at ten second intervals for a total of twenty four recordings for each behavior scored. After the session, the rat was removed from the chamber and returned to its home cage. The chamber was then cleaned using a weak vinegar solution, and the collecting pan was removed and cleaned. This process was then repeated for the second rat.

**Training:** Each rat was then placed in a bucket near the infusion pump where infusions of APV or saline were administered (see *Drug Infusion* above). The infusions were only carried out during week one. Rats were placed in chambers and a computer program MED- PC IV, which delivered the footshocks, was operated. Footshocks were initiated at one for four minutes. No recordings were taken. After the session was completed, each rat was returned to its home cage and the chamber was cleaned as described above in the Pre-training procedure. This process was also repeated for the second rat.

**Post-Training:** See pre-training procedure. Results were recorded.

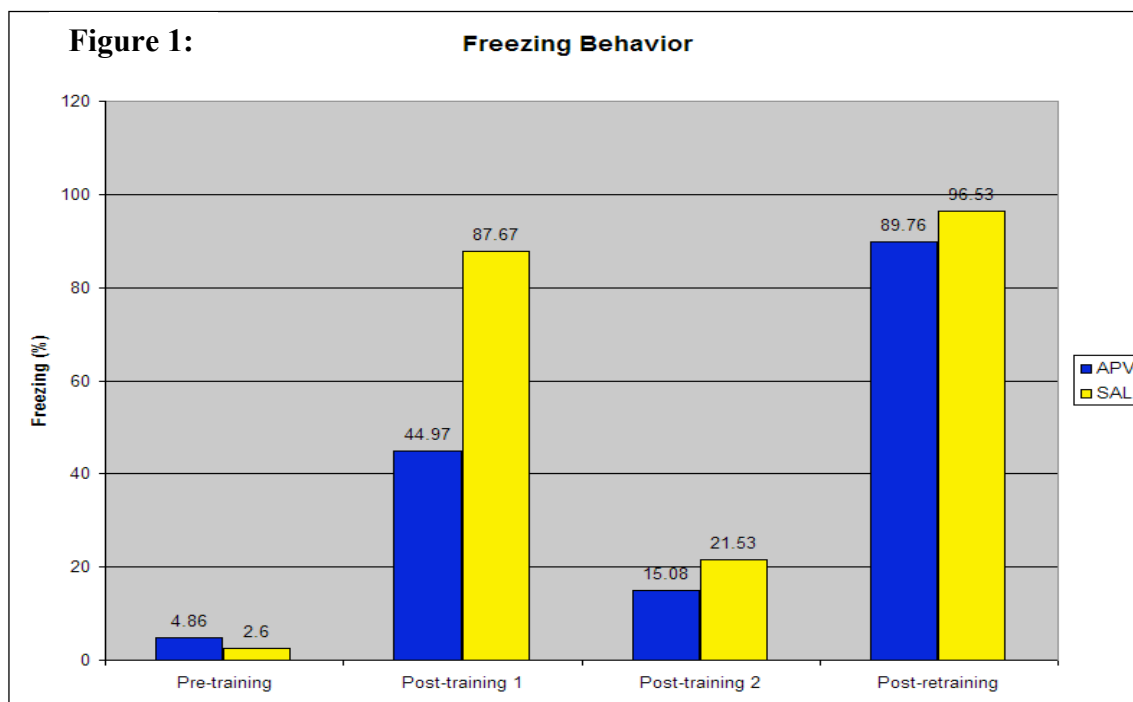
## **Week 2:**

These same procedures were repeated again two weeks later in the second session. The procedures were equivalent to the procedure the first session described above except for the absence of drug infusion for the second session.

## **Results and Figures**

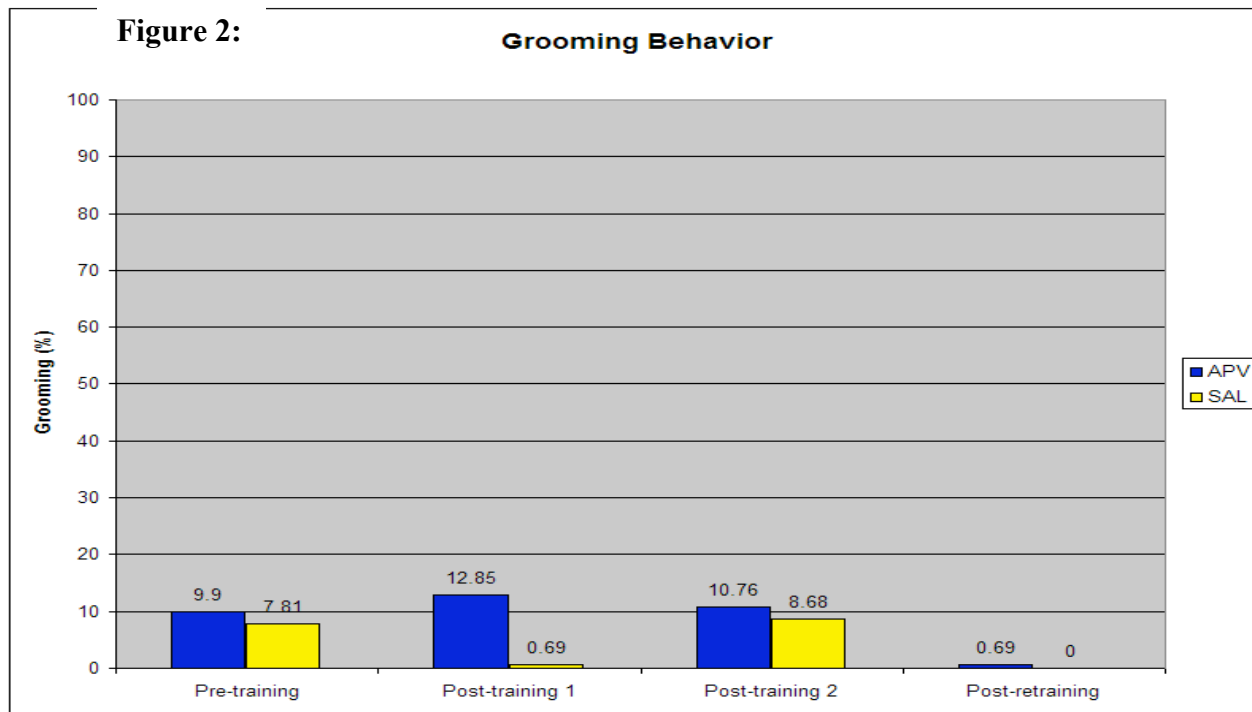
### **Freezing:**

Before training the freezing behavior of both groups of rats did not differ significantly and was very low. However, freezing behavior increased greatly in the first post-training session for both the saline group (2.6→87.67%) and the APV treated group (4.86→44.97%). APV only increased freezing behavior by about half of the increase seen in the saline controls. Two weeks later in the second post-training session, the freezing behavior decreased to a great extent in both saline (87.67→21.53%) and APV groups (44.97→15.08%), yet the freezing behavior of APV treated rats was still lower than that of the saline group. After the retraining session, both groups increased dramatically in the amount of time spent freezing (96.53 % for saline, and 89.76% for APV), and there was not a great difference between the two groups (Figure 1).



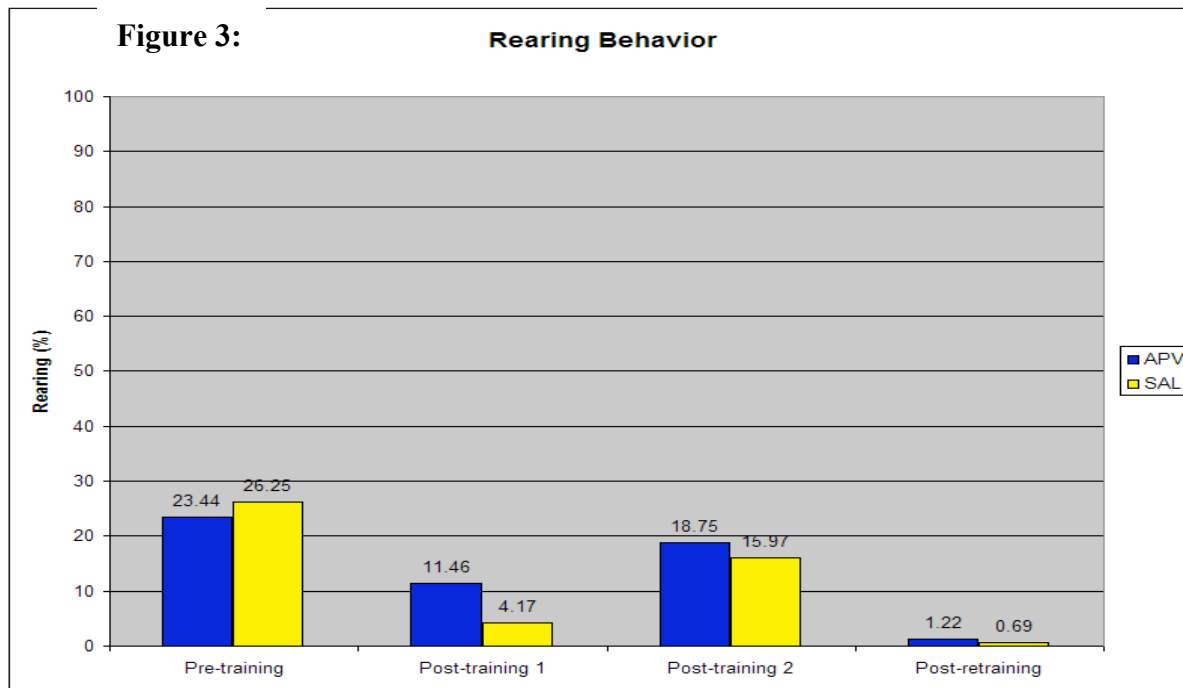
### Grooming:

In the pre-training session, there was no considerable difference between the groups grooming behavior (9.9% for APV and 7.81% for saline). In the first post-training session, the levels of grooming in the APV treated group increased slightly to 12.85% whereas that of the saline group significantly decreased to 0.69%. Two weeks later in the second post-training session, the levels of grooming in both groups returned to levels exceptionally close to the levels seen in pre-training (10.76% for APV, and 8.68% for saline), and there was no significant difference between the two groups. However, in the post-retraining both groups declined vastly: 0.69% of the APV group and 0% of the saline group showed grooming behavior (Figure 2).



### Rearing:

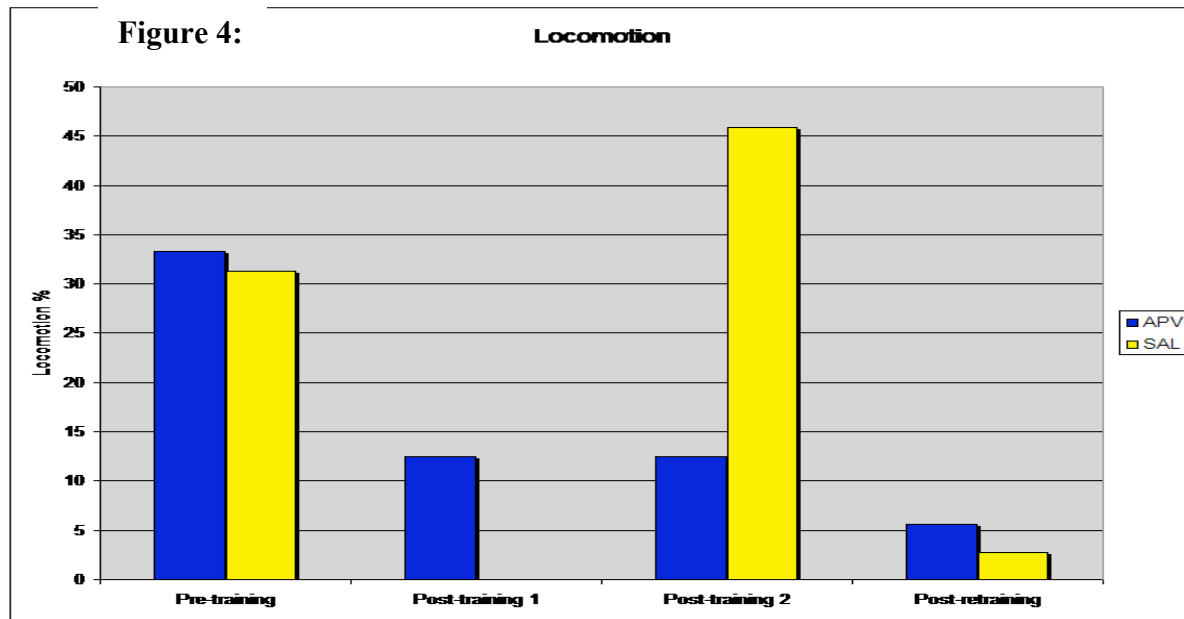
Before training, rearing behavior did not differ significantly between the two groups (23.44% for APV and 26.25% for saline). After training rearing declined in both groups, yet it declined much more in the saline treated group (26.25→4.17%) compared to the APV group (23.44→11.46%). In the second post-training session, rearing behavior increased again in both the saline group (4.17→15.97%) and the APV group (11.46→18.75%). Again, rearing behavior was less in the saline treated group. In the post-retraining session, rearing for both groups declined remarkably: 1.22% in APV treated rats and 0.69% in the saline group (Figure 3).



### Locomotion:

In the pre-training session, no significant differences were seen between the control group and the experimental group (33.3% for APV and 31.25 for saline). In the post-training 1 session, locomotion declined in both groups. In the APV treated group locomotion decreased by almost one third (33.3→12.5%). The decrease observed within the saline treated group was markedly greater as no locomotion was recorded in the post-training group (31.25→0%). In the second post-training session, locomotion did not change from the first pre-training session for the APV treated group (12.5%). However, locomotion increased greatly in the saline treated group (0→45.83%). The locomotion observed in the rats in the post-training 2 (45.83%) session was also much greater than the pre-training session (31.25%). After retraining, locomotion decreased in both the APV treated group (12.5→5.56%) and the saline controls (45.83→2.78%) (Figure 4).





## Discussion

By analyzing the results, we found some of the information to be expected based on our background knowledge of the biology of the brain, but there were also some surprising outcomes. As anticipated, both rats had low levels of fear behavior in the pre-training trial because the chamber had not yet been paired with the aversive stimulus, the shock (4.86 APV rats and 2.6 SAL rats for freezing percentage). With classical conditioning, the conditioned stimulus must be paired with the unconditioned stimulus in order for an association, and in this point of the learning, that had not been done yet. Along with the absence of freezing and other fear responses during the pre-training, the rats also showed similar levels of grooming and rearing, because they were not preoccupied with fear of the chamber yet.

The division in behaviors became apparent in post-training week 1 when rats in the APV group spent a considerably less amount of time freezing than the SAL group, and thus more time grooming and rearing (freezing percent: 44.97 vs. 87.67). This finding is very similar to Maren's study on APV infusion, where the experimental group showed that the drug "severely blunted the acquisition of immediate postshock freezing" (Maren et al., 1996). There are many explanations

for why the APV rats showed less fear response after being shocked at the same intensity as their SAL counterparts. Perhaps the drug interfered with the acquisition of the fear learning at the time of training by blocking the NMDA receptors that are crucial to learning. If the rat could not encode the experience properly, then it could not consolidate or recall it at a later time because the association wouldn't have entered into memory. Possibly by blocking the receptors during the experience, the drug also disrupted the expression of fear, shown by less freezing than the control group. A way to test if it is both acquisition and expression is to place the rat back in the cage when it is drug free. If it does not show the fear response still, it is clear that it did not acquire the learning in the first place. If the rat did instead demonstrate freezing, that would show acquisition happened but the drug simply interfered with exhibiting the response.

As with any experiment, a few of the results were somewhat unexpected and warranted a closer look as to the reason they did not match our predictions. We thought that the testing in post-training week 2 would yield similar results with respect to comparing freezing levels between the rats. Nevertheless, both the APV and SAL groups showed a sharp decrease in freezing behaviors during this testing (APV 15.08% and SAL 21.53%). Since the SAL group was so low, it brings up the question of whether or not we actually blocked long term memory of fear in the APV group. This result suggests that similar factors could have played a role in decreasing freezing in week 2, even though one group received drugs and the other didn't. For instance, perhaps the time span between the training and testing was too long for the rats to remember the chamber. If they could not pair the environment with the shock, they would have no reason to freeze. It is possible that if the time span was the same, but the intensity of the shock was greater, then more long term potentiation may have been able to take place, and thus better learning.

A small number of rats were also scored on their locomotion during the task, but the SAL group showed much more movement during post-training week 2 than the APV group, which was the opposite of what was predicted. If the SAL group demonstrated a greater fear response because of better acquisition of the association, then they should be freezing, not exploring the cage. Given that the sample size was so small compared to the other results, however, it is likely that the variation is misleading because of the limited information available. If all groups had been able to score on locomotion, a more accurate representation might have been possible. Considering possible problems with the experimental design also prompts new ideas for future experiments. While there was some concern about the amount of time between training and post-training trials, maybe upcoming tests could study the effect of spaced training over a few weeks instead of a mass training in one session. Spaced trials could produce longer lasting memory and better recall. The results also brought to our attention how important it is to follow lab protocols. During the post-training week 2, when SAL groups also decreased freezing (an unexpected result), it is possible that not all groups put their rat in the chamber at the same time. By remaining in the environment during a time with no shocks, it is possible the rats learned that the chamber was not frightening after all. In addition, this could have interfered with the pairing we were trying to do, because the rat may not have learned as easily that each time he is in the chamber he will get shocked.

In the future, it would be interesting to test the effect that various intensities would have on the rate on learning. I would predict as the intensity if the shock increased, the level on sensitization in the rat would increase, as well as long term memory of the situation. Additionally, other drugs which target NMDA receptors could be used, and at varying amounts. Furthermore, drugs that facilitate NMDA receptors could also be used in an experiment such as

this. In another study examining fear conditioning in the amygdala, the rats were infused with corticosterone, which enhanced memory of fear learning (Roosendaal et al., 2006).

### References

- Maren, S., Aharonov, G., Stote, D.L., & Fanselow, M.S. (1996). N-Methyl-D Asparate receptors in the basolateral amygdala are required for acquisition and expression of conditional fear in rats. *Behavioral Neuroscience*, 6, 1365-1374.
- Roozendaal, B., Hui, G., Hui, I., Berlau, D.J., McGaugh, J.L., & Weinberger, N.M. (2006). Basolateral amygdala noradrenergic activity mediates corticosterone-induced enhancement of auditory fear conditioning. *Neurobiology of Learning and Memory*, 86, 249-255.