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Kimberly Schauder April 6, 2011

Fear and safety signal learning and conditioned inhibition using a visual version of the AX+BX-fear-potentiated startle paradigm in monkeys

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Abstract

Fear and safety signal learning and conditioned inhibition using a visual version of the AX+BX- fear-poteniated startle paradigm in monkeys

By Kimberly B. Schauder

Fear learning and conditioned inhibition have long been studied in animal models to better understand the behaviors and neurobiology of anxiety disorders. Recently, the conditional discrimination, or AX+BX-, paradigm has proven consistent and effective across rodent, nonhuman primate and human species. This study employed a new version of this paradigm, in which the traditional multimodal cues were replaced by entirely visual cues so that repeated-measure experimental designs can be possible. Seven rhesus macaques were trained and tested on at least one set of this strictly visual AX+BX- paradigm. Results showed that this visual paradigm was comparable to the traditional AX+BX- paradigm in terms of fear and safety signal learning as well as fear modulation. Additionally, this study provided evidence that this paradigm can be used across multiple sets and explored the possibility for use of a shortened training procedure that yields the same results. Future research needs to confirm these preliminary findings before this paradigm is utilized in longitudinal and developmental studies.

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Fear and safety signal learning and conditioned inhibition using a visual version of the AX+BX- fear-potentiated startle paradigm in monkeys

In healthy humans, it is normal and adaptive to develop a fear response in unsafe situations. However, individuals with anxiety disorders experience excessive fear and constant worry. Thus, anxiety disorders are characterized by a maladaptive fear response in inappropriate circumstances. For example, in panic disorder, individuals live in constant fear that something bad will happen to them; those diagnosed with posttraumatic stress disorder (PTSD) have recurrent flashbacks to traumatic episodes thus causing worry about reliving those experiences.

Fear-potentiated startle paradigms have become an important method for studying fear learning, retention, and modulation in order to better understand these disorders. Fear potentiated startle is a body reaction used to assess conditioned fear, whereby an organism responds with increased startle amplitude to a startle cue that has previously been paired with an aversive stimulus as compared when the same startle cue has not been paired with an aversive stimulus (Davis, et al., 2003). This potentiated startle measure can then be used to quantify one's level of anxiety (Grillon, et al., 1991). Paradigms have been developed for humans (Grillon, et al., 1991; Grillon & Davis, 1997), nonhuman primates (Antoniadis, et al., 2007) and rodents (Brown, et al., 1951; Davis, 1986).

Most recently, the paradigm that yields the most consistent results across species is the conditional discrimination, or AX+/BX-, paradigm, which allows fear potentiation, acquisition, modulation, and extinction to be independently assessed and evaluated. In this paradigm, first developed in rats (Myers & Davis, 2004), two cues, A and X presented together are paired with an aversive event (shock in rats, air blast in monkeys and humans) and cues B and X signal that no aversive event will occur. Cue A develops the highest level of fear because it is a consistent

signal for an aversive event, cue B becomes inhibitory because now in compound with the X cue it predicts the lack of an aversive event and X is also somewhat fearful but not as much as the A cue. Transfer of inhibition by the B cue is tested when, for the first time, it is put in compound with the A cue and leads to a reduction of fear to the A cue. Currently, the rodent and monkey AX+BX- paradigm uses a visual (light), an auditory (tone), and a tactile (fan) cue to act as the A, B and X stimuli.

This has proven successful in both humans (Jovanovic, et al., 2005) and rhesus macaques (Winslow, et al., 2008). In humans, individuals with different types of anxiety disorders show both impaired baseline startle amplitude to certain stimuli and fear modulation as assessed by this paradigm. For example, individuals with social anxiety disorder show abnormal fear potentiation to socially relevant stimuli (Lissek, et al., 2008), and individuals with PTSD show increased potentiation to stimuli that remind them of a recently traumatic event in their lives. Furthermore, although individuals with PTSD are able to successfully acquire fear to various stimuli, they show a deficit in fear modulation (Jovanovic, et al., 2009) and extinction (Norrholm, et al., 2009). It is therefore believed that a core problem in individuals with PTSD, and other anxiety disorders is the inability to recognize and interpret safety cues in new situations to inhibit fear (Cannistraro & Rauch, 2003). Furthermore, impaired fear inhibition has been recognized as a biomarker of PTSD, but not depression (Jovanovic, et al., 2010), which is an important finding given the comorbidity of these two disorders.

The implementation of various fear conditioning paradigms in monkeys and rats has led to important discoveries about the neural circuitry of fear learning (for review see (Davis, 1992; Kalin & Sheltona, 2003; LeDoux, 1998). Results from rat and primate studies indicate the central

role of the amygdala in the process of fear acquisition, and in rats, fear expression as well (Antoniadis, et al., 2007; Antoniadis, et al., 2009; LeDoux, 1998). Other brain regions, such as the hippocampus and areas of the pre-frontal cortex, have been studied extensively in rats and are just beginning to be investigated in monkeys. The rodent hippocampus is critical for contextual fear conditioning (Phillips & LeDoux, 1992) and the ventromedial prefrontal cortex for fear extinction (Morgan & LeDoux, 1995).

Because the AX+BX- paradigm is a much more recent development, the majority of studies implementing this paradigm focus on normal/control animals rather than evaluating the biological and neural underpinnings of fear learning and conditioned inhibition. To date, there is one study in rats investigating the role of estrogen on fear learning and inhibition and one lesion study in monkeys investigating the effect of neonatal lesions of the amygdala, hippocampus, and orbital frontal cortex. Toufexis, et al. (2007) found that estrogen disrupts the inhibition of fear in females. More recently, Kazama (2010) showed that monkeys with neonatal amygdala damage were able to successfully demonstrate fear learning. This study showed retarded but not abolished fear learning suggesting that the amygdala may process fear more quickly, but it is not the only region capable of the fear learning process. Additionally, hippocampal and OFC lesions in monkeys did not seem to affect fear learning, safety signal learning, fear modulation, nor extinction (Kazama, et al., 2010).

Clearly, more studies need to be conducted in order to better understand the neurobiology of fear learning, inhibition, and extinction. However, the use of lights, tones, and fans as the traditional stimuli for this paradigm limit the types of studies that can be carried out. Once a stimulus has been associated with a fearful situation, that stimulus cannot be re-used for future investigations within the same animal. In order to broaden the study of fear learning, it is

important to develop a paradigm to enable repeated-measure designs. With a valid repeated-measure paradigm, within-subject comparisons across development, cortical inactivation experiments, and pharmacological treatment studies become possible.

In this study, the traditional light, tone and fan cues were replaced by various picture cues presented via a computer screen, which can be substituted with novel images for each additional experiment. For example, pictures V, Y, Z can be used as stimuli A, B, X in the first experiment and then replaced by pictures D, E, F in the next experiment, G, H, I in the next experiment and so on. Monkeys attend closely to picture cues and are able to discriminate between pictures as early as one month of age as evidenced by visual paired comparison preferential looking tasks (Bachevalier, et al., 1993; Pascalis & Bachevalier, 1999). However, in monkeys neither pictures nor multiple stimulus sets have been used in the context of fear conditioning. The overall goals of this study were to test the validity of this strictly visual paradigm as a) a model of fear and safety signal learning, b) a model of fear modulation, and c) for use in repeated-measure designs. This study addressed all of these goals, but left some important questions unanswered that future studies need to explore.

Hypotheses and Predictions

Because the AX+BX- paradigm using lights, tones and fans has been successfully used with monkeys before and because monkeys attend closely to picture cues, we predicted normal adult rhesus macaques would successfully learn fear and safety signals in this purely visual AX+BX- paradigm. Learning time should be comparable to the previous paradigm (3 days of training for each phase; (Winslow, et al., 2008), although it is possible it will take slightly longer to learn because our paradigm involves discrimination between two visual stimuli rather than between stimuli of different sensory modalities. If our results supported this hypothesis, then it

can be concluded that normal adult rhesus macaques trained and tested using an entirely visual version of the AX+BX- paradigm can be considered good models of fear and safety signal learning. If monkeys were unable to successfully learn fear and safety signals when presented as pictures, then it is possible that monkeys were generalizing their fear within a single modality in a fear conditioning context and therefore cannot be used as a model of healthy human fear and safety signal learning.

If monkeys were able to complete the three training rounds as expected, they were tested for transfer of inhibition. Because this task is almost identical to the multi-modal version of the AX+BX- paradigm, normal monkeys should also demonstrate successful modulation of fear. In fact, humans can learn these associations quite quickly using strickly visual cues (colored cues on a computer screen – Jovanovic et al., 2005) although multiple discrimination with different sets of cues has not yet been tested. Lastly, if monkeys are able to successfully learn more than one set of these visual stimuli, then this paradigm can be used in the future in repeated-measure designs assessing fear learning and modulation. The learning of a second set of stimuli could either be easier or more difficult. After training through one set of stimuli, the animals could learn the second set faster because they have developed a learning set and simply have to learn new picture associations and discriminations rather than relearning the entire task. Alternatively, animals could over-generalize their fear, making learning new associations and discriminations more difficult. However, if the pictures in one set are vastly different from pictures used in previous sets, then learning should be comparable across sets.

Methods

Subjects

Seven rhesus macaques (2 males, 5 females) ranging between 6 and 11 years of age were used in the current study. All were born at the Yerkes National Primate Research Center Field Station (Lawrenceville, GA) where they were reared by their mothers in social groups. After being transferred to the Yerkes Main Station (Atlanta, GA) in adulthood, they were either single or pair-housed allowing visual contact to other monkeys and humans. The behavioral training/testing (see below) began approximately one month after the animals had been transferred. At the beginning of testing, animals weighed between 7-10 kg.

Design

All animals underwent one or two sets of the behavioral testing paradigm, each set lasting approximately 1.5 months. Individual training/testing sessions lasted 30 minutes to 1 hour (depending on the phase; see below) and were each separated by 48 hours absent of any testing. This study adopted the AX+/BX- paradigm for studying fear conditioning in macaques (Winslow, et al., 2008). This paradigm consists of three pre-training phases and three training phases followed by a single test phase. All seven phases consisted of 20-120 trials, in which startle amplitude is recorded as the maximum startle elicited in the last 600ms of each trial.

Originally, this task was administered using pictures from a bank of images that monkeys have shown successful discrimination in visual paired comparison and preferential looking paradigms (Bachevalier, et al., 1993; Pascalis & Bachevalier, 1999). Four monkeys (RNu16, RPo16, RHu16, RNp16) were trained using these pictures before we concluded that the complexity of these images was potentially inhibiting the monkeys from successfully completing the task. Therefore, simpler images were selected first to re-test the monkeys previously tested using the complex pictures and second to test the remaining three monkeys (RUd16, RNa16, RSz16) for the first time. Finally, one of the three monkeys (RNa16) that received the simple

pictures for the first round of testing was given a second round using a different set of simple shapes. Two additional animals (RUd16 and RSz16) were presented a modified version of a second set after completion of the first full set of the simple shape paradigm (see Table 1). This modified version consisted of pretraining, AX+BX- training, and testing, omitting training on A+ and A+B-.

Apparatus and Stimuli

Subjects were trained to sit in a nonhuman primate restraint chair (Med Associates, St. Albans, VT) and subsequently placed in a primate startle box (Industrial Acoustic Company, New York, NY). The startle box (see Figure 1) was a ventilated, sound-attenuated steel chamber with a piece of plexi-glass on one wall to allow visual access to a monitor that displayed the picture cues. Inside the startle box, there was a platform to which the primate chair was fastened. The platform contained a force transducer in order to obtain an electrical signal from the startle-induced movement of the animal. This signal was then amplified, converted to a digital format, and saved to a computer for later analysis (Version 2.1, Med Associates, St. Albans, VT). A 65dB background noise was produced by a speaker placed 30cm behind the animal's head and was continuously on throughout the duration of each experimental session.

In this classical conditioning paradigm, two unconditioned stimuli were used. A 700-msec, 100 PSI airblast generated by an air compressor and delivered through four nozzles directed toward the monkeys' face and a 50-msec (95dB or 105dB) white noise startle cue produced by the same speakers that generated the background noise. Three different picture cues, A, B, and X served as the conditioned stimuli: aversive conditioned stimulus, safety conditioned stimulus, and neutral conditioned stimulus, respectively. All pictures were 3x3 inch pictures displayed on a black background on PowerPoint slides. The complex pictures were drawn from

the bank of images previously described (Bachevalier, et al., 1993; Pascalis & Bachevalier, 1999), and the simpler pictures were colored basic shapes (red square, blue circle, green star, etc.) created from the shape tool on PowerPoint. Examples of both complex and simple pictures are shown in Figure 2. Cues for both the complex and simple pictures conditions were counterbalanced across subjects so that each image served as different cues (A, B, or X) across subjects.

Behavioral Testing

Pre-training phases. Before any conditioning, animals underwent three pre-training phases to obtain each animal's baseline startle response and to ensure lack of fear-potentiated startle in the presence of any of the stimuli prior to conditioning. Animals were presented with only startle noises in phase I, only picture cues in phase II, and both startle noises and picture cues in phase III.

Phase I/Baseline Acoustic Startle. A baseline acoustic startle curve was obtained to ensure that each animal startled to startle cues in the appropriate manner: higher startle to louder startle noises. This 60 trial session lasted 35 minutes and included measures of baseline activity level (10 trials) and startle response to various decibel intensity startle noises (95dB, 100dB, 110dB, 115dB, and 120dB; 10 trials each). All trials were presented in a pseudorandom order. Animals were run on this phase for 2 days to ensure normal baseline startle.

Phase II. Following the baseline acoustic startle phase, animals underwent a picture habituation phase to allow the animals to become accustomed to viewing the pictures while sitting in the startle box. This 20-minute phase contained a total of 30 trials in which all of the different picture cues were presented in the absence of any startle noise. In all phases the

duration of each picture was xx seconds. Animals were run on this phase for two days, and no startle data was collected because no startle noises were presented.

***Phase III*.** Next, animals were presented with the picture cues in the presence of the startle noises to assess any unconditioned effects of the picture cues prior to conditioning. This phase lasted 35 minutes and consisted of a 10-minute habituation period followed by 60 trials, 30 trials with the startle noise alone and 30 trials with one of the to-be-conditioned picture cues (A, B, X, AX, BX, AB; 5 trials each) in the presence of the startle noise. For each cue-startle trial, the startle noise was presented four seconds after the picture cue was displayed.

Percent fear potentiated startle (%FPS) was calculated for each of the picture cues as follows:

%FPS = [(picture with noise)-(noise alone)/noise alone] x 100.

Animals were run on this phase for two days or until fear-potentiated startle to all picture cues was below 30%.

Training phases. The three training phases were used to train the animals to associate certain pictures with specific states of fear or safety. Training used classical conditioning to associate a specific picture cue to an unconditioned response: aversive, safety, or neutral. Each training phase became increasingly more complex, starting with one cue (A) in phase I, two cues (A, B) in phase II, and compound cues (AX, BX) in phase III. All training phases began with 10 minutes of habituation followed by a series of stimulus trials.

***Phase I/A+ Training/Testing*.** The A+ phase used classical fear conditioning to establish an association between the A picture cue and a fearful situation by pairing the A picture cue with the aversive airblast. Each A+ training session lasted 40-minutes and contained 28 stimulus trials, four of which directly paired the A picture cue with the airblast to facilitate fear learning.

These four trials were scheduled so that one occurred at the beginning of the training session, one at the end, and the remaining two dispersed in between. The airblast was delivered four seconds after picture inset. The other 24 stimulus trials included 12 trials of the A picture cue in the presence of the startle noise and 12 trials of the startle noise presented alone. The startle noise was presented four seconds after the onset of the picture for the picture-startle trials. %FPS was calculated for the A picture cue according to the previously defined formula (see pre-training phase III). A+ training lasted a minimum of two days and until their %FPS was 100% above their pre-training startle response to the A cue.

Phase II/A+B- Training/Testing. This phase introduced the safety signal, B, to train the animals to continue associating the A cue with a fearful situation and additionally, to associate the B cue with a safe situation. In this phase, A was still paired with the airblast on four of the stimulus trials in the same manner that it was in the A+ training, but B was never paired with the airblast. Each A+B- training session lasted 50-minutes and contained 40 stimulus trials. Stimulus trials included presentation of the A picture cue paired with the airblast (4 trials), the A picture cue in the presence of the startle noise (12 trials), the B picture in the presence of the startle noise (12 trials), and the startle noise alone (12 trials). Delivery of the airblast and startle noise was four seconds after the picture cue was displayed. A+B- training lasted a minimum of two days and until there was 100% difference in the %FPS between the A and B picture cues.

Phase III/AX+BX- Training/Testing. This final training phase introduced paired picture cues to force the animal to learn using pairs of cues. Previous studies in humans indicated that presentation of the AB cue in the transfer test was treated as a completely novel cue rather than as a combination of the previously learned A and B cues. Kim, this is not quite right, we need to discuss. Thus, it was important to eliminate the adoption of this alternative strategy by training

the animals to discriminate between complex cues. This phase included a series of stimulus trials in which the X (neutral) picture cue was presented with either the A (aversive) or B (safety) cues. This phase was identical to the A+B- training phase except the A picture cue was replaced with the AX picture cue (A and X presented side-by-side) and the B picture cue was replaced by the BX picture cue (B and X presented side-by-side) thus making all cues compound. AX+BX- training lasted a minimum of two days and until there was a 100% difference in %FPS between the AX and BX picture cues (this is a little rough).

Transfer test Phase. After training was completed, animals received a final 1- hour long testing session to determine whether they showed successful transfer of inhibition when presented with the aversive (A) and safety (B) signals together for the first time. All stimulus trial types were presented in this 48-trial test: 2 trials of the AX picture cue paired with the airblast, 2 trials of the AX picture cue in the presence of the startle noise, 10 trials of the A picture cue in the presence of the startle noise, 2 trials of the BX picture in the presence of the startle noise, 10 trials of the B picture in the presence of the startle noise, 10 trials of the AB picture in the presence of the startle noise, and 12 trials of the startle noise alone. %FPS was calculated for the A, AX, B, BX, and AB cues.

Data Analysis

Data analysis included 3 parts. First, a "sessions to criterion parameter" was used to assess learning in each of the three training phases. This learning was then confirmed with paired t-tests using log-transformed %FPS values.

$$\%FPS = [(\text{picture with noise})-(\text{noise alone})/\text{noise alone}] \times 100.$$

Previous studies (Winslow, et al., 2008) indicated that fear-potentiated startle values are not typically normally distributed, thus a logarithmic base 10 transformation normalized the data before comparisons were made.

Second, several paradigm comparisons were conducted using the number of days taken for the animals to reach criterion on each training phase. Non-parametric statistics were used to a) compare the multimodal paradigm to the visual paradigm (complex and simple combined), b) compare the multimodal paradigm to the complex version of the visual paradigm, c) compare the multimodal paradigm to the simple version of the visual paradigm, and d) compare the simple and complex versions of the visual paradigm. Lastly, repeated measures ANOVAs using log transformed %FPS values from the test phase were used to analyze conditioned inhibition. If these tests were significant, paired t-tests were conducted to analyze differences between each pair of stimuli.

Results

Fear/Safety Signal Learning using a Visual Version of AX+BX- Paradigm

Although some animals were trained using complex shapes and others with simple shapes, all seven animals were able to pass the A+ and A+B- training phases on their first set of picture stimuli. Six of the seven animals were also able to learn the AX+BX- discrimination. Table 2 provides the number of sessions each animal took to pass each training phase. Although a sessions to criterion parameter was used to assess learning, a t-test using the log transformed %FPS values on the passing day of each training phase (see Table 3 and Figure 3) confirmed that startle to the aversive cue was significantly higher after training ($t(6) = 8.928$, $p = 0.000$), and that fear/safety-signal discrimination training resulted in increased startle to the A cue compared

to the B cue both in the A+B- training ($t(6) = 4.604$, $p = 0.004$) and AX+BX- training ($t(5) = 5.359$, $p = 0.003$).

Paradigm Comparisons for Fear and Safety Signal Learning

Comparisons of days to criterion for each training phase between sham-operated animals trained using the light, tone and fan cues from a previous study (Kazama, et al., 2010) and the animals trained on the picture cues (simple and complex combined) in the present study revealed differences that approached significance for the A+ (Mann Whitney-U, $p = 0.073$) and AX+BX- (Mann Whitney-U, $p = 0.073$) training phases. However, when only the animals trained on the complex version of the visual paradigm were compared with the multi-modal paradigm, there were significant group differences for the AX+BX- training phase (Mann Whitney-U, $p = 0.029$), in which the multi-modal paradigm took significantly fewer days to master than the complex version of the visual paradigm. Comparisons of days to criterion between the animals trained on the multi-modal paradigm to the animals trained on the simple shapes version of the visual paradigm showed no group differences in any of the learning phases ($p > 0.1$). Nonetheless, when comparisons between the complex and simple versions of the visual paradigm were made, no group differences were found (Mann-Whitney U, $ps > 0.1$). For a summary of the paradigm comparison data see Table 2 & 4 and Figure 4. Additionally, see Figure 5 for the learning curves for each of the three training phases.

Modulation of fear in presence of safety signal (AB probe test)

A repeated measures ANOVA with paradigm group (simple or complex; between subjects) and trial type (A, B, AX, BX, AB; within subjects) as main factors revealed no differences based on paradigm group ($F(1,4) = 3.011$, $p = 0.158$), trial type ($F(4,16) = 0.813$, $p =$

0.535), nor an interaction between the two ($F(4,16) = 1.996$, $p = 0.144$; see Table 5 and Figure 6).

A repeated measures ANOVA including only the first set of the three subjects that were trained on the simple shape version revealed no differences based on trial type ($F(4,8) = 0.309$; $p = 0.225$), indicating that startle amplitude did not differ according to the stimulus presented. However, a subsequent repeated measures ANOVA including only those animals tested on simple shapes with paradigm group (full or shortened; between subjects) and trial type (A, B, AX, BX, AB; within subjects) as main factors revealed significant differences based on trial type ($F(4,16) = 4.057$; $p = 0.019$; see Figure 7) indicating that startle amplitude was dependent on which stimulus was presented. No differences based on paradigm group ($F(1,4) = 0.121$; $p = 0.745$) were found, however an interaction approaching significance ($F(4,16) = 2.544$; $p = 0.080$) indicated that startle amplitude on various trial types depended on paradigm group. Paired t-tests comparing each trial type showed animals startled significantly more to the AX cue compared to the A, BX, and AB cues (see Table 6).

Visual paradigm for use in repeated measures designs

Three of the animals (RNu16, RPo16, RHu16) first trained on the complex version of the paradigm were trained on a second set using the simple shape paradigm. All of these animals had difficulty learning this set and therefore were never tested for transfer (see Table 7).

One animal (RNa16) completed two sets of the simple shape paradigm. She was able to successfully learn all training phases and showed conditioned inhibition in the test phase for both sets. Two additional animals (RUd16 and RSz16) completed one full set and a second modified set of the simple shape paradigm. Both subjects showed learning across both sets and RSz16

showed conditioned inhibition in both sets, but RUd16 only showed conditioned inhibition on the second set. A summary of these results can be found in Table 8 and Figure 8.

Modified Version of Visual AX+BX- Paradigm for Fear/Safety Signal Learning and Fear Modulation

Two animals were trained on a second set using the modified version of the visual paradigm, which included only AX+BX- training, thus excluding A+ and A+B- training. RUd16 and RSz16 showed similar learning using the AX+BX- training between the first (full paradigm) and second (modified paradigm) sets. RSz16 showed successful fear modulation in both the first set trained using the full paradigm and the second set trained using the modified paradigm. Interestingly, although RUd16 did not show successful fear modulation in the first AB probe test when using the full paradigm, she did show fear modulation when trained using the modified version of the paradigm.

Discussion

Results suggest five main conclusions. First, a strictly visual version of the AX+BX- paradigm in rhesus macaques can be used as an animal model of healthy human fear and safety signal learning. Second, the simple shape version of this paradigm is more comparable to the previously used multimodal paradigm in terms of number of sessions to criterion in the three learning phases. Third, the simple shape version has the potential to be used as a model of fear modulation. Four, the modified version of the visual AX+BX- paradigm appeared to be learned at the same rate if not faster than the full version of the paradigm. Lastly, these preliminary results explore the possibility that this paradigm can be used for multiple sets within the same animal, thus making repeated measures designs to study fear conditioning in monkeys a reality.

Fear/Safety Signal Learning using a Visual Version of AX+BX- Paradigm

Animals trained on both the complex and simple sets of the visual paradigm were able to learn fear and safety signals. Regardless of paradigms, animals demonstrated an increased startle response to the aversive stimulus (A+/AX+) compared to the unconditioned stimulus (A pretraining) or safety signal (B-/BX-) on the passing day of each phase. Successful learning was defined as a 100% FPS difference between the unconditioned or safety signal and the aversive signal. This study, along with several others using this passing criterion in the AX+BX- paradigm (Kazama, et al., 2010; Winslow, et al., 2008), demonstrates the validity of this method to assess learning. Although animals took a variable number of days to pass each phase, the day that was considered "passing" did in fact represent learning for each phase.

Paradigm Comparisons for Fear and Safety Signal Learning

Although animals were generally able to learn using both paradigms, the simple visual version was more comparable to the previously used multimodal paradigm than the complex visual version. Because the overall goal was to develop a paradigm identical to the multimodal paradigm with the additional feature of being able to be used repeatedly, the simple visual version can be considered a better paradigm to study fear and safety signal learning. In fact, this is typically used in rats (Myers and Davis, Toufexis et al.,) and humans (several Jovanovic et al.)

Previous studies using the lights, tones and fans as cues have shown that normal monkeys can learn both fear and safety signals in two to three days of training (Kazama, et al., 2010; Winslow, et al., 2008). By switching to a strictly visual paradigm, the animals are forced to discriminate within a single modality. Within a fear learning context, there is evidence that fear memories are formed by convergence of multiple sensory inputs (for reviews see Fanselow & LeDoux, 1999; Maren & Quirk, 2004). So by restricting the stimuli to strictly the visual

modality, it is possible that fear memories are more difficult to form. Nonetheless, when the pictures were simple shapes of a single color, monkeys were able to learn the fear and safety associations almost as fast as in the multimodal paradigm.

Additionally, multiple technology failures with the startle program during the testing could have resulted in the decreased rate of learning in this study. To minimize these effects, only days when the system was fully functioning were included in the analyses. Nevertheless, the animals could have experienced setbacks in learning as a result of inconsistencies caused by the technological problems. Technology failures occurred fairly consistently across both the complex and simple versions of the paradigms, so comparisons between these versions are still valid, as both were affected in the same way.

Although technology failures and the change from a multimodal to a visual paradigm could have contributed to the differences in learning observed across paradigms, it seems likely that these differences can mostly be attributed to failure to discriminate between the complex pictures. The complex pictures (as depicted in Figure 2) contained many shapes and colors and are known to be easily discriminated by monkeys even at a very early age (Bachevalier, et al., 1993; Pascalis & Bachevalier, 1999). However, in these studies, animals were able to make the discrimination by passively looking at complex stimuli, whereas in the present study, some of these neutral complex stimuli became aversive through conditioning. Therefore, animals could have overgeneralized their fear, thus leading to poor discrimination between the cues. All animals trained using the complex shapes took longer than the minimum of two days on at least two of the training phases, and one animal never learned the final discrimination between AX and BX.

In this strictly visual paradigm, it is also possible that the X cue may not have been salient enough to cause B to produce the same amount of conditioned inhibition as seen in the multimodal version of the paradigm. Because X is presented with A in the AX trials, X becomes somewhat excitatory by itself. Thus, when the BX trials are presented, B becomes inhibitory by way of being presented with the excitatory X cue and being followed by no airblast. Therefore, if the X cue is not salient enough because it is presented in the same modality as both the A and B cues, then discrimination would be more difficult and the amount of potential conditioned inhibition would be less.

When the cues were changed to simple shapes in a single color, animals seemed to discriminate between the pictures much more easily. Because there were only very minimal differences in the number of sessions to criterion in the learning phases between the multimodal and simple version paradigm, but much greater differences between the multimodal and complex version paradigm, we believe that the main problem was in the discrimination of the pictures in this fear conditioning context. This conclusion is further confirmed by the results of the fear modulation section of this experiment (see below).

Modulation of fear in presence of safety signal (AB probe test)

Two out of the three animals trained using the simple shapes showed successful fear modulation in their set one test phase, whereas none of the animals trained using the complex shapes showed this conditioned inhibition. This could be due to several reasons including slightly different criterion parameters and discrimination problems caused by the complexity of the images in the complex version. It is likely that both of these reasons contributed to the overall problem being that the animals trained in the complex version may have never actually learned to discriminate between the fear and safety signals.

Animals trained using the complex version remained on each training phase according to the criterion described in the methods (minimum of 2 days and until they showed a 100% FPS difference between the unconditioned or safety cue and the aversive cue). One animal was unable to learn the AX+BX- discrimination and therefore was never tested for fear modulation. However, the three animals that did reach criterion on all training phases and were tested for fear modulation did not show conditioned inhibition as predicted. Additionally, they did not demonstrate the learning discriminations during the actual probe test. For example, we would expect high startle to A+ and AX+ and low startle to B- and BX- in the test phase if the animals actually learned to discriminate. These three animals did not show this learning in the test phase despite having previously reached criterion on the training phases. Therefore, the startle data to the AB cue (measuring conditioned inhibition) cannot really be trusted, because it is not likely that the animals actually learned the discrimination.

For this reason, when the simple visual version was used, the passing criterion was also extended to two consecutive days of the 100% FPS difference to ensure learning. All data was analyzed using the less stringent criterion to standardize between the two paradigms, but this new criterion proved successful as evidenced by the predicted learning discriminations shown in the test phase. Therefore, the fear modulation data in the animals trained using the simple shapes is much more reliable. Two of these three animals showed conditioned inhibition when presented the AB cue in the test phase. This is consistent with the human data indicating only 66% of healthy humans show conditioned inhibition (Jovanovic, et al., 2005). Furthermore, all animals showed conditioned inhibition in their second set of simple shapes.

Although any differences between trial types on the test phase of the first set failed to reach significance, it still seems that the simple shape version can be used as model of fear

modulation. With such a small sample size (n=3) and such high variability in startle amplitude, there was not enough power to see any significant differences. Our sample size was small because, originally, all seven animals would have been trained using the complex version of the paradigm. Upon realizing that the complex version was not successful, only three of the seven animals remained naïve and were switched to the simple visual paradigm. Although all animals were trained on the simple shapes, only those that had not previously received training on the complex shapes showed success in the simple shape version (see next section for explanation).

Differences between trial types on the test phase reached significance when data from both sets one and two of the animals trained only on the simple shapes were included. Subsequent pairwise comparisons showed that animals startled significantly more to the AX+ cue compared to the A+, BX-, and AB cue. This indicates good discriminatory ability between AX and BX and successful fear modulation. The unexpected difference between A+ and AX+ suggests that AX+ is more aversive than A+. Because two of the animals were trained using the shortened paradigm (AX+BX- training only), AX+ would be expected to be more aversive than A in these animals. Although no differences based on paradigm type (full or shortened) were found, the data from these two animals could be causing this unexpected difference between A+ and AX+. Furthermore, the expected difference between A+ and B- was not seen, which further confirms the above explanation. Because results are indicating that the shortened version of the paradigm may be better (see below for explanation), future studies need to analyze these trial type differences in the test phase using only data from the shortened paradigm. If good discrimination between AX+ and BX- and fear modulation persist, then this paradigm can be considered an effective model of fear modulation.

Even if future studies confirm this finding, it is still unknown whether modulation is due to conditioned inhibition or external inhibition (Jovanovic, et al., 2005). In order to investigate this distinction, an additional trial type of AC must be added to the test phase. If animals show decreased startle to AB, but not to AC, then it can be concluded that the animals are using a conditioned inhibition strategy, and the decreased startle to AB can be attributed to B, the safety signal, inhibiting the startle to A, the aversive signal. If animals show decreased startle to both AB and AC, then it is likely that the results can be explained better by external inhibition, a phenomenon caused by decreased startle resulting from the addition of a neutral stimulus. Although past studies in rats (Myers & Davis, 2004) have shown that training on compound cues (AX, BX) reduced external inhibition, the AC test trial has never been added when testing monkeys, and thus the effects of external inhibition have never been tested directly in monkeys.

Visual paradigm for use in repeated measures designs

Results from this study explore the possibility that the simple shape version of the visual AX+BX- paradigm can be used in repeated measures designs, at least for two sets. Although none of the animals first trained on the complex shapes and subsequently trained on the simple shapes successfully showed conditioned inhibition, those animals that started training on the simple version showed promise to be trained in a second set of the AX/BX paradigm. Only one animal completed two full sets of simple shapes, but two additional animals were trained on one set using the full paradigm followed by a second set using a modified version of the simple shape paradigm.

The three animals that were first trained on the complex shapes and subsequently trained on the simple shapes were unable to successfully learn the fear and/or safety cues. Upon training these animals on the simple shapes paradigm, one animal was dropped after 12 days of

unsuccessful learning on the A+ phase and the other two animals were dropped after unsuccessful safety signal learning. As previously stated, the complex shapes contained many different shapes and colors, so it is likely that the animals were generalizing their fear to all pictures displayed. If this were the case, we would not expect animals exposed to these complex shapes to be able to learn subsequent associations, even if the new pictures were the simple shapes. The difficulty in learning on the simple shapes paradigm after already being exposed to the complex shapes provides further evidence that the complex shapes were too complex to use in this fear-conditioning paradigm, especially because the overall goal of this paradigm development was to open up the possibility of repeated-measure designs.

The animals that were first exposed to simple shapes, and thus never exposed to the complex shapes, show promise for this paradigm's potential use in repeated-measure design. However, when looking at the differences between two sets, there does seem to be some generalization across trial type in the second set compared to the first, although fear modulation is still clear in both sets. Because the test phase (where the trial type comparisons are taken from) only compares the startle to the first presentation of each trial type, these data cannot always be trusted. RNa16 startled the most to the first presentations of each trial type, so overall, her generalization seems slightly less than depicted in Figure 6. Because of time constraints, only three animals in the current study provide evidence for this possibility. Thus, future studies need to further investigate this finding by not only expanding the number of subjects but also testing more than two sets to ensure the success of this paradigm over five or six sets.

Modified Version of Visual AX+BX- Paradigm for Fear/Safety Signal Learning and Fear Modulation

Because two animals were able to learn and show fear modulation on the modified version of this paradigm, we concluded that this shorter version may be sufficient for studying fear learning in monkeys. Currently, rat and human studies that employ this AX+BX- paradigm use the shortened version and only use the habituation, AX+BX- training, and test phase in their experimental design (Jovanovic, et al., 2005; Myers & Davis, 2004). When this paradigm was first adopted for monkeys, they were unable to learn the discrimination in the AX+BX- training phase and therefore a progressive training schedule was developed (Winslow, et al., 2008). In the current study, the shortened paradigm was found to lead to similar learning times as the full paradigm, but also led to fear modulation in the one animal that did not show fear modulation when trained using the full paradigm. This added benefit suggests that this shortened paradigm may be better when using entirely visual cues. It is possible that the combination of a strictly visual paradigm with a progressive training schedule confused the animals or led them to disregard the X cue.

Overall Conclusions and Future Directions

From this study, we conclude that monkeys trained on the simple shape version of the visual AX+BX- paradigm can be used as models of healthy human fear and safety signal learning as well as fear modulation. Although this study provides evidence that this paradigm may be useful for repeated-measure designs, there is not enough evidence at this time to draw firm conclusions. Future studies need to confirm this possibility and investigate whether this paradigm can be used across five or six sets and still show successful learning, discrimination, and modulation.

If this paradigm proves successful, we will evaluate the role of specific orbital frontal fields in the expression of inhibition using a previously learned stimulus set and in the

acquisition and expression of new fear and safety associations using a new stimulus set. Although neonatal lesions of OFC areas 11 and 13 have recently been found to have little effect on fear and safety signal learning and conditioned inhibition, the strong connections between the amygdala and OFC make other OFC areas regions of interest for fear modulation (Kazama, et al., 2010). If specific fields of the OFC are found to play a role in this process in adult monkeys, a further study will investigate the developmental aspect of safety signal learning and conditioned inhibition following juvenile monkeys from ten months to three years of age.

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Table 1

Subject training progression

Subject Training Progression

	Complex Set	Simple Set 1	Simple Set 2
RNu16	X	X	
RPo16	X	X	
RHu16	X	X	
RNp16	X		
RUd16		X	X*
RNa16		X	X
RSz16		X	X*

X indicates subject went through the full training paradigm (A+, A+B-, AX+BX-) for that set
X* indicates subject went through a modified version of the training paradigm (AX+BX- only) for that set

Table 2

Sessions per learning stage for each version of the AX+BX- paradigm

		Sessions per learning stage – Set 1		
Paradigm version	**Subject**	**A+**	**A+B-**	**AX+BX-**
Multi-modality	Neo-C1	2	2	2
	Neo-C2	2	2	2
	Neo-C3	2	2	2
	Neo-C4	2	2	2
	Mean	**2**	**2**	**2**
	SEM	**0**	**0**	**0**
Visual: Complex	RNu16	4	5	12
	RPo16	6	2	4
	RHu16	2	5	9
	RNp16	3	5	3
	Mean	**3.75**	**4.25**	**7**
	SEM	**0.43**	**0.375**	**1.41**
Visual: Simple	RUd16	2	2	2
	RNa16	3	2	2
	RSz16	3	2	4
	Mean	**2.67**	**2**	**2.67**
	SEM	**0.19**	**0**	**0.38**
Combined visual	**Mean**	**3.29**	**3.29**	**5.14**
	SEM	**0.20**	**0.23**	**0.64**

Sessions to criterion reported for the first set that each subject was exposed to. If trained on multiple sets, only the first set is reported here.

Table 3

Log transformed % Fear Potentiated Startle on Passing Day of Each Training Phase

	Pretrain	A+		A+B-			AX+BX-	
Subject	**A**	**A**	**A**	**B**	**A-B**	**AX**	**BX**	**AX-BX**
RNu16	1.94	2.30	2.52	2.22	2.42			
RPo16	1.58	2.34	2.50	2.17	2.42	2.53	2.32	2.37
RHu16	1.98	2.77	3.05	2.76	2.81	2.96	2.79	2.61
RNp16	1.61	2.36	2.73	2.47	2.53	2.68	2.52	2.38
RUd16	1.81	2.33	3.12	2.43	3.05	2.93	2.49	2.80
RNa16	2.03	2.80	3.03	2.75	2.79	3.44	3.06	3.24
RSz16	1.89	2.31	2.80	2.72	2.32	2.79	2.33	2.70
Mean	**1.84**	**2.46**	**2.82**	**2.50**	**2.62**	**2.89**	**2.59**	**2.68**
SEM	**0.07**	**0.08**	**0.10**	**0.09**	**0.10**	**0.13**	**0.12**	**0.13**

All values are logarithmic base 10 transformed %FPS values. Only values from the first set that each animal was exposed to are reported here.

Table 4

Paradigm comparison p values across all three training phases

Paradigm Version	**A+**	**A+B-**	**AX+BX-**
Visual vs Multimodal	**0.073**	0.315	**0.073**
Complex vs Multimodal	0.114	0.114	**0.029**
Simple vs Multimodal	0.229	1.000	0.629
Complex vs Simple	0.400	0.114	0.114

All comparisons were made using non-parametric statistics (Mann Whitney-U) to compare sessions to criterion of the various paradigm versions.
Values reaching and approaching significance at the $p = 0.05$ level are bolded.

Table 5

Log transformed % Fear Potentiated Startle of various trial types on test phase

		A	B	AX	BX	AB
	RPo16	1.73	2.31	2.04	1.85	2.10
	RHu16	2.02	1.49	1.69	1.73	1.95
Complex	RNp16	2.17	1.84	2.46	2.68	2.66
	Mean	**1.97**	**1.88**	**2.06**	**2.09**	**2.24**
	SEM	**0.13**	**0.24**	**0.22**	**0.30**	**0.22**
	RUd16	2.87	2.32	3.57	2.74	2.18
	RNa16	3.31	3.2	3.17	3.33	2.68
Simple	RSz16	2.26	2.61	2.45	1.28	1.85
	Mean	**2.81**	**2.71**	**3.06**	**2.45**	**2.24**
	SEM	**0.30**	**0.26**	**0.33**	**0.61**	**0.24**

Values presented represent the logarithmic base 10 transformed %FPS value of the first presentation of each trial type on the test phase of the first set that each animal was exposed to.

Table 6

Pairwise comparisons of various trial types on simple shapes paradigm test phase

Trial Type	*t*	*p*
A vs B	1.11	0.318
AX vs BX	**3.705**	**0.014**
A vs AB	0.778	0.472
B vs AB	-0.014	0.99
AX vs AB	**3.657**	**0.015**
BX vs AB	-0.263	0.803
A vs AX	**3.003**	**0.03**
B vs BX	0.154	0.884

Bolded entries signify significant paired t-tests.

Table 7

Summary of results by subject

	Complex		Simple Round 1		Simple Round 2	
	Learning	**Transfer**	**Learning**	**Transfer**	**Learning**	**Transfer**
RNu16	-		-			
RPo16	+	-	-			
RHu16	+	-	-			
RNp16	+	-				
RUd16			+	-	+*	+
RNa16			+	+	+	+
RSz16			+	+	+*	+

+ represents successful learning across all three training stages (learning) or successful fear modulation as measured in the test phase (transfer)
- represents unsuccessful learning in all or some of the training stages (learning) or unsuccessful fear modulation as measured in the test phase (transfer)
+* represents successful learning in the modified version of the training paradigm (AX+BX- training only)

Table 8

Log transformed % Fear Potentiated Startle on Various Trial Types on Test Phase across Two Sets

Subject		A	B	AX	BX	AB
RNa16	**Set 1**	2.87	2.32	3.57	2.74	2.18
	Set 2	3.31	3.20	3.17	3.05	2.68
RUd16	Set 1	2.52	2.63	2.98	2.04	2.74
	Set 2*	2.51	1.97	3.19	2.34	2.52
RSz16	**Set 1**	2.26	2.61	2.45	1.28	1.85
	Set 2*	2.14	1.91	3.00	2.88	2.69

Values presented represent the logarithmic base 10 transformed %FPS value of the first presentation of each trial type
Bolded entries represent evidence of conditioned inhibition.
* signifies training on modified version of the paradigm (including only AX+BX- training)

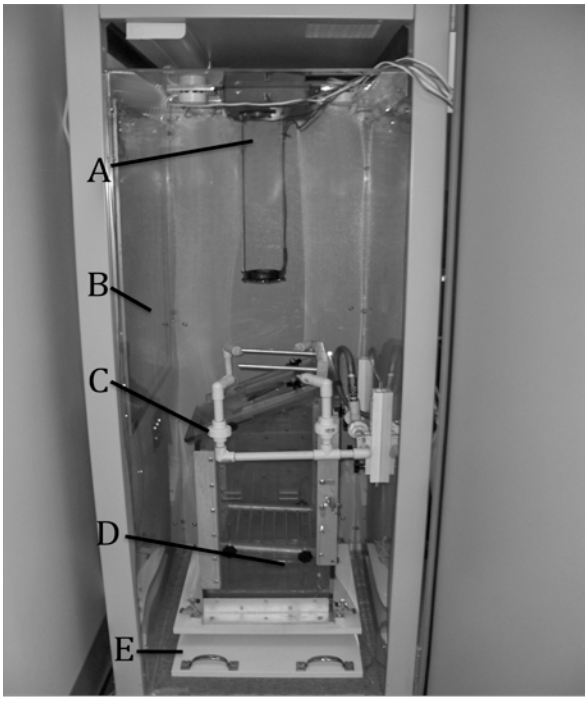

Figure 1. Primate startle box
Photograph of primate startle chamber. A. Speaker B. Picture display screen C. Air delivery nozzle D. Primate chair E. Load cell

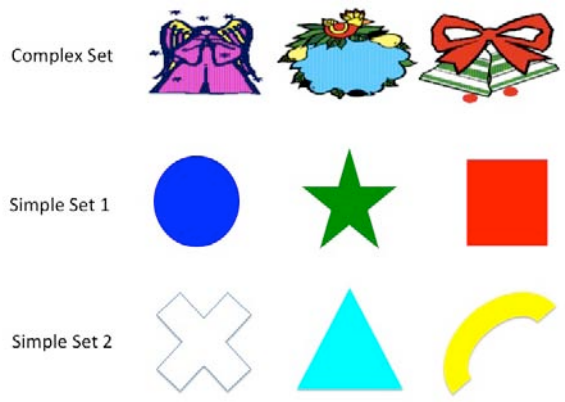

Figure 2. Visual stimuli.

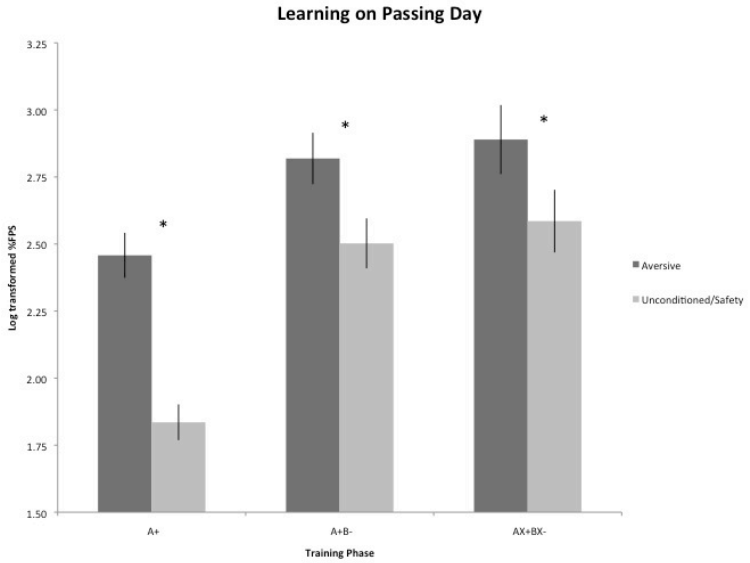

Figure 3. Comparison between the startle response to the unconditioned or safety signal and the aversive signal. * indicates significant difference at the p = 0.05 level between these two cues.

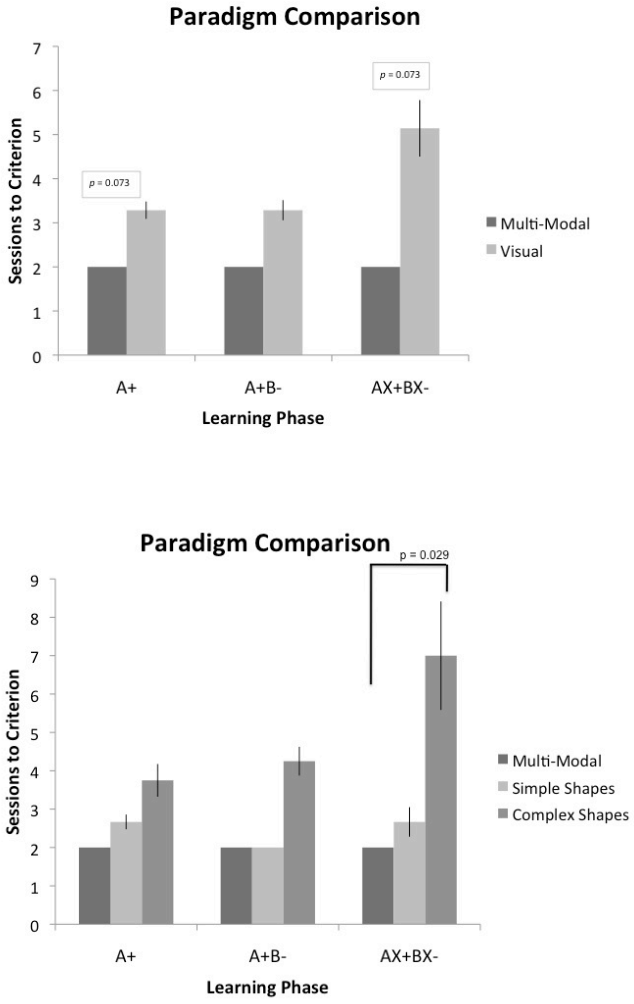

Figure 4. A) Comparison between the visual and multimodal paradigm. B) Comparison between all three paradigms.

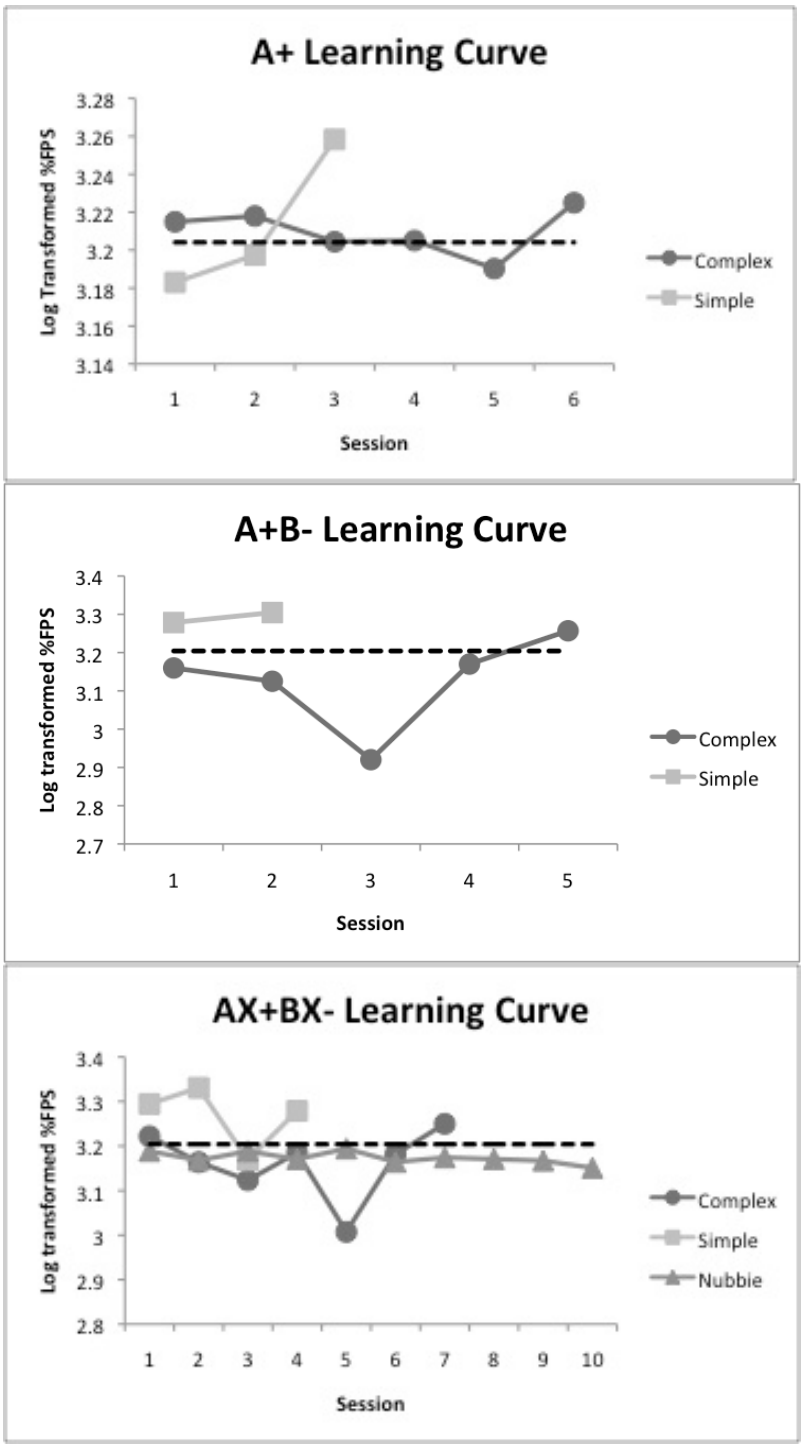

Figure 5. Complex and simple shape learning curves for A) A+ training phase, B) A+B- training phase, and C) AX+BX- training phase. RNu16 never passed AX+BX- training and therefore was excluded from the complex shapes learning curve and depicted on an independent curve.

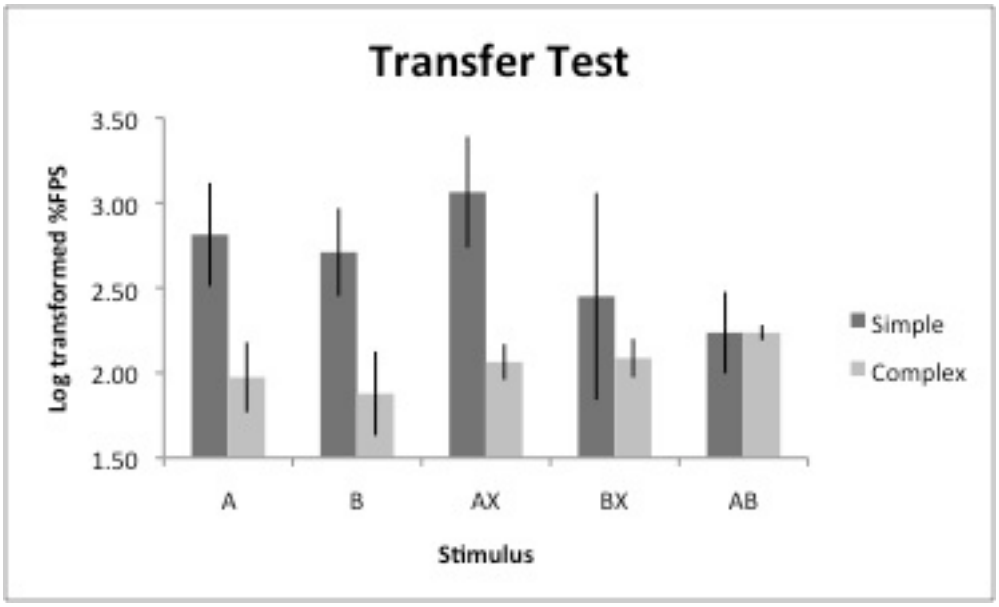

Figure 6. Averaged log transformed potentiated startle response to the first presentation of each trial type during the test phase of each animal's first set of stimuli. n=3 for both simple and complex groups.

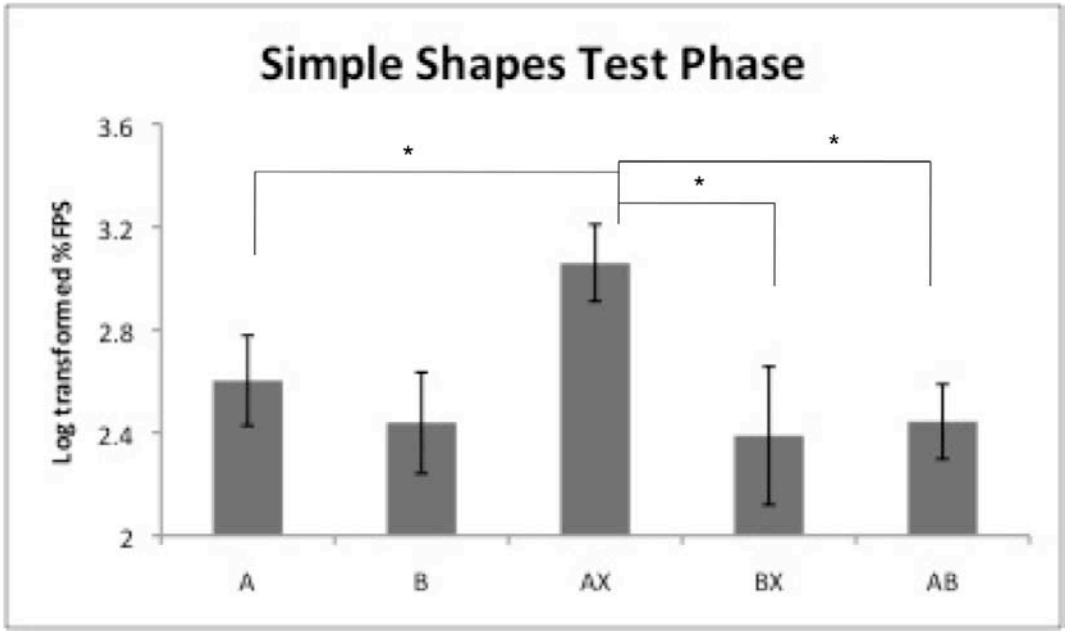

Figure 7. Averaged log transformed potentiated startle response to the first presentation of each trial type during the test phase for the simple shapes paradigm. Data from sets 1 and 2 for the three animals only trained using simple shapes are included.

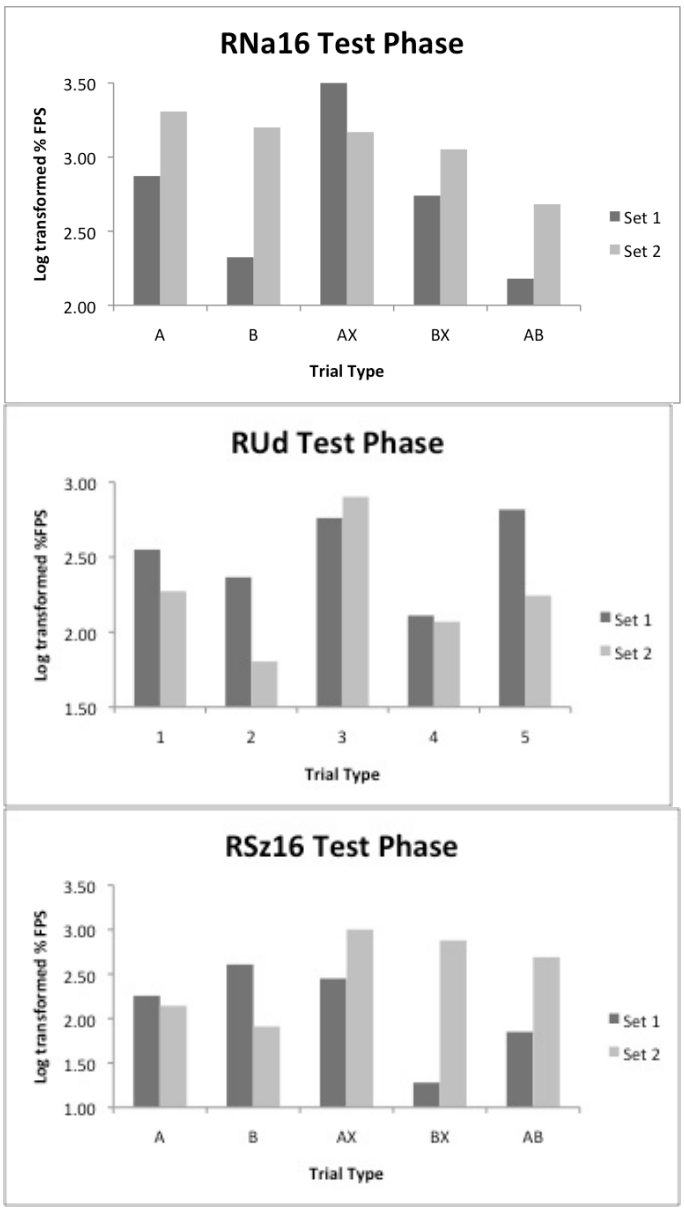

Figure 8. Log transformed potentiated startle response to the first presentation of each trial type in the test phase across two sets of simple shapes. RNa16 received two full sets of the paradigm; RUd16 received one full set and a second modified version of the paradigm.