THE INVOLVEMENT OF AMYGDALA NEURONS AND AMYGDALOID DOPAMINERGIC AND GLUTAMATERGIC RECEPTORS IN THE ACQUISITION AND REINSTATEMENT OF FEAR-POTENTIATED STARTLE IN RATS

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METHODS AND RESULTS

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7. **Experiment 1A**

**ANTAGONISM OF AMYGDALOID D\textsubscript{2} DOPAMINE RECEPTORS WITH RACLOPRIDE BLOCKS THE ACQUISITION OF FEAR-POTENTIATED STARTLE IN RATS.**

**Rationale**

Research indicates that DA receptors located in the amygdala (Meador-Woodruff, et al., 1989, Meador-Woodruff, et al., 1991; Boyson, et al., 1986; Wamsley, et al., 1989; Fuxe, et al., 2003) play an important role in conditioned fear learning and expression Guarraci, et al., 1999; 2000; Nader and LeDoux, 1999b; Greba and Kokkinidis, 2000; Waddington-Lamont and Kokkinidis, 1998) and the involvement of the mesoamygdaloid DA system in fear learning and expression has been well documented (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Waddington-Lamont and Kokkinidis, 1998; Nader and LeDoux, 1999b; Guarraci, et al., 1999; 2000; Greba and Kokkinidis, 2000; Gifkins, et al., 2002; Rosenkranz and Grace, 2002). Previous work revealed that the acquisition and the expression of FPS could be blocked by peripheral and intra-amygdaloid administration of the DA D\textsubscript{1} receptor antagonist SCH 23390 (Greba and Kokkinidis, 2000; Waddington-Lamont and Kokkinidis, 1998). Experimental work by Guarraci and associates (2000) has shown that conditioned defensive freezing behaviour is blocked by intra-amygdalar infusions of the DA D\textsubscript{2} receptor antagonist eticlopride. Also, unpublished work demonstrated that FPS acquisition and retention could be disrupted by peripheral administration of the DA D\textsubscript{2} receptor antagonist raclopride (Greba and Kokkinidis, 1999 unpublished observation; also see Davis, et al., 1993). Based on the above findings, the present study sought to examine the involvement of DA D\textsubscript{2} amygdaloid receptors in the acquisition of FPS. Prior to undertaking this research there had been no scientific examination of the role of amygdaloid DA D\textsubscript{2} in the acquisition of FPS.
7.1: Method

7.11: Subjects

A total of sixty-eight naive, male Wistar rats bred and housed in our laboratory at the University of Canterbury served as subjects in these experiments. Forty-eight rats were used for the larger acquisition experiment and the remaining twenty rats were used in a control experiment to test whether drug infusion affected baseline acoustic startle amplitudes. On average, rats weighed 350 grams at the beginning of the experiment (range 265-370 grams). A constant temperature of 20° Celsius (±1° Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulation took place during the light portion of this cycle.

7.12: Apparatus

Five identical cages (15 cm X 9.5 cm X 10 cm), constructed from stainless steel wire mesh mounted on a Plexiglas frame, served as fear conditioning and startle testing apparatus. Each individual cage was suspended 4 cm above the centre of a strip of piezo-electric film (29 cm X 23 cm) and housed inside a sound-attenuating Styrofoam chamber (33 cm X 26 cm X 27 cm, interior dimensions). The piezo-electric strip was surrounded with insulating foam and covered with cardboard and a Mylar sheath. Fluctuations in voltage amplitudes resulting from kinetic energy or movement on the piezo were transferred into a signal that was filtered, amplified, and then measured by a specially designed sample-and-hold circuit interfaced to a 386 microcomputer (5 mV peak voltage amplitude equals 100 units). A 6.3 volt light located 4 cm above the centre of each stainless steel wire mesh cage provided the conditioned stimulus (CS).

White-noise bursts (100 ms duration, 5ms rise-decay time) were delivered via a high frequency speaker (10 cm in diameter) located in the side-wall of each Styrofoam test chamber. The white-noise burst served as the acoustic startle stimulus and was generated by a white-noise-generator (Med Associates, Fairfield, VT). A Simpson sound level meter set to the A scale (model 860; Elgin, IL) was used to measure the average ambient noise level in each chamber and this was found to be 55dB. Electrical footshock served as the
unconditioned stimulus (UCS) in the experiment and was administered through a second Mylar sheath covered with strategically placed strips of aluminium tape.

Aluminium tape (Manco Inc., Avon, OH) was applied to each half of the Mylar sheath and was separated by a glass rod embedded in a silicone barrier. The glass rod embedded in silicone served as an insulated border between the positive and negatively charged portions of aluminium tape. This barrier divided the surface of the Mylar sheath along its central length so as to prevent rat urine and faeces from short circuiting the stimulator. The stainless steel wire mesh walls and Plexiglas frame of the cages were nickel-coated (CG Electronics, Rockford, IL) and connected to the aluminium taped Mylar. An 800 μA current (monopolar square wave, 200 Hz frequency, 0.8 ms pulse-width and 500 ms in duration) was delivered through the taped aluminium Mylar sheath, thus electrifying the floor and the nickel-coated Plexiglas and stainless steel mesh walls of the cage. This design attempted to ensure that the footshock delivered to the subjects during fear conditioning was inescapable. The footshock was provided by a programmable constant current stimulator (Schnabel Electronics, Saskatoon, SK, Canada) connected to the 386 microcomputer. Specially designed software (Dr. Ping Chen, Saskatoon, SK, Canada) was used to control the stimulus presentations.

7.13: Stereotaxic Surgery

Once anesthetised (sodium pentobarbitone 95.0 to 110 mg/kg), rats were mounted in a stereotaxic device (Stoelting; Wood Dale, IL). In order to achieve flat skull the incisor bar was adjusted until the dorsoventral measurements at bregma and lambda were equal. 22-gauge stainless steel guide cannulae (C313; Plastics-One, Roanoke, VA) were then bilaterally implanted so that each guide cannula would be fixed 1.0mm above the medial portion of the basolateral amygdala (BLA). Implantation co-ordinates were AP -2.80 mm from bregma, L ±4.80 mm from the sagittal suture, and D.V. -7.40 mm from the skull surface. These co-ordinates were obtained from the rat brain atlas of Paxinos and Watson (1986).

Cannulae were secured to the skull surface via a head cap constructed from dental acrylic. This head cap was attached to four stainless steel self-tapping jeweller’s screws (3.20 mm in length, Lomat Precision Tools, Montreal Quebec, Canada) embedded in the
skull surface. Specially cut dummy cannulae with stylets (C313DC; 28 gauge; Plastics-One, Roanoke, VA) which protruded 0.25 mm below the tip of each guide cannula were inserted and screwed onto the secured cannulae. This prevented foreign particles from entering the guide cannulae and damaging the amygdaloid nuclei. Following surgery, rats were placed into a recovery cage until the anaesthetic wore off and were then returned to normal group housing conditions. Rats were allowed a 12 to 14 day recovery period to ensure their wounds were adequately healed before any behavioural testing or experimental manipulation commenced.

7.14: Procedure

7.15: Baselining

After recovery from surgery, acoustic startle was assessed for each rat over 7 blocks of 20 white-noise trials. Thus, each rat received a total of 140 white-noise trials with an interstimulus interval (ISI) of 30 seconds. During the first 4 baselining blocks the noise intensity levels were adjusted (range = 86.0-103 dB) for each animal to establish a stable acoustic startle response (between 50 and 500 units). The noise intensity measured in decibels and the mean acoustic startle scores recorded on the 7th block of 20 white-noise trials were used as the animal’s noise intensity and baseline acoustic startle scores. Once an animal’s noise-intensity level had been determined this level was used for all subsequent acoustic startle testing. Animals were then matched on decibel and startle scores and assigned to one of four drug infusion groups (N=12 per group). Thus ensuring each experimental group was equated on average startle score and decibel level. This baselining protocol not only served to acclimatised rats to the startle apparatus but also helped reduce any between-subjects variability in startle responding that occurs when rats are first exposed to white-noise trials. Hence, the main aim behind the baselining methods employed here and throughout the remainder of this thesis was to establish stable acoustic startle responding for all rats.
7.16: Drug Infusion

Forty-eight hours after baselining, pre-drug and pre-shock baseline startle was measured by using one block of 20 noise trials (30-s, ISI). This brief exposure to the startle generating white-noise bursts provided a noise-alone baseline startle score just prior to drug infusion and fear-training/testing. The last four noise-alone trials of this baseline score were later used to determine the level of shock sensitised startle exhibited by rats treated with various doses of raclopride.

Ten minutes later rats were bilaterally infused with either saline (0.5 µl/side, pH = 7.3), or 2.0, 4.0, or 8.0 µg of S(-) raclopride-L-tartrate (Sigma Aldrich, New South Wales, Australia) into the BLA. All doses of S(-) raclopride-L-tartrate were dissolved in physiological saline (pH = 7.3). Prior to the drug infusion procedure, the infusion pumps were primed to ensure that the drug or vehicle solution was being properly released from the infusion cannula. To facilitate the infusion process rats were wrapped in a soft towel and hand held. After removing the dummy stylets (C313DC; Plastics-One) a preloaded 28-gauge stainless steel internal infusion cannula (C313I; Plastics-One) was inserted into each guide cannula. Once in place the infusion cannula extended 1.0 mm below the tip of the guide cannula. Drugs were bilaterally infused via polyethylene tubing (PE 20) that connected the internal infusion cannula to a 2.0-µl Hamilton syringe. Two Stoelting infusion pumps (model 310; Wood Dale, IL) were used to deliver the appropriate volume of drug or vehicle solution (0.5 µl per side) into the BLA over a 60 second period. The infusion cannulae were left in place for an additional 2 minutes after the drug infusion procedure to allow the drug ample time to diffuse into the surrounding brain tissue. Each infusion cannula was slowly removed and the dummy stylets were reinserted.

7.17: Fear Conditioning and Fear Testing

Rats were then returned to their prescribed startle chambers and remained undisturbed for 5 minutes during an adaptation period. Rats were then subjected to the first of five blocks of 8 light (CS) plus footshock (UCS) pairings. The light (CS) was presented for 3.5 s and the footshock (UCS) occurred during the last 500 ms interval of light onset. The duration of the 800 µA footshock was 500 ms, and the inter-trial interval was 45 s. Each
subject's reactivity to the footshock was measured for 100 ms after shock onset. Collection of the shock reactivity data was important in that it made it possible to assess the impact of intra-amygdaloid raclopride infusion on shock reactivity during fear conditioning.

The acquisition of fear-potentiated startle (FPS) was assessed five minutes after each fear conditioning block ended. This test consisted of four noise-alone and four CS + noise trials (30 s, ISI) presented in a random order. The light was presented for 3.5 s and the white-noise burst occurred during the last 100 ms of the light interval. Each rat’s predetermined noise intensity level was used to assess any augmentation of acoustic startle that took place during each fear-potentiated startle (FPS) test in the acquisition phase of this experiment. The entire fear conditioning-testing procedure involved a total of 40 CS (light)-shock pairings divided into 5 fear-conditioning blocks and five FPS tests (4 noise-alone vs. 4 light + noise). This experimental design made it possible to assess the effects raclopride had on the acquisition of FPS over time while the rats were still in the drug state.

Subjects were retested 48h later in a drug-free state to measure the level of fear retention. To achieve this, rats were presented with 20 noise-alone trials immediately followed by the random presentation of 10 noise-alone and 10 CS + noise trials (30 s, ISI). This retention test was administered to rule out the possibility that any disruption in conditioned fear acquisition produced by raclopride could not be attributed to a drug-induced performance deficit during the acquisition testing phase. Furthermore, it is possible that weak CS-UCS fear associations can still be formed even in the face of challenge imposed by intra-amygdaloid raclopride application, and these associations may influence levels of conditioned fear during later testing. Thus, the retention test was necessary in order to provide proof that antagonism of D2 dopamine (DA) amygdaloid receptors impair conditioned fear acquisition and prevent long-term fear memories from becoming established.

7.18: Histology

A lethal dose of sodium pentobarbitone was administered to sacrifice each animal. A 0.9 percent saline solution followed by a FAM solution consisting of formalin (10%), glacial acetic acid (10%) and methanol (80%) was perfused intracardially in order to fix the brain tissue. Each animal’s brain was then removed from the skull. To ensure the tissue
was well preserved the brains were stored in the FAM mixture. Two days later each brain was transferred to a 70% sucrose solution. Several weeks later brains were sliced into coronal sections (50 μm) using a cryostat (Bright Model OTF) and mounted on gel-coated slides. Cresyl violet was used to stain the sections and these were then microscopically examined to ascertain the location of the guide cannulae. This histological practice made it possible to establish the final composition of each experimental group in this study.

7.19: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, including, acoustic startle amplitude data obtained during baselining, shock reactivity data gathered during fear conditioning, and the level of FPS measured during the acquisition and retention experimental phases. Differences between group means were assessed using Newman-Keuls multiple comparisons ($\alpha = 0.05$). Within-group Newman-Keuls post hoc tests were used to assess differences between the two levels of the factor Stimulus Condition (i.e. CS + noise trials versus noise-alone trials) for each group when a main effect was found by the ANOVA. When Newman-Keuls post hoc tests were used to make between-group comparisons with interactions between two or more factors a pooled error term or a larger mean square error term was used to calculate the critical ranges required for statistical significance (Winer, 1962; Winer, Brown and Michels, 1991). This statistical practice of pooling individual error terms for each factor that contributes to an interaction sets a more conservative critical range for the Newman-Keuls post hoc tests. This technique is scientifically valid because it recognizes that each factor that contributes to a significant interaction on an ANOVA also brings along its own level of error and degrees of freedom. Thus, when interactions occurred and between-group comparisons needed to be made, the Newman-Keuls tests in this thesis relied on a pooled error term to help establish the critical ranges that are typically used to denote statistical significance (see Winer, 1962; Winer, Brown and Michels, 1991).

Baseline startle was assessed prior to drug infusion and light + shock presentation. Following the drug infusion, a small fear acquisition test was presented 5 min after each session of eight light + shock pairings. Thus, in addition to examining drug effects on the
development of FPS, this experimental procedure also allowed for the assessment of the shock sensitisation of acoustic startle. This involved comparing the preshock-predrug mean baseline startle amplitudes obtained on the last 4 noise trials during the baseline startle procedure to the postshock startle amplitude means obtained from the 4 noise-alone trials presented during each acquisition test.

Shock reactivity is a reflexive response (i.e. jumping or flinching) in reaction to the aversive footshock. Reactivity levels were measured during light + shock pairings and this was useful for examining the effects of raclopride on each animal’s behavioural reaction to the aversive qualities of the footshock. Shock reactivity data were collected during the first 100 ms of each electric shock presentation. The shock reactivity amplitudes were averaged for each of the five fear conditioning blocks (8 light plus shock pairings per block) that were administered to the subjects during the acquisition phase of the experiment. These five averages were then analysed statistically to determine whether or not there were any group differences.

The fear retention test conducted 48 hours after the acquisition phase served two purposes. First, it made it possible to assess whether rats which displayed conditioned fear during the acquisition testing phase retained this fear 48 hours later. Second, it meant that any experimental groups that did not display FPS in the drug-state could still be assessed for FPS in a drug-free state. Thus, there was the possibility that animals could acquire fear but be unable to express it while in the drug state. The retention test occurred while the animal was in a drug-free state so that any possible effect of raclopride on the conditioned fear could be examined with a greater degree of certainty.

As was alluded to earlier, it is possible that intra-amygdaloid raclopride may prevent rats from exhibiting conditional fear during the acquisition phase of the experiment by causing impairments in the ability to performance a fear response. Thus, it is possible that raclopride-treated rats could still have formed weak CS-UCS fear associations during Pavlovian fear conditioning but been unable to express potentiated startle to the CS during acquisition testing. To test this possibility out and provide more definitive scientific evidence regarding the role played by D2 DA amygdaloid receptors in fear learning and memory formation it was necessary to administer a retention test 48 hours later. The advantage of the retention test is that it simply rules out the possibility that drug-induced
performance deficits can be held responsible as the underlying factor that influences FPS scores during acquisition testing.

**7.2: Results and Discussion**

**7.2.1: Histology Results**

Figures 8 to 11 depict the guide cannulae locations and approximate locations of infusion cannula for the 3 raclopride drug infusion groups (N=12 each) and the saline control group (N=12). Based on the examination of the cannulae placements it was determined that no animals needed to be excluded from the study. Most guide cannulae were located in an area approximately 1.0 mm above the medial portion of the BLA. When considering these histological results it should be noted that the infusion cannulae protruded 1.0 mm below the end of the guide cannula. This ensured that most infusions would have been made into the basolateral amygdaloid complex. However, the present study cannot rule out the possibility of unintentional drug spread to the central amygdala. Nevertheless, it should be noted that both the basolateral and central amygdala contain substantial populations of D2 receptors (Scibilia, Lachowicz and Kilts, 1992; Meador-Woodruff, et al., 1991). Thus, raclopride would have interacted with DA receptors in amygdaloid nuclei that have been shown to play a prominent role in conditioned fear learning and expression (Davis, 1992a,b,c; Miserendino, et al., 1990; Sananes and Davis, 1992; Kim and Davis, 1993a,b; Campeau and Davis, 1995a; Falls and Davis, 1995; LeDoux, et al., 1988; LeDoux, 1993; 2000; Maren, 1999; Fendt and Fanselow, 1999; Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; 2000; Greba and Kokkinidis, 2000).

The possibility of dorsal spread of raclopride to the ventral portions of the caudate putamen cannot be ruled out from our histological results but this seems unlikely since lidocaine infusions of 1 µl into cortical tissue has an approximate radial spread of 0.63 mm to 0.75 mm from the tip of the infusion needle and since infusions of 2 µl of lidocaine inactivates cortical neurons up to 0.80 mm from the infusion needle tip (Malpel! and Schiller, 1979; Sandkühler, Maisch and Zimmersmann, 1987; Martin 1991; Tehovnik and Sommer, 1997). In general, it seems that most drugs diffuse in all directions away from the
infusion cannula forming a sphere of tissue around the infusion needle that has been saturated by the drug (see Tehovnik and Sommer, 1997). Some traces of the drug flow upward towards the guide cannula along the length of the infusion needle, while other amounts move in a ventral and lateral direction away from infusion needle tip. In an attempt to reduce the likelihood of the drug diffusing dorsally the infusion needles were left in place for 2 min after the drug infusion was completed. Also, it should be noted that the volume of 0.5 µl per hemisphere is an acceptable standard amount used in the behavioural neuroscience field when carrying out pharmacological manipulations of the amygdala in rats (see Maren, et al., 1996b; Greba and Kokkinidis, 2000; Guarraci, et al., 1999; 2000; Fendt, 2001; Walker and Davis, 2000; Goosens and Maren, 2003). This means that the volume of drug and/or vehicle solution used in the experiments contained within this thesis are in line with what is accepted as standard practice for direct pharmacological manipulations of the amygdala in rats.
Figure 8. Schematic depictions of guide cannula locations represented as filled circles (•) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the saline control group ($N=12$) of the fear-potentiated startle acquisition studies of Experiment 1A. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 9. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the 2.0 µg raclopride group (N=12) of the fear-potentiated startle acquisition studies of Experiment 1A. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 10. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the 4.0 μg raclopride group (N=12) of the fear-potentiated startle acquisition studies of Experiment 1A. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom: - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 11. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the 8.0 μg raclopride group (N=12) of the fear-potentiated startle acquisition studies of Experiment 1A. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
7.22: Baselining and Noise Intensity Level Results

ANOVA was used to statistically examine white-noise intensity levels and baselining acoustic startle responses for the saline control and raclopride (2.0 µg, 4.0 µg, and 8.0 µg) groups. ANOVA of the noise intensity levels yielded a non-significant result \( F(3,44) = 0.3420, \ p = 0.7950 \) [n/s] indicating that all groups were equated on the white-noise intensity levels prior to either drug infusion or fear conditioning and testing. ANOVA of baseline startle data obtained on the seventh baselining block yielded a non-significant result \( F(3,44) = 0.5593, \ p = 0.6446 \) [n/s] suggesting that the saline and raclopride experimental groups were adequately matched on baseline acoustic startle responses before drug infusion and fear conditioning/testing commenced. Figures 12 and 13 display the mean decibel levels and baseline startle amplitudes for the saline and raclopride groups (2.0 µg, 4.0 µg and 8.0 µg) respectively. As can be clearly seen in Figure 13, all experimental groups had similar levels of baseline startle responding before drug infusions and fear training and testing began.
White-noise Intensity Used to Induce Acoustic Startle

Figure 12. Mean white-noise intensity level in decibels that was used to induce acoustic startle responses in the saline control group (N=12) and the three raclopride drug groups (2.0 µg, 4.0 µg and 8.0 µg; N=12 each). Presented above are the mean decibel scores along with the S.E.M. ± for each drug treatment group.
Figure 13. Mean (S.E.M. ±) acoustic startle amplitudes for the saline group (N=12) and the three raclopride groups (2.0 µg, 4.0 µg, or 8.0 µg; N=12 per group). Baseline startle amplitudes were obtained from the seventh baselining block which occurred 48 hours prior to either drug infusion or fear conditioning and testing.
7.23: Fear Acquisition Testing Results

Results from the acquisition phase of this experiment demonstrate that rats infused with raclopride failed to develop robust FPS. In contrast, saline-infused animals developed robust FPS responding as training and testing continued over time. ANOVA (4 Drug Dose Treatment x 5 Test Block x 2 Stimulus Condition) of the acoustic startle data obtained from the noise-alone and CS + noise trials presented during the acquisition phase of this experiment, with repeated measures on the stimulus condition factor (light + noise vs. noise-alone) and test block, found significant main effects of Drug Treatment, $F(3,44) = 3.54, p < 0.03$; Stimulus Condition, $F(1,44) = 11.84, p < 0.002$; as well as a Drug Treatment x Stimulus Condition interaction, $F(3,44) = 5.13, p < 0.004$; and a Stimulus Condition x Test Block interaction, $F(4,176) = 3.03, p < 0.02$. A within-group Newman-Keuls comparing the 4 CS + noise to the 4 noise-alone trials for the saline group revealed that this group showed robust FPS during acquisition testing phase, whereas raclopride-infused drug groups did not.

As can be seen in Figure 14 the saline control group displayed FPS on blocks 1, 3, 4, and 5 while rats infused with raclopride into the BLA showed marked deficits in fear acquisition. The present results from the saline group seem to suggest that animals can acquire fear following a single block of 8 fear conditioning trials. However this view may be presumptuous since the saline rats failed to demonstrate a significant CS-enhancement of startle on the second acquisition test block. Regardless of the non-significant result observed on the second test block, rats in the saline group showed significant and robust levels of FPS on test blocks 3 to 5. On the other hand, the three groups of animals infused with raclopride (2.0, 4.0 and 8.0 μg doses) did not show FPS on any of the five fear acquisition test blocks.

With respect to the significant Drug Group X Stimulus Condition interaction, saline animals generally exhibited higher mean startle scores on the 4 CS + noise test trials than did rats that received intra-amygdaloid raclopride infusions but this effect seemed to be dependent on the dose of raclopride infused. For example, Newman-Keuls between-group analyses of the Drug Group X Stimulus Condition interaction revealed that the saline group had significantly higher mean startle scores on the 4 CS + noise trials on each of the test blocks (1 to 5) when compared to the 8.0 μg raclopride group. In a similar fashion, the
Newman-Keuls post hoc tests also revealed that saline animals had significantly higher startle amplitudes on the 4 CS + noise trials on test blocks 1, 2, and 4 relative to the 4.0 μg raclopride group. Newman-Keuls tests that compared the saline group to the raclopride 2.0 μg group on 4 CS + noise trials across test blocks revealed that the saline group only had significantly higher mean startle amplitudes on test blocks 1 and 4 (see Figure 14a depicting the between-group differences on the CS + noise trials). These results demonstrate that raclopride infusion into the BLA interferes with the acquisition of FPS and seems to do so in a dose-dependent fashion. This view is further supported by results obtained from difference score data that were calculated and then collapsed over the five test blocks.
Figure 14. Mean (S.E.M. ±) of acoustic startle amplitudes recorded on each 4 noise-alone and 4 CS + noise trial test-block as a function of infusion of saline (N=12) or raclopride (2.0 µg, 4.0 µg or 8.0 µg; N=12 each) into the basolateral amygdala and fear conditioning. A fear conditioning block (8 light + footshock pairings) preceded each of the five test-blocks allowing for a total of 40 fear conditioning trials to be administered across the five train-test blocks. (* P<0.05 CS + noise relative to noise-alone trials). Please note that Noise 1 corresponds to CS + Noise 1, Noise 2 corresponds to CS + Noise 2 etc... for each test block of the acquisition experiment.
Acquisition of FPS

5 Blocks of 4 CS + Noise Trials Depicting the Between-Group Differences on this Stimulus Type

Figure 14 (a). Mean (S.E.M. ±) of acoustic startle amplitudes recorded on each 4 CS + Noise trial type test block as a function of infusion of saline (N=12) or raclopride (2.0 μg, 4.0 μg, or 8.0 μg; N=12) into the basolateral amygdala and fear conditioning. As can be seen with this between-group comparison on just the CS + Noise stimulus type, the saline rats exhibited higher startle responding on the CS + Noise stimulus trials on most of the test blocks than did the raclopride-infused rats. Double star symbols (*) placed over each raclopride group’s bar graph represents a statistically significant difference on CS + Noise trials on each individual test block (* P < 0.05 relative to the saline group’s CS + Noise scores on each test block). Clearly, the mean startle scores on the CS + Noise trial type for the raclopride-infused rats were significantly smaller than that obtained from the saline-treated rats across most of the test blocks. This further illustrates that pretraining intra-BLA raclopride infusions prevented CS-induced startle from developing.
7.24: Difference Score Analysis

Difference scores for each test block were obtained by subtracting the mean startle amplitude obtained on the 4 noise-alone trials from the mean startle amplitude obtained on the 4 light + noise trials (Difference Score = [4 light + noise] - [4 noise-alone]). This difference score was calculated for each of the 5 test blocks and then collapsed in order to calculate a grand mean of the difference scores for each drug treatment group. ANOVA of the difference score revealed an effect of Drug Treatment, $F(3,44) = 5.13$, $p < 0.004$.

Appropriate Newman-Keuls tests were conducted for the effect of Drug Treatment and it was revealed that a robust level of fear acquisition following the 40 fear conditioning trials was exhibited by the saline group only. In contrast, the three raclopride groups did not display statistically significant levels of fear acquisition. However, as can be seen in Figure 15 the group of rats infused with 2.0 µgs of raclopride did display a higher mean difference score than those infused with either 4.0 or 8.0 µgs of raclopride, although this result was not statistically significant. Nevertheless, this result is interesting in that it suggests some fear learning may have occurred in the 2.0 µg group despite the challenge imposed on amygdaloid D2 receptors by this lower dose of raclopride. These results suggest that higher doses of raclopride may be necessary to completely block the acquisition of FPS, whereas lower doses may only have a dampening effect on this type of fear learning. This notion is supported by statistical analysis carried out on the data obtained from the fear retention test conducted 48 hours after the fear-conditioning/testing procedures in the acquisition phase of the experiment.
Figure 15. The grand mean (S.E.M. ±) of the difference scores ([CS+ noise] - [noise-alone]) collapsed over the five fear acquisition test blocks after intrabasolateral amygdaloid infusion of either saline (N=12) or raclopride (2.0 μg, 4.0 μg and 8.0 μg, N=12 each; ★ P<0.05 relative to saline control group).
7.25: Fear Retention Test Results

Fear retention data were collected 48 hours after the fear conditioning/testing procedure and are based on the mean acoustic startle amplitudes from the 10 noise-alone and the 10 CS + noise trials for each of the drug infusion groups. The results of this fear retention data is presented in Figure 16. These results provide further confirmation that raclopride prevented the acquisition of FPS in rats. ANOVA (4 Drug Treatment X 2 Stimulus Condition) of this data yielded main effects of Drug Treatment, $F(3,44) = 5.20, p < 0.004$; and Stimulus Condition, $F(1,44) = 13.59, p < 0.0007$, as well as a significant Drug Treatment X Stimulus Condition interaction, $F(3,44) = 6.33, p < 0.002$. These results support the notion that raclopride did not cause a drug-induced performance deficit during acquisition training and testing but rather interfered with CS-UCS fear learning and memory formation since raclopride-treated rats that exhibited deficits in FPS during the acquisition phase did so during retention testing as well. However, the deficit in FPS exhibited by rats was dependent on the dose of raclopride administered into the amygdala.

Newman-Keuls post hoc comparisons revealed higher mean startle amplitudes on the CS + noise trials relative to the noise-alone trials in the saline and 2.0 µg raclopride groups. This result suggests that rats in these two groups did acquire fear and were able to express this response 48 hours later. In marked contrast to the saline and 2.0 µg-infused rats, no FPS was exhibited on the retention test by the groups infused with either 4.0 or 8.0 µg of raclopride prior to fear conditioning (see Figure 16). Furthermore, Newman-Keuls statistical evaluation of the Drug Group X Stimulus Condition interaction revealed that the saline group’s mean startle amplitude on the 10 CS + noise trials of the retention test was significantly higher than that exhibited by the 4.0 µg and 8.0 µg raclopride groups (see Figure 16 double star symbols). This result indicates that raclopride infusion into the BLA prior to conditioning significantly and specifically disrupted CS-induced fear learning and thus prevented rats from exhibiting FPS on the retention test. This means that raclopride-infused rats failed to form strong CS-UCS associations during Pavlovian fear conditioning. Hence, the absence of FPS during acquisition testing in rats treated with higher doses of raclopride (i.e. 4.0 and 8.0 µg groups) can not be attributed to a drug-induced performance deficit because if the animals in question did form a memory trace of the fear conditioning event they should have been able to exhibit FPS during retention testing when in the non-
drug state. Since FPS during retention testing failed to materialize in the two higher dose raclopride groups and only marginally surfaced in the 2.0 μg raclopride group it is more likely that the blockade of D₂ amygdaloid receptors prevented stable and enduring CS-UCS fear association from being formed.

A closer examination of the retention test data for the noise-alone trials demonstrates that acoustic startle responding was slightly higher in both the saline and 2.0 μg raclopride groups when compared to the groups infused with either 4.0 or 8.0 μg of raclopride. Although these differences in noise-alone startle amplitude were not statistically significant they may be important as they may be indicative of the increased arousal and hypervigilence normally displayed during exposure to fear eliciting stimuli. Since the 4.0 μg and 8.0 μg raclopride infusion groups failed to acquire FPS during the acquisition phase and were unable to express fear on the retention test, one possible explanation for the depressed noise-alone startle observed in these groups on the retention test is that these animals lacked emotional arousal simply because they failed to make the necessary connection during fear-conditioning that the CS predicts danger. Thus, noise-alone levels and FPS levels were elevated in the saline and 2.0 μg treatment groups but not in the two higher raclopride dose groups. The dampened baseline startle observed on the retention test in the 4.0 μg and 8.0 μg groups may be related to raclopride’s blocking effect on fear learning during the acquisition phase of the experiment. It is also possible that the 4.0 μg and 8.0 μg doses of raclopride had an effect on the animal’s motor responding. However, the shock reactivity results do not support this supposition (see Shock Reactivity Results section below).

ANOVA of the retention test difference score (Difference Score = [10 CS + noise] - [10 noise-alone]) data revealed a main effect of Drug Treatment, F(3,44) = 6.33, p < 0.002. This result is consistent with the previous statistical analysis and confirms that infusing raclopride into the BLA prior to fear conditioning blocked FPS, whereas infusion of saline or a low dose of raclopride had no effect (see Figure 17). For example, rats in the 4.0 μg and 8.0 μg treatment groups showed no evidence of FPS following administration of the retention test, suggesting that higher doses of raclopride infusion into the BLA prior to fear conditioning led to a significant impairment of the neurobiological processes associated with fear learning. Further statistical examination employing Newman-Keuls post hoc tests
revealed that rats infused with the lowest dose of raclopride (2.0 µg) into the BLA before fear conditioning exhibited a statistically significant level of FPS responding on the retention test. However, it is important to point out that the difference score analysis demonstrates that the actual level of fear retention expressed by the 2.0 µg group was still significantly lower than the level of fear expressed by the saline group. Also, although the animals infused with 2.0 µg of raclopride displayed a conditioned fear response 48 hours later, this dose of raclopride still appeared to impair fear learning although to a lesser degree than that observed in the 4.0 and 8.0 µg groups. The results from the acquisition and retention tests suggest that raclopride infusion into the BLA blocks the acquisition of FPS in a dose-dependent fashion (see Figures 14 to 17). Thus, pretraining raclopride infusion into the BLA complex blocked the acquisition of FPS and by default any formation of long-term fear memories associated with the CS as rats infused with the higher doses of this DA D<sub>2</sub> antagonist also failed to exhibit FPS responding on the retention test.
Figure 16. Mean (S.E.M. ±) acoustic startle amplitudes and difference score ([10 CS + noise] - [10 Noise-alone]) results for saline control (N=12) and raclopride (2.0, 4.0 and 8.0 µg) groups (N=12 each) on the retention test conducted 48 hours after bilateral basolateral amygdaloid infusions and 5 fear conditioning/test blocks (★ P<0.05 CS + Noise relative to the Noise-Alone trial type; ★ P<0.05 difference scores of raclopride drug groups 2.0, 4.0 and 8.0 µg relative to the saline group's difference score). The double star symbols (★★) placed over the raclopride 4.0 µg and 8.0 µg groups denote a statistically significant between-group difference on the CS + Noise trial type relative to the saline group (★★ P<0.05 relative to the saline group's CS + Noise mean startle score on the retention test).
Retention Test Difference Score Results

Figure 17. Mean (S.E.M. ±) difference scores ([10 CS + noise] - [10 noise-alone]) of the saline and raclopride (2.0, 4.0 and 8.0 μg) groups (N=12 each) on the retention test administered 48 hours after basolateral amygdaloid drug infusion and fear conditioning/testing ( ★ P<0.05 relative to the saline-control group).
7.26: Shock Reactivity Results

During each of the 5 fear conditioning blocks movement amplitudes during shock presentation were collected and analysed. This was done to determine whether or not raclopride infusion into the BLA impaired the subject's perception of the footshock. A 4 Drug Treatment X 5 Shock ANOVA found no drug-induced group differences reflecting the fact that animals infused with saline and raclopride exhibited similar levels of reactivity during footshock administration (see Figure 18). However, ANOVA did find a main effect of Shock, $F(4,176) = 17.03, p < 0.0001$. As can be seen in Figure 18 all groups exhibited a decrease in shock reactivity over time. Thus, repeated exposure to footshock led to a decrease in shock reactivity and this decrease could not be attributed to intra-amygdalar raclopride infusion since the ANOVA failed to find a significant effect of Drug $F(3,44)= 0.179, p= 0.9100 \ [n/s]$, or a significant Drug X Shock Block interaction $F(12,176)= 1.27, p= 0.2357 \ [n/s]$. Therefore, it is safe to conclude that raclopride infusion into the BLA did not interfere with the capacity of rats to exhibit reflexive reactions (i.e. defensive jumping and flinching) in response to the footshocks administered during Pavlovian fear conditioning.
Figure 18. The mean movement amplitude (S.E.M ±) recorded during the presentation of 5 blocks of 8 light + footshock fear conditioning trials to rats that were infused with either saline or one of three doses of raclopride (2.0 µg, 4.0 µg or 8.0 µg) into the basolateral amygdala.
7.27: Shock Sensitisation Results

Laboratory rats that are exposed to footshock in the same experimental context where baselining startle amplitudes are measured often show subsequent significant increases in startle amplitudes. This phenomenon is called the shock sensitisation of startle and is thought to be indicative of rapid contextual conditioning to the experimental environment (Richardson and Elsayed 2000). Shock sensitisation has been used as a measure of fear expression in experiments examining the neurobiological substrates involved in fear learning and expression (Gifkins, Greba and Kokkinidis, 2002, Hitchcock, Sananes and Davis 1989). Research results from this experiment show that blockade of amygdaloid DA D2 receptors with low to moderate doses of raclopride (i.e. 2.0 and 4.0 μg) did not block the shock sensitisation of acoustic startle. Nevertheless, the highest dose of raclopride applied to the basolateral amygdaloid region did seem to dampen the shock sensitisation of startle.

Figure 19 depicts the shock sensitisation results from the present experiment. A 4 Drug Treatment X 2 Shock Test (preshock vs. postshock) ANOVA of the startle amplitudes obtained on the last 4 noise trials during predrug-preshock baselining (Preshock noise) and the 4 noise-alone trials following the first block of 8 CS + shock (Postshock; Noise 1) presentations revealed a shock sensitisation effect on startle $F(3,44) = 54.99, p < 0.0001$. In essence, this result demonstrated that footshock administration increased acoustic startle responding. This ANOVA also revealed a main effect of Drug Treatment, $F(3,44) = 2.79, p = 0.051$. This result indicates that the 8.0 μg raclopride infusion into the BLA appeared to dampen the shock sensitisation of acoustic startle and this effect can be observed in the lower startle amplitudes expressed by the 8.0 μg raclopride group relative to the saline controls.

To better assess the effects of raclopride infusion on the shock sensitisation of acoustic startle, a second ANOVA (4 Drug Treatment X 6 Shock Test Block) comparing the preshock baseline startle (Preshock Noise) to the acoustic startle scores on the 4 noise-alone trials obtained from each of the five fear acquisition tests (Postshock Noise-alone test blocks; Noise 1 to Noise 5) was performed. This statistical analysis compared the mean 4 preshock noise-alone startle response amplitudes to the mean of each of the 4 postshock noise-alone startle amplitudes. ANOVA of this data revealed an effect of shock session only, $F(5,220) = 13.47, p < 0.0001$, indicating that footshock administration increased
acoustic startle amplitudes in all groups (see Figure 19). No Drug Treatment X Shock Test interaction (\(F(15,220) = 0.89, [n/s]\)) was observed. This result confirms that all groups displayed increased startle responding after exposure to footshock during the acquisition phase of the experiment.

The experimental design provided five blocks of data thus making it possible to assess the effects of intraamygdaloid raclopride infusion on the shock sensitisation of acoustic startle over time. To achieve this goal the simple main effects were assessed (Winer, 1962; Winer, Brown and Michels, 1991). Newman-Keuls tests revealed that rats in the saline, 2.0 and 4.0 \(\mu\)g groups all displayed the shock sensitisation of acoustic startle. It is important to note that the 2.0 and 4.0 \(\mu\)g doses of raclopride did not impair the animal’s ability to express the shock sensitisation fear effect. Thus rats in the 2.0 \(\mu\)g group expressed a shock sensitisation effect on test blocks 1, 2 and 4 while the 4.0 \(\mu\)g group showed shock sensitisation on all 5 test blocks. Similarly, the saline group exhibited a shock sensitisation effect on test blocks 1 to 4. These results are important in that they indicate that lower doses of raclopride did not block the non-specific fear arousing effects produced by footshock. The present finding is generally consistent with that reported by Guarraci and colleagues (2000) who found that contextual defensive freezing shortly after footshock administration was not suppressed by eticlopride microinfusion into the amygdala.

Animals in the 8.0 \(\mu\)g infusion group did show footshock-associated increases in startle amplitudes however, these increases were not significantly different from their preshock baseline startle levels on any of the test blocks. One interpretation of this result is that infusion of the 8.0 \(\mu\)g dose dampened the shock sensitisation of startle by specifically inhibiting the action of amygdaloid D2 receptors and thus interfering with the fear-enhancing properties of footshock. Since the 8.0 \(\mu\)g dose blocked conditioned fear learning and dampened the shock sensitisation of acoustic startle following infusion into the amygdala it is possible that D2 amygdaloid receptors may ultimately contribute to the neurobiological processes underlying CS-UCS learning. This supposition is supported by the fact that the D2 DA receptor antagonist eticlopride blocked conditioned defensive freezing behaviour in rats when it was infused into the amygdaloid complex (Guarraci, et al., 2000).
Figure 19. Mean (S.E.M. ±) acoustic startle amplitude of the last 4 noise-alone trials prior to fear conditioning (Preshock Noise) for the raclopride (2.0 µg, 4.0 µg, and 8.0 µg) and saline basolateral amygdala infusion groups versus the average startle amplitudes obtained on each of the 5 test blocks containing 4 noise-alone trials presented after light + footshock pairings (depicted as Noise 1 to Noise 5). The symbol (*; P<0.05) represents significant differences of the post-shock acoustic startle means (Noise 1 to 5) relative to the preshock startle means for each drug treatment condition.
8. Experiment 1B
RACLOPRIDE INFUSION INTO THE BLA AND ITS EFFECTS ON BASELINE STARTLE AMPLITUDES

Hemsley and Crocker, (1999) reported that raclopride has the potential to increase muscle rigidity. Thus, the observed deficit in shock sensitisation in Experiment 1A may have been due to some impairment in the animal’s sensorimotor responding following the intraamygdalar infusion of the 8.0 μg dose of raclopride. Although this seems unlikely, given that all groups showed similar levels of shock reactivity during fear conditioning, the possibility that sensorimotor responding was impaired in the raclopride 8.0 μg group could not be ruled out in Experiment 1A. To address this issue, a control experiment was carried out to determine whether raclopride infusion into the amygdala would have any effect on sensorimotor processing as measured using baseline startle responding.

8.1: Method

8.12: Subjects
Twenty naïve male, Wistar rats with guide cannula bilaterally implanted 1.0 mm above the medial portion of the BLA were used in this experiment. All particulars concerning housing, surgery, startle apparatus, baselining and infusion procedures were the same as for Experiment 1A.

8.13: Procedure
Twelve to fourteen days after surgery rats were baselined using 7 blocks of 20 noise-alone trials (30 s; ISI) and then assigned to one of two groups (N=10 each) with similar averaged startle amplitudes and decibel levels. Forty-eight hours later rats were assessed for predrgu baseline startle by administering 20 white noise trials (30 s; ISI) at the animal’s predetermined decibel level obtained during baselining. Animals were then bilaterally infused with either saline (0.5 μl/side) or raclopride (8.0 μg/0.5 μl per side) and placed back into the test chambers. Following a 5 min habituation period, rats were retested for
baseline startle while in the drug state (postdrug) by administering 40 white noise trials (30 s; ISI).

8.2: Results and Discussion

8.21: Histological Results

Histological examination of the coronal brain sections revealed that the guide cannulae were located just dorsal to the medial portion of the BLA. As in experiment 1A infusion cannula extended 1.0 mm ventrally beyond the end of the guide cannula and this meant infusions were located in or near the basolateral amygdaloid complex (see Figure 20 and 21). The saline control group had an N= 9 as one of the saline rats lost its headcap during the infusion process and thus was excluded from the experiment.
Figure 20. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the saline control group (N=9) of Experiment 1B that examined raclopride's effects on acoustic startle responding. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 21. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 8.0 µg group (N=10) of Experiment 1B that examined raclopride's effects on acoustic startle responding. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom: -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
8.22: Experimental Results

The main finding of this control experiment was that an 8.0 μg dose of raclopride infused into the amygdala did not reduce acoustic startle amplitudes in laboratory rats. A 2 Drug Treatment X 2 Test (predrug vs. postdrug) ANOVA with repeated measures on the latter factor was carried out on the startle scores obtained from the saline and raclopride-infused animals. This ANOVA found a significant effect of Test, F(1,17) = 18.6, p < 0.0004. There was no significant Drug X Test interaction, [F(1,17) = 1.0, p < 0.3], and this highlights the fact that there were no significant between-group differences on acoustic startle responding before or after drug infusion (see Figure 22). This indicates that the 8.0 μg raclopride infusion had no effect on startle amplitude. Newman-Keuls post hoc tests carried out on the main effect of Test (predrug vs. postdrug startle) revealed that the saline and raclopride 8.0 μg groups both exhibited a significant increase in startle amplitudes from predrug to postdrug testing blocks (see Figure 22) however, there were no significant between-group differences that could be attributed to the effect of drug infusion since there was no significant effect of Drug F(1,17)= 1.00 p=0.3297 [n/s] or Drug Group X Test interaction F(1,17)=0.068, p=0.7971 [n/s]. Generally speaking, both experimental groups exhibited an approximately equal increase in acoustic startle responding after infusion of either saline or raclopride into the amygdala. This increase in acoustic startle amplitudes may simply be a reaction by the animals to the infusion procedure as there is usually some minimal distress caused by such a procedure. Given the lack of between-group differences in the present experiment it seems that a high dose of raclopride infused into the BLA does not interfere with normal sensorimotor responding.
The Effects of Intra-Amygdalar Raclopride Infusion on Baseline Startle

Figure 22. Mean (S.E.M. ±) acoustic startle amplitudes on the preinfusion and the postinfusion acoustic startle tests for animals infused with either saline or an 8.0 μg dose of raclopride into the basolateral amygdala (* P<0.05 relative to predrug infusion startle).
9. Experiment 1C
LIGHT CONTROL EXPERIMENT EXAMINING THE EFFECTS OF LIGHT-ALONE ON ACOUSTIC STARTLE RESPONDING

Although light + shock presentation produced robust FPS and shock sensitisation in saline-infused rats in Experiment 1A, it is also conceivable that exposure to light-alone without footshock administration may potentiate startle amplitudes. For example, Walker and Davis (1997a) found that exposing animals to long-term bright light conditions in the startle apparatus prior to acoustic startle testing significantly enhanced startle amplitudes in these animals relative to control rats that were not exposed to the bright light treatment. This finding indicates that the light may not be a totally neutral stimulus and the effect it has on startle responding may depend on its intensity and duration. To determine whether or not the light used in the acquisition experiments was a neutral stimulus, it was necessary to run an additional control experiment that examined the effects of light-alone exposure on acoustic startle. Thus, the aim of this experiment was to rule out the possibility that repeated exposure to the light-alone somehow contributed to the FPS effect observed in Experiment 1A.

9.1: Method

9.11: Subjects
A total of 13 naive male, Wistar rats were used in this light control experiment. All particulars concerning animal housing and testing periods were the same as described earlier. The only difference is these rats did not undergo surgery and formed an unoperated control group.

9.12: Apparatus
All particulars concerning the design of the startle testing apparatus were the same as described earlier.
9.13: Procedure

Rats were assessed for baseline startle using 7 blocks of 20 white noise-alone trials (30 s; ISI). Noise intensity levels were adjusted during the first 4 baselining blocks so stable baseline startle responding was established for each rat. Forty-eight hours after baselining rats were placed into their specified test chambers and after a 5 min habituation period were exposed to 40 light-alone (no shock) trials. The duration of the light was 3.5 s and the interstimulus interval for the light presentation was 45 s. No footshock was presented at any time during this training sequence. Movement amplitudes were measured for 100 ms prior to light presentation (prelight movement) and for 100 ms after the onset of the light (light movement). After the 40 light-alone trials, rats were removed from the test chamber and returned to their home cages. Forty-eight hours later rats were tested for FPS. This test involved first exposing rats to 20 noise-alone trials (30 s; ISI) followed by a random presentation of 10 noise-alone and 10 light + noise trials (30 s; ISI).

9.2: Results and Discussion

The main finding of this control experiment was that the light-alone presentations did not cause FPS when rats were tested 48 hours later. A dependent T-test was used to analyse the movement data obtained during the 40 light-alone presentations (prelight movement vs. light movement). The T-test of this data approached but failed to reach significance $t(1,12)=2.04, p=0.06$. As shown in Figure 23 movement amplitude measured during the period when the light was on was higher than movement amplitudes measured during the prelight period. This indicates that light presentation caused a slight increase in movement (prelight movement mean $= 28.7$, SEM $\pm 3.1$; light movement mean $= 36.7$, SEM $\pm 6.1$). This increase in movement reactivity during the light period may be indicative of anxiety or agitation produced by light onset. Alternatively, it may also indicate that rats were exhibiting a normal orienting response towards the light and assessing its relevance.

The dependent T-test of the final test data comparing the 10 light + noise trials to 10 noise alone trials found no significant differences in acoustic startle amplitudes between these two stimulus conditions, $t(1,12)=0.81, p=0.433$ [n/s], (light + noise mean $= 171.2$
SEM ± 35.5; noise-alone mean = 149.7 SEM ± 28.8). These results demonstrate that the light used in this experiment was indeed a neutral stimulus because presentation of the light prior to the noise burst during final testing failed to induce FPS in rats previously exposed to 40 light-alone trials (see Figure 24). Thus, light presentation in and of itself did not cause the observed FPS effect in the saline group rats in Experiment IA, but rather, the repeated pairing of light and footshock over time facilitated learning by strengthening the CS-UCS association and causing the once neutral stimulus (light) to quickly become a fear eliciting stimulus.
Figure 23. Mean movement amplitudes (S.E.M. ±) recorded 100 milliseconds before light-alone onset (pre-light movement in total darkness) and 100 milliseconds after the onset of the 3.5 second light presentation (post-light movement) that illuminated the test chamber. The premovement and post movement means were calculated from 40 light-alone presentations (no foot-shock).
Startle Responding to the Presentation of a Non-Conditioned Light-alone Stimulus

Figure 24. Mean startle amplitudes (S.E.M. ±) for 10 light + noise trials and 10 noise-alone trials that were used to assess the effects of light only (no footshock) presentations made 48 hours earlier on fear-potentiated startle. The mean difference scores (S.E.M. ±) ([10 light + noise] - [10 noise-alone]) demonstrate that 40 light only presentations do not produce fear-potentiated startle when they are not repeatedly paired with aversive footshock.
10. Experiment 1D

THE EFFECTS OF D₁ VERSUS D₂ AMYGDALOID DOPAMINE RECEPTOR ANTAGONISM ON THE ACQUISITION OF FEAR-POTENTIATED STARTLE IN RATS

The role of D₁ and D₂ DA amygdaloid receptors in fear acquisition and expression has been examined recently (Guarrachi, Frohardt and Kapp, 1999; Greba and Kokkinidis, 2000; Guarrachi, Frohardt, Falls and Kapp, 2000; Greba, Gifkins and Kokkinidis, 2001; Nader and LeDoux, 1999) however, a comparison between raclopride and SCH 23390 and their relative effectiveness in blocking the acquisition of FPS has not been made. In experiment 1A raclopride dose-dependently blocked the acquisition FPS and interfered with the formation of long-term conditioned fear memories. Additionally, the DA D₁ receptor blocker SCH 23390 has been shown to produce similar effects on FPS when administered either peripherally or directly into the BLA prior to fear training (Greba and Kokkinidis, 2000).

The D₁ receptor antagonist SCH 23390 and the D₂ receptor antagonist raclopride tend to bind selectively to the D₁ or D₂ receptor sites respectively (Protaias, Chagraoui, Arbaoui and Mocaer 1994; Kopp, Lindefors, Brene, Hall, Persson and Sedvall, 1992; Sidhu, Vanoene, Dandridge, Kaiser and Kebabian, 1986; O’Boyle and Waddington, 1987; Iorio, Barnett, Leitz, Houser and Korduba, 1983; Dawson, Gehlert, Yamamura, Barnett and Wamsley, 1985; Andersen, 1988). In addition, research indicates that there is a rich population of D₁ and D₂ receptors located in the basolateral and central lateral amygdaloid nuclei, many of which receive innervation from DA neurons located in the VTA (Boyson, McGonigle and Molinoff, 1986; Dawson, Barone, Sidhu, Wamsley and Chase, 1988; Meador-Woodruff, Mansour, Healy, Kuehn, Zhou, Bunzow, Akil, Civelli and Waston, 1991; Scibilia, Lachowicz and Kilts, 1992; Oades and Halliday, 1987; Swanson, 1982; Boyson, McGonigle, and Molinoff, 1986). This experiment sought to replicate the deficits in fear learning that were produced by the D₂ DA receptor antagonist raclopride (see Experiment 1A) as well as those reported previously using the D₁ receptor antagonist SCH 23390 (Greba and Kokkinidis, 2000; Guarrachi, Frohardt, Falls and Kapp, 2000; Guarrachi, Frohardt, and Kapp, 1999) after direct infusion into the amygdala. To accomplish this goal
it was desirable to use effective doses of the D₂ receptor antagonist raclopride (4.0 μg) and
the D₁ receptor antagonist SCH 23390 (2.0 μg) to evaluate their impact upon the
acquisition and retention of FPS following infusion into the amygdala. These drugs and the
selected doses were chosen because they have previously been shown to be effective in
blocking fear acquisition and expression when infused into the amygdala (Greba, Gifkins
and Kokkinidis 2001; Greba and Kokkinidis 2000; Guarraci, Frohardt and Kapp, 1999;
Frohardt et al., 2000; Waddington-Lamont and Kokkinidis, 1998). It is helpful to ascertain
the potency of the drug required to block FPS as far as dopaminergic antagonists are
concerned because this may provide some insight in terms of how particular amygdaloid D₁
and D₂ receptor populations are involved in the information processing and memory storage
that takes place during fear learning and expression.

10.1: Method

10.1.1: Subjects

A total of 39 naive, male Wistar rats served as subjects in this experiment. On average
the rats weighed 480 grams at the beginning of the experiment and were approximately 3.5
months old. A constant temperature of 20°C Celsius (±1°C Celsius) was maintained and food
and water were available for each animal on a continuous basis. Animals were group
housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at
8:00 pm. All behavioural testing and experimental manipulations occurred during the light
portion of this cycle.

10.1.2: Procedure

All particulars concerning stereotaxic surgery protocol, behavioural testing apparatus,
drug infusion and fear conditioning method employed were the same as those described in
experiment 1A. The only differences were the baselining protocol in terms of noise
intensity employed and in the number of trials used to assess FPS during the retention test
carried out 48 hours after the acquisition phase of the experiment. Thus, 14 to 16 days after
recovery from stereotaxic surgery that involved bilateral cannulation in a location 1.0 mm
above the BLA, all rats were baselined using a noise intensity of 95 decibels. This noise
intensity level was used during all baselining and subsequent fear tests in this experiment. The 95 decibel noise intensity level was chosen for this particular experiment because it was near the overall average white-noise level that was used to induce stable baseline acoustic startle responding in rats belonging in Experiment 1A and it also eliminated the need to make fine adjustments between baselining blocks.

During baselining each rat was placed in the startle apparatus and after a 5 min habituation period was administered 7 blocks of 20 noise-alone trials. This resulted in each rat being exposed to a total of 140 white-noise bursts. The interstimulus interval (ISI) between each noise burst was 30 seconds. Based on acoustic startle scores obtained on the seventh baselining block, rats were then assigned to one of three drug infusion groups (saline, raclopride 4.0μg, or SCH 23390 2.0μg; N=13 each) with similar averaged acoustic startle response amplitudes. Forty-eight hours after baselining rats were again placed in the startle apparatus and after a 5 minute habituation period exposed to 20 noise-alone trials in order to evaluate pre-drug and preshock baseline startle for the fear acquisition test.

10.13: Drug Infusion

Ten minutes later rats were wrapped in a soft cloth towel, and their dummy stylets were removed (C313DC; Plastic-One). A preloaded 28-gauge stainless steel internal cannula (C313I; Plastic-One) was then inserted into each guide cannula. Depending on drug group assignment, rats received a bilateral infusion of saline, raclopride (4.0μg dose), or SCH 23390 (2.0μg dose) into the basolateral region of the amygdala. The bilateral infusions were administered concurrently over a 1 min interval with the aid of two Stoelting infusion pumps (model 310). Each internal infusion cannula was left in place for an additional 2 min after the infusion pumps completed their cycle so as to ensure that the drug or vehicle solution adequately diffused into the desired brain tissue. The internal cannulas were slowly removed and a dummy stylet was reinserted into each guide cannula.

Animals were placed into their respective startle chambers, and after a 5 min habituation period, they were exposed to the first of five fear conditioning/test blocks. Each fear conditioning block was made up of eight CS-UCS (light + footshock) pairings and all particulars concerning light and shock duration, shock intensity, and inter-trial interval were the same as those used in Experiment 1A. Five minutes after each fear conditioning
block rats were assessed for the acquisition of FPS using the random presentation of four noise-alone trials and four CS + noise trials (30 s ISI). The noise intensity was set at 95 decibels for all animals and the light duration and sequencing of the noise burst was the same as described previously. This entire fear conditioning-testing procedure involved a total of 40 CS + shock presentations and five FPS tests. Each FPS test occurred 5-min after a fear conditioning block consisting of 8 light + shock pairings.

Fear retention was assessed 48 hours later when the animals were in a drug-free state. The retention test consisted of 20 noise-alone baseline trials (95 dB; 30 s ISI) followed 1 min later by the random presentation of 4 noise-alone and 4 CS + noise trials (95 dB; 30 s ISI). As was the case in Experiment 1A, the retention test served two purposes. The first allowed it to determine whether rats which exhibited conditioned fear during the acquisition testing phase retained the fear 48 hours later. Second, it ensured that any groups that did not display FPS in the drug-state could be assessed for FPS in the drug-free state. As will be recalled from Experiment 1A, there was a concern that drug infusion into the amygdala may prevent rats exhibiting FPS during the acquisition phase by somehow impairing the ability to perform a fear response. Thus, it is possible that rats can still form a weak CS-UCS fear association during Pavlovian fear conditioning but be unable to perform this response while in the drug state. To rule out this possibility, and provide solid evidence that D1 and D2 amygdaloid DA receptors are involved in Pavlovian fear learning and memory formation, the retention test was once again incorporated into this paradigm’s testing regime.

10.14: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, these include; acoustic startle amplitude data obtained during baselining and testing, shock sensitisation and shock reactivity data gathered during fear conditioning, and the level of FPS measured during the acquisition and retention experimental phases. This statistical procedure involved conducting a 3 Drug Group X 5 Test Block X 2 Stimulus Condition (4 noise-alone versus 4 light + noise trials) ANOVA. This ANOVA compared the saline control group to the 4.0μg raclopride and the 2.0μg SCH 23390 drug groups. Where appropriate Newman-Keuls multiple comparisons (α = 0.05) were used to assess
differences between group means. Within-group Newman-Keuls post hoc tests were used to assess differences between the two levels of the factor Stimulus Condition (i.e. CS + noise trials versus noise-alone trials) for each group when a main effect was found by the ANOVA. When Newman-Keuls post hoc tests were used to make between-group comparisons with interactions between two or more factors a pooled error term or a larger mean square error term was used to calculate the critical ranges required for statistical significance (Winer, 1962; Winer, Brown and Michels, 1991). Thus, as in Experiment 1A, Newman-Keuls post hoc tests based on either a pooled error term or a larger mean square error term were employed when applicable.

ANOVA was also used to statistically assess group differences that had been collapsed into a grand mean across the five acquisition tests. Finally, the difference scores (Difference Score = [4 CS + noise]-[4 noise alone]) obtained on the retention test were examined using an ANOVA. In general the statistical analysis used and the dependent measures assessed were identical to those evaluated in experiment 1A with the only exceptions being the number and type of drug groups being examined. Once again, Newman-Keuls multiple comparisons were used to determine precisely where differences occurred between the various group means.

10.2: Results and Discussion

10.21: Histology Results

All particulars concerning the histological procedures were identical to those described earlier. Histological examination of the coronal brain sections revealed that most of the guide cannulae were located just dorsal to the medial portion of the BLA with the infusion cannulae extending 1.0 mm ventrally into the BLA (see Figures 25 to 27). In just a few cases some cannulae were located more medial in an area above the central lateral amygdala or in an area between the basolateral and central amygdaloid nuclei which is the amygdalostratal transition area, a nuclear region thought to be involved in initiating rapid reflexive responses during fear-induced behaviour (Wang, 2002; Killcross, et al., 1997). These animals were kept in the statistical analysis because the 0.5µl volume infused would still have the capacity to diffuse into the basolateral and central lateral amygdaloid region,
two areas that have a rich distribution of $D_1$ and $D_2$ DA receptors (Boyson, McGonigle and Molinoff, 1986; Dawson, Barone, Sidhu, Wamsley and Chase, 1988; Meador-Woodruff, Mansour, Healy, Kuehn, Zhou, Bunzow, Akil, Civelli and Watson, 1991; Scibilia, Lachowicz and Kilts, 1992). Moreover, the more medial placement of the cannula in some animals tended to occur in just one hemisphere, whilst the other cannula in the contralateral hemisphere was generally more accurately placed above the basolateral complex.
Figure 25. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the saline group (N=13) of the fear-potentiated startle acquisition control Experiment ID. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 26. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 4.0 μg group (N=13) of the fear-potentiated startle acquisition control Experiment 1D. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 27. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the SCH 23390 R+ 2.0 μg group (N=13) of the fear-potentiated startle acquisition control Experiment 1D. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ±4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
10.22: Baselining Results

An ANOVA was used to statistically examine baseline startle responses for the saline, raclopride (4.0 μg) and SCH 23390 (2.0 μg) experimental groups. The ANOVA yielded a non-significant result F(1,36) = 0.122, p = 0.8850 [n/s], indicating that all three groups were matched on baseline startle responding prior to any experimental manipulation that involved either drug infusion or fear conditioning/testing (see Figure 28).

10.23: Fear Acquisition Results

The major finding was that both raclopride (4.0 μg) and SCH 23390 (2.0 μg) blocked the acquisition of FPS following infusion into the basolateral amygdaloid complex. ANOVA (3 Drug Group X 5 Test Block X 2 Stimulus Condition) of the fear acquisition tests that compared the saline control group to the 4.0 μg raclopride and 2.0 μg SCH 23390 groups yielded a main effect of Stimulus Condition (4 noise alone vs. 4 light + noise trials) F(1,36)=25.76, p< 0.00002, as well as a significant Drug Group X Stimulus Condition interaction F(2,36)=9.90, p<0.0004 and a Stimulus Condition X Test Block interaction F(4,144)=2.60, p<0.04. Newman-Keuls post hoc comparisons carried out on the main effect of Stimulus Condition (5 blocks of 4 noise-alone versus 4 light + noise trials) revealed that rats infused with saline exhibited significant FPS on test trials 2 to 5 (see Figure 29). In marked contrast, animals administered the 4.0 μg dose of raclopride or the 2.0 μg dose of SCH 23390 into the BLA failed to show significant fear effects on any of the five test block trials during the acquisition phase of this experiment (see Figure 29).

With respect to the significant Drug Group X Stimulus Condition interaction, saline animals exhibited significantly higher mean startle scores on the 4 CS + noise test trials than did rats that received intra-amygdaloid raclopride or SCH 23390 infusions. For example, Newman-Keuls analysis of the Drug Group X Stimulus Condition interaction revealed that saline animals had significantly higher mean startle scores on the 4 CS + noise trials on test blocks 2, 3 and 4 than did animals infused with raclopride. In a similar fashion, the Newman-Keuls revealed that saline animals had significantly higher scores on the 4 CS + noise trials on test blocks 2 to 4 than did the SCH 23390 2.0 μg-infused group (see Figure 29a). Taken together, these results indicate that acoustic startle responding during CS + noise presentations was significantly depressed in the raclopride and SCH
23390 groups relative to the saline control rats. This means that only the saline rats exhibited conditioned fear during the acquisition phase of the experiment.

The present results involving raclopride replicated those obtained in experiment 1A while the SCH 23390 results are consistent with previous reports highlighting the importance of D₁ amygdaloid receptors in fear acquisition and expression (Greba and Kokkinidis, 2000; Guarraci, Frodhardt and Kapp, 1999; Waddington-Lamont and Kokkinidis, 1998). Hence, the results obtained here clearly show that a 4.0 µg dose of raclopride or a 2.0 µg dose of SCH 23390 infused into the BLA blocks the acquisition of FPS. This supposition is supported by results obtained from difference score data that were calculated and then collapsed over the five test blocks.
Baseline Startle Scores

![Bar graph showing baseline startle amplitudes](image)

**Figure 28.** Mean (S.E.M. ± ) baseline startle amplitudes recorded on the seventh baselining block for the saline, raclopride 4.0 μg, and SCH 23390 2.0 μg experimental groups (N=13 per experimental group).
Figure 29. Mean (S.E.M. ±) acoustic startle amplitudes recorded on each 4 noise-alone and 4 CS + noise trial test block as a function of infusion of saline, raclopride (4.0 μg) or SCH 23390 (2.0 μg) into the basolateral amygdala and fear conditioning. A fear conditioning block (8 light + footshock pairings) preceded each of the five test blocks allowing for a total of 40 fear conditioning trials to be presented across the five train-test blocks (★ P<0.05 CS + noise relative to noise-alone trials). Please note that Noise 1 corresponds to CS + Noise 1, Noise 2 corresponds to CS + Noise 2 etc... for each test block of the acquisition experiment.
Acquisition of Fear-Potentiated Startle

**Figure 29 (a).** Mean (S.E.M. ±) of acoustic startle amplitudes recorded on each 4 CS + Noise trial type test block as a function of infusion of saline (N=13), raclopride (4.0 µg; N=13) or SCH 23390 (2.0 µg; N=13) into the basolateral amygdala and fear conditioning. As can be seen with this between-group comparison on just the CS + Noise stimulus type, the saline-infused rats exhibited higher startle on the CS + Noise stimulus type trials on most of the test blocks than did rats infused with either raclopride or SCH 23390. Double star symbols (☆☆) placed over the raclopride and SCH 23390 group's bar graph represents statistically significant differences on the CS + Noise trials on each individual test block (☆☆ P < 0.05 relative to the saline group's CS + Noise scores on each test block). Clearly, the mean startle scores on the CS + Noise trial type for the raclopride and SCH 23390-infused rats were significantly smaller that obtained from the saline-treated rats across most of the test blocks. This further demonstrates that pretraining intra-BLA raclopride or SCH 23390 prevents CS-induced startle in rats.
10.24: Acquisition Difference Score Results

As in Experiment 1A, difference scores for each test block were obtained by subtracting the mean startle amplitude obtained on the 4 noise-alone trials from the mean startle amplitude obtained on the 4 light + noise trials (Difference Score = [4 light + noise]-[4 noise-alone]). This difference score was calculated for each of the 5 test blocks and then collapsed in order to calculate a grand mean of the difference scores for the saline, raclopride 4.0 μg and SCH 23390 2.0 μg drug treatment groups. ANOVA of the mean difference scores collapsed across the 5 test blocks during the acquisition phase yielded a significant effect F(2, 36) = 8.81, p < 0.0008; ([Saline Mean = 253.5, SEM = ± 55.0, SD = 198.4; N = 13] versus [Raclopride 4.0 μg; Mean = 47.7, SEM = ±17.4, SD= 62.9; N = 13]) versus [SCH 23390 2.0 μg; Mean =54.4, SEM = ±36.4, SD= 131.2; N = 13]). Newman-Keuls post hoc tests revealed that the saline-infused rats had a greater magnitude of conditioned fear after 40 light + footshock trials than either the raclopride (4.0 μg) or SCH 23390 (2.0 μg) group. These results are depicted in Figure 30 and clearly show that saline animals exhibited a significant fear effect, whereas rats treated with either raclopride or SCH 23390 did not. The differences in fear acquisition between the saline, raclopride 4.0 μg, and SCH 23390 2.0 μg groups could not be explained by any between-group differences in baseline startle responding since an ANOVA of the baseline startle amplitudes for each group revealed a non-significant result as reported earlier. As can be seen by the startle amplitude means listed below, all three experimental groups were properly equated on baseline startle amplitude prior to drug manipulation ([Saline Mean = 184.4, SEM = ± 38.2, SD = 137.9; N = 13] versus ([Raclopride 4.0 μg; Mean = 170.9, SEM = ± 25.8, SD = 92.9; N = 13]) versus ([SCH 23390 2.0 μg; Mean = 162.5, SEM = ± 29.3, SD = 105.8; N =13]). Thus, variability in acoustic startle responding was not the primary factor contributing to the deficits in fear conditioning displayed by the raclopride and SCH 23390-infused rats.
Figure 30. The grand mean (S.E.M. ±) of the difference scores ((CS + noise)-(noise-alone)) collapsed over the five fear acquisition test blocks after the intra-basolateral amygdaloid infusion of saline, raclopride (4.0 μg) or SCH 23390 (2.0 μg). The significance level ★ P<0.05 is relative to the saline control group (N=13 per experimental group).
10.25: Fear Retention Test Results

It is possible that rats infused with either raclopride (4.0 μg) or SCH 23390 (2.0 μg) made a CS-UCS association during the acquisition phase but were unable to express the conditioned fear response while in the drug state. As will be remembered from Experiment 1A, the retention test conducted 48 hours after acquisition experimentation made it possible to assess FPS when rats were not in a drug state. If raclopride and SCH 23390-infused rats did form CS-UCS fear associations it would be expected that previously acquired fear would resurface during retention testing and rats would express FPS. The results obtained during the retention testing phase revealed that this did not occur. Only saline treated rats exhibited FPS, whereas the raclopride and SCH 23390 rats did not.

A 3 Drug Group X 2 Stimulus Condition ANOVA carried out on the retention data yielded a main effect of Stimulus Condition (4 light + noise trials vs. 4 noise-alone trials) F(1,24) = 4.86, p < 0.04, as well as a significant Drug Group X Stimulus Condition interaction F(1,24) = 5.02, p < 0.02. Newman-Keuls post hoc comparisons on the main effect of Stimulus Condition revealed that saline rats exhibited a significant fear effect as measured by the observed increase in mean startle amplitudes on the 4 CS + noise trials versus the 4 noise alone trials. In contrast, the Newman-Keuls analysis did not find any significant differences on the 4 CS + noise trials versus the 4 noise-alone trials for rats belonging to either the 4.0 μg raclopride or 2.0 μg SCH 23390 groups. Thus, a drug-induced performance deficit during the acquisition phase was ruled out. This means that the raclopride or SCH 23390 infusion into the BLA 48 hours earlier affected the ability of animals to express conditional fear during retention testing presumably by disrupting the formation of CS-UCS associations at the level of the amygdala.

Newman-Keuls analysis on the significant Drug Group X Stimulus Condition interaction confirmed this view by demonstrating that the saline groups’ mean startle amplitude on the 4 CS + noise trials was significantly different to the mean amplitude of the 4 noise-alone trials and to the mean startle amplitude of the raclopride and SCH 23390 groups on the 4 CS + noise trials. This meant that the mean startle amplitude measured for the saline group during CS + noise trials on the retention test was significantly larger than those measured for the raclopride 4.0 μg or SCH23390 2.0 μg drug groups (see Figure 31). This result indicates that raclopride or SCH 23390 infusions into the basolateral complex
48 hours earlier and just prior to fear acquisition training and testing significantly impaired the ability of rats to express FPS during the retention test.

These findings strongly suggest that SCH 23390 or raclopride infusion into the basolateral complex prior to fear conditioning likely antagonized DA D₁ and D₂ amygdaloid receptors and disrupted neurochemical cascades that are involved in the formation of associative learning and fear-memory storage. As a result, fear expression on the retention test was absent in rats that were administered intraamygdalar infusions of SCH 23390 or raclopride forty-eight hours earlier simply because no CS-UCS associations were formed during training. Thus, the SCH 23390 and raclopride-infused animals could not retrieve a fear memory that had not been adequately formed during training. This is in marked contrast to the saline-infused rats that clearly were able to retrieve fear memories and express robust FPS during the retention test. The results presented here replicated those previously reported (Greba and Kokkinidis, 2000; see Experiment 1A) and further highlight the importance of DA D₁ and D₂ amygdaloid receptors in fear learning and retrieval (also see Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; Guarraci, et al., 2000; Nader and LeDoux, 1999).
Retention Test FPS Results

Figure 31. Mean (S.E.M. ±) acoustic startle amplitudes and difference score ([4 CS + noise]-[4 noise-alone]) results for the saline, raclopride (4.0 µg) and SCH 23390 (2.0 µg) groups on the retention test conducted 48 hours after bilateral basolateral amygdaloid infusions and 5 fear conditioning/testing blocks ( ★ P<0.05 CS + noise relative to the noise-alone trial type; + P<0.05 difference scores of raclopride 4.0 µg or SCH 23390 2.0 µg drug group relative to the saline group's difference score). Each experimental group contained N=13 rats. Double star symbols ( ★★ ) placed over the raclopride and SCH 23390 groups denote a statistically significant between-group difference on the CS + Noise trial type relative to the saline group ( ★★ P<0.05 relative to the saline group's CS + Noise mean startle scores on the retention test).
10.26: Retention Test Difference Score Results

Difference scores were calculated for each animal belonging to the saline, raclopride (4.0 µg) or SCH 23390 (2.0 µg) experimental groups. This data was obtained during the retention test and the formula used to calculate a difference score is as follows; \[ \text{Difference Score} = (4 \text{ light + noise trials}) - (4 \text{ noise-alone trials}) \]. An ANOVA was used to compare the difference scores of the three experimental groups. The ANOVA of the difference scores yielded a significant result \( F(2,36) = 5.02, p < 0.02 \). Newman-Keuls tests carried out on this effect revealed that rats in the saline control group showed a significantly greater magnitude of FPS in the presence of the CS than did the animals that received intra-amygdalar infusions of either raclopride or SCH 23390. Figure 32 depicts the mean acoustic startle difference score and the standard error of the mean of saline control rats relative to animals infused with either raclopride (4.0 µg) or SCH 23390 (2.0 µg). The mean difference score for the saline group was 165.2 (SEM ± 50.9; SD = 183.6; N=13), whereas the raclopride and SCH 23390 groups’ mean difference scores were only 21.1 (SEM ± 32.7; SD = 117.9; N=13) and -20.4 (SEM ± 44.7; SD = 161.4; N=13) respectively. This result indicates that either raclopride or SCH 23390 infusion into the basolateral complex prior to fear conditioning severely impaired the ability of rats to express FPS on the retention test. This deficit in fear retention is likely due to the selective blockade of amygdaloid D₁ and D₂ DA receptors during fear training since saline-infused rats expressed robust FPS on the retention test. This finding adds credence to the idea that these drugs selectively antagonized DA D₁ and D₂ amygdaloid receptors and interfered with the neurochemical cascades that are involved in the formation of associative learning and fear-memory storage. As a result, it is quite possible that fear expression was absent in rats that were administered intraamygdalar infusions of raclopride or SCH 23390 forty-eight hours earlier simply because either no CS-UCS associations were formed during Pavlovian fear conditioning or these associations were very labile. Thus, the raclopride or SCH 23390-treated animals could not retrieve fear-memories that either were poorly developed and labile or simply did not exist. This would explain why saline-infused rats expressed significant levels of FPS on the retention test, whereas raclopride (4.0 µg) and SCH 23390 (2.0 µg)-infused rats did not.
Retention Test FPS Difference Score
Results

Figure 32. Mean (S.E.M. ±) difference scores ([4 CS + noise]-[4 noise-alone]) of the saline, raclopride (4.0 µg) and SCH 233990 (2.0 µg) groups (N=13 per group) on the retention test administered 48 hours after basolateral amygdaloid infusion and conditioning/testing ( ★ P<0.05 relative to the saline control group).
10.27: Shock Reactivity Results

On the whole, shock reactivity amplitudes were unaffected by the infusion of either raclopride or SCH 23390 into the basolateral amygdaloid complex. ANOVA of the shock reactivity data obtained from the 5 fear conditioning blocks failed to yield main effect of Drug Group $F(2,36) = 0.939$, $p = 0.400$ [n/s] and Shock Block $F(4, 144) = 1.56$, $p = 0.1872$ [n/s]; indicating that the saline, raclopride (4.0 $\mu$g) and SCH 23390 (2.0 $\mu$g) rats had similar levels of shock reactivity during fear conditioning. This was confirmed by the fact that the ANOVA found no significant Drug Group X Shock Block interaction $F(4,144) = 1.25$, $p = 0.2733$ [n/s]. The mean movement amplitudes recorded during shock administration for the saline (N=13), raclopride 4.0 $\mu$g (N=13), and SCH 23390 2.0 $\mu$g (N=13) are depicted in Figure 33. These results indicate that a 4.0 $\mu$g dose of raclopride or a 2.0 $\mu$g of SCH 23390 infused into the BLA had no effect on the animals’ ability to perceive the aversive qualities of the footshock. Thus, the observed blockade of fear learning to a specific CS during the acquisition phase of the experiment could not be attributed to raclopride or SCH 23390 diminishing the aversive qualities associated with footshock since raclopride, SCH 23390 and saline infused rats did not differ on mean shock reactivity scores on any of the 5 conditioning blocks. Therefore, any impairment to fear acquisition and then retention 48 hours later was more likely caused by the specific action of raclopride acting on D2 dopaminergic amygdaloid receptors and SCH 23390 acting on D1 dopaminergic amygdaloid receptors while fear training was in progress.

This would mean that raclopride or SCH 23390 infusions into the basolateral amygdaloid nucleus disrupted the formation of CS-UCS associations and therefore prevented fear learning during acquisition as well as fear expression during retention testing. The lack of a FPS effect on the retention test is not really all that surprising and points toward raclopride and SCH 23390 preventing the long-term fear associations from becoming established. Even if one assumes that the amygdala had the capability to overcome the interfering effects produced by either the 4.0 $\mu$g dose of raclopride or the 2.0 $\mu$g dose of SCH 23390 and still form weak CS-UCS associations one would still expect conditioned fear to resurface during the retention test. Evidently this did not happen, so it is likely that a drug-induced performance deficit during fear acquisition testing can be ruled out by the present results. Thus, the most parsimonious explanation is that the 4.0 $\mu$g dose
of raclopride and the 2.0 μg dose of SCH 23390 blocked fear acquisition and most likely prevented CS-UCS presentations from being consolidated and stored as long-term fear memories.

Nevertheless, it is important to recall that in Experiment 1A a smaller dose of raclopride (i.e. a 2.0 μg dose) infused into the BLA prior to fear-training did not seem to totally inhibit FPS during the acquisition phase of the experiment. As a consequence, the 2.0 μg raclopride rats in Experiment 1A were able to express a FPS effect that was significantly greater in magnitude than that expressed by the 4.0 and 8.0 μg raclopride drug groups on the retention test (see Figures 14 to 15 in Experiment 1A for a review). In general, the results obtained in this experiment regarding raclopride are consistent with those observed in Experiment 1A and seem to indicate that the level of fear impairment during the acquisition and retention phases may depend to a large degree on the dose of raclopride infused into the BLA. Since doses lower than 2.0 μg have not been tested using SCH 23390, it is difficult to tell if a similar dose-response function exists for this D₁ receptor antagonist in this particular paradigm. However, it is noteworthy to report that a dose-dependent blockade of conditioned fear responding has been observed with this particular D₁ receptor antagonist in a defensive freezing paradigm (see Guerraci, et al., 1999).
Figure 33. The mean movement amplitudes (S.E.M. ±) recorded during the presentation of 5 blocks of 8 light + footshock fear conditioning trials to rats that were infused with either saline, raclopride (4.0 µg) or SCH 23390 (2.0 µg) into the basolateral amygdala (N=13 per group).
**10.28: Shock Sensitisation Results**

The shock sensitisation results obtained in this experiment indicate that raclopride and SCH 23390 infusion into the amygdala had no impact on the shock sensitisation of acoustic startle. The mean startle amplitude obtained from the last 4 predrug-preshock noise-alone trials for the saline, raclopride, and SCH 23390 groups were compared to the mean startle amplitudes obtained from the 4 noise-alone postdrug-postshock trials on each of the 5 test blocks. A 3 Drug Group Treatment X 5 Shock Test ANOVA of this data yielded a main effect of Shock Test only $F(5,180) = 14.84$, $p < 0.0000001$, indicating that the saline, raclopride (4.0 µg) and SCH 23390 (2.0 µg) groups showed a shock sensitisation effect as indicated by an increase in noise-alone startle after shock presentation. The ANOVA results for the Drug Treatment X Shock Test interaction was not significant $F(10,180) = 1.08$, $p = 0.3769$ [n/s]. Newman-Keuls post hoc comparisons revealed that saline rats showed a significant shock sensitisation effect on postshock test blocks 1 to 5 (i.e. Noise 1 to Noise 5) when comparisons were made with the preshock/predrug mean startle amplitude (Preshock Noise). In a similar fashion, the animals treated with a 4.0 µg dose of raclopride into the BLA exhibited a shock sensitisation of startle on test blocks 1 to 5 (Noise 1 to Noise 5) when comparisons were made to the preshock/predrug (Preshock Noise) mean startle amplitudes. Finally, Newman-Keuls multiple comparisons revealed that the SCH 23390 (2.0 µg) treated group also exhibited shock sensitization on test blocks 1 to 5 (i.e. Noise 1 to Noise 5). The shock sensitisation results for the saline, raclopride (4.0 µg) and SCH 23390 (2.0 µg) rats are depicted in Figure 34 and indicate that raclopride or SCH 23390 infusions into the BLA did not block the shock sensitisation of acoustic startle.

The results reported here replicate those obtained with 4.0 µg of raclopride used in Experiment 1A and together they suggest that blockade of amygdaloid D2 receptors with raclopride prevents the formation of CS-UCS associations during Pavlovian fear conditioning but has no impact on the shock sensitisation of startle that may rely more heavily on arousal and short-term memories associated with the aversive qualities of the footshock and non-specific contextual cues (Richardson and Elsayed, 1998; Davis, 1989; 1992; Willick and Kokkinidis, 1995; Gifkins, Greba and Kokkinidis, 2001; Gifkins, Greba and Kokkinidis, 2002). Put simply, the shock sensitisation of startle which relies on
increased arousal and the memories associated with the aversive qualities of footshock and non-specific contextual cues paired with the footshock was not blocked by the 4.0 µg dose of raclopride in the present experiment. Thus, blockade of dopaminergic D₂ amygdaloid receptors with a sufficient dose of raclopride only seems to prevent the acquisition of FPS and any formation of long-term fear memories but leaves some behavioural correlates of fear intact. This finding is consistent with other research that demonstrates a prominent role for amygdaloid DA D₂ receptors in fear learning and expression (Guarraci, et al., 2000; Nader and LeDoux, 1999; see Experiment 1A).
Figure 34. Mean (S.E.M. ±) acoustic startle amplitude of the last 4 noise-alone trials prior to fear conditioning (Preshock Noise) for saline, raclopride (4.0 μg) or SCH 23390 (2.0 μg) basolateral amygdala infusion groups versus the average startle amplitudes obtained on each of the 5 test blocks containing 4 noise-alone trials presented after light + footshock pairings (depicted as Noise 1 to Noise 5). The symbol ( ★ P<0.05) represents significant differences of the post-shock acoustic startle means (Noise 1 to 5) relative to the preshock startle means for all experimental groups (N=13 per group).
The SCH 23390 2.0 μg shock sensitisation results reported here are also consistent with previous work that has demonstrated that peripheral and intra-amygdalar administration of SCH 23390 fails to impair shock induced increases in acoustic startle responding (Greba and Kokkinidis, 2000). It appears that the blockade of amygdaloid D1 DA receptors with SCH 23390 prevents the formation of CS-UCS associations during Pavlovian fear conditioning but has no impact on the shock sensitisation of startle. This means that shock sensitisation possibly represents a response that is driven by general arousal and a short-term memory that is associated with the aversive qualities of the footshock and the non-specific cues of the experimental context. Thus, a memory for the painful footshock and the context is established and stored in conjunction with the CS-UCS associations that are being formed during fear acquisition training and testing. Over a period of time, and as the fear-training and testing continues, CS-evoked startle in non-drugged animals eventually becomes greater than the noise-alone startle, however, the noise-alone startle in both saline and drug-treated rats is still usually much higher than baseline startle scores obtained prior to footshock administration. Thus, it appears that rats treated with an intra-amygdalar infusion of either SCH 23390 or raclopride fail to make CS-UCS connections or discriminate adequately between contextual/nociceptive fear cues and salient visual cues that predict footshock. As a result, raclopride and SCH 23390-treated animals do not exhibit a robust or seemingly coherent and planned fear reaction to a specific visual stimulus or cue that predicts shock, but rather seem to react to stimuli in a non-discriminating manner. Thus, as can be seen in Figures 29 and 33, both raclopride and SCH 23390-infused rats show reasonably high startle responses during both noise-alone and CS + noise presentations, however these startle responses are generally equal in magnitude and tend to cancel each other out and as a result produce only negligible fear effects. This would indicate that in addition to impairing CS-UCS associative fear learning, raclopride and SCH 23390 infusions into the amygdala may have also interfered with the ability of rats to discriminate between sensory stimuli that predict danger (i.e. CS[light] + noise) and stimuli that predict relative safety (i.e. darkness prior to noise burst). It is possible that this added deficit could have served to further compound the associative learning difficulties observed in the raclopride and SCH 23390-treated rats during the acquisition phase of FPS testing.
In stark contrast, saline-infused rats tend to form strong CS-UCS associations and seem to make accurate discriminations between various sensory stimuli presented to them during training and testing. As a result, saline-infused rats developed asymptotic levels of FPS as training and testing continued over time, whereas raclopride and SCH 23390-infused rats did not. This raises the interesting possibility that in addition to preventing the formation of CS-UCS associations, DA amygdaloid receptor antagonism could also impair the ability of animals to make accurate discriminations between stimuli. This notion is supported by the fact that DA receptor agonists and antagonists have been shown to be involved in enhancing or inhibiting learning in behavioural tasks that require animals to make discriminations between various sensory stimuli or cues that signal the onset of aversive events (Doty and Doty, 1966; Evangelista and Izquierdo, 1971; Haycock, et al., 1977; Johnson, 1969; White and Viaud, 1991) or food reward (Krivaneck and McGaugh, 1969 in Carr and White, 1984). In fact, levels of DA and its metabolite DOPAC have been shown to increase significantly in the basolateral amygdala region while rats learn and perform an operant discrimination task (Hori, Tanaka, and Nomura, 1993). This may explain why raclopride and SCH 23390-treated animals express some types of fear by exhibiting the shock sensitisation of startle which is not as cue specific, but not other forms such as CS-potentiated startle that require more complex sensory discrimination and associative learning processes that likely rely on more complex amygdaloid neural circuitry. Thus, blockade of dopaminergic D₁ and D₂ amygdaloid receptors with a sufficient dose of SCH 23390 or raclopride prevented the acquisition of FPS, a finding which is consistent with other research that demonstrates a prominent role for both amygdaloid DA D₁ and D₂ receptors in fear learning and expression (Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 2000; Greba and Kokkinidis, 2000; Greba, Gifkins and Kokkinidis, 2001; Nadar and LeDoux, 1999).

In summary, the result presented here suggests that raclopride (4.0 μg) or SCH 23390 (2.0 μg) infusions made into the basolateral amygdaloid region 48 hours earlier and just prior to fear acquisition training and testing significantly impaired the ability of rats to express FPS during the retention test. These results replicated those of earlier studies (Greba and Kokkinidis, 2000; Guarraci, et al., 1999) and further highlight the pivotal role played by mesolimbic DA systems, and DA D₁ and D₂ amygdaloid receptors in fear
learning and expression (Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Greba and Kokkinidis, 2000; Gifkins, Greba and Kokkinidis, 2002). Moreover, the observed blockade of fear learning to a specific CS during the acquisition phase of the experiment could not be attributed to raclopride or SCH 23390 diminishing the aversive qualities associated with footshock since all three experimental groups did not differ on mean shock reactivity scores on any of the 5 conditioning blocks.

In addition, the observed deficit of FPS during acquisition testing could not be attributed to a drug-induced performance deficit because raclopride and SCH 23390-infused rats failed to exhibit FPS on the retention test. So if raclopride and SCH 23390-treated rats did manage to form any CS-UCS fear associations, they certainly did not become unmasked during retention testing when rats were in a non-drug state. Thus, any impairment to fear acquisition and then retention 48 hours later was more likely caused by the specific action of raclopride and SCH 23390 acting on dopaminergic amygdaloid receptors while fear training was in progress. Simply stated, a 2.0 µg dose of SCH 23390 or a 4.0 µg dose of raclopride infused into the basolateral amygdaloid nucleus prior to fear-training sufficiently disrupted the formation of CS-UCS associations and therefore prevented fear acquisition during testing. This deficit in conditioned fear was obviously long-lasting and robust since it seemed to carry over to the retention test. So it is quite possible that DA receptors in the amygdala are important for the formation of long-term conditioned fear memories as well.

11. Experiment 1 E

SCH 23390 4.0µg DRUG CHALLENGE INFUSION INTO THE BASOLATERAL AMYGDALA AND ITS EFFECTS ON BASELINE STARTLE AMPLITUDES

11.11: Rationale

Dopaminergic receptor antagonists have been shown to produce impairments in locomotor and involuntary movement activity and high peripherally administered doses that cross the blood brain barrier can have neurotoxic effects resulting in neurological impairments and sensorimotor deficits (Ungerstedt, Ljungberg, and Schultz, 1978;
Ljungberg and Ungerstedt, 1978). For instance, neuroleptic drug treatment in humans can cause extrapyramidal side effects and tardive dyskinesia. Many of the symptoms include; repetitive bodily movements, involuntary muscle spasms, aberrant postures of limbs, trunk and tongue, and Parkinson-like tremors and rigidity of muscles and limbs (Klein and Davis, 1969; Ungerstedt, et al., 1978; Julien, 1996; Kandel, 2000 in Kandel, Schwartz and Jessell, 2000). However, it is unclear whether DA D_{1} receptor antagonists actually contribute to motor impairments since research by Gerhardt, Gerber and Liebman (1985) demonstrated that SCH 23390 did not enhance the ability of haloperidol to induce dyskinetic responses. So it is questionable whether the benzazepine antipsychotic SCH 23390 actually produces any motor impairment that would disrupt acoustic startle responding in a meaningful way.

Nevertheless, a control experiment was carried out to determine whether SCH 23390 infusion into the amygdala would disrupt the involuntary reflexes needed to perform a normal startle response. This experiment was carried out to ensure that the blockade of FPS produced by intra-BLA SCH 23390 in Experiment 1D was not simply caused by a drug-induced motor impairment. Thus although, Experiment 1D used a medium dose of SCH 23390 (i.e. a 2.0 μg dose) that seemed to be effective in blocking the acquisition of FPS in rats, there is the possibility that this drug may have prevented animals from expressing fear simply by diminishing the animal’s capacity to startle to acoustic stimuli in general (i.e. motor impairment). To rule this possibility out, a control experiment was undertaken to examine the effects of intra-amygdaloid SCH 23390 administration on baseline acoustic startle responding. This experiment entailed subjecting rats to a 4.0 μg SCH 23390 drug challenge to study the drug’s effect on baseline acoustic startle responding after intra-BLA infusion. Although this dose was twice as large as the dose used in Experiment 1D, it was chosen because previous research demonstrated that a 4.0 μg dose of SCH 23390 blocked fear acquisition and expression following infusion into the BLA (Greba and Kokkinidis, 2000; also see Waddington-Lamont and Kokkinidis, 1998) and because SCH 23390 effects on baseline acoustic startle have not been properly assessed after intra-amygdalar administration.
11.12: Subjects

Twenty-two naive male, Wistar rats with guide cannula bilaterally implanted 1.0 mm above the medial portion of the BLA were used in this experiment. All particulars concerning housing, surgery and baselining were the same as those described in Experiment 1D. The white noise intensity level during baselining and drug testing was set at 95 decibels.

11.13: Procedure

Fourteen days after surgery rats were baselined using 7 blocks of 20 noise-alone trials (30 s; ISI; 95 dB) and then assigned to one of two groups (N=11 each) with similar averaged startle amplitudes. This assignment was based on the mean acoustic startle data obtained from each animal’s score on the seventh block of 20 noise-alone baselining trials. Forty-eight hours later rats were assessed for predrug baseline startle by administering 20 white noise trials (30 s; ISI) at the prescribed intensity (i.e. 95 dB). Animals were then bilaterally infused with the appropriate drug {either saline (0.5 μl/side) or 4.0 μg/0.5 μl challenge dose of SCH 23390} and placed back into the test chambers. Following a 5 min habituation period, rats were retested for baseline startle while in the drug state (postdrug) by administering 40 white-noise trials (30 s; ISI; 95 dB). All particulars concerning the infusion process and histology were identical to those described earlier. The only difference was that the animals in this control experiment were deliberately challenged with a 4.0 μg dose of SCH 23390 infusion into the amygdala.

Microscopic examination of brain sections revealed that guide cannulae were located just dorsal to the medial portion of the BLA or in an area between the central lateral and basolateral amygdaloid nuclei in the rostral half of the amygdala (see; Figure 35 and 36). The infusion cannula extended 1.0 mm ventrally beyond the guide cannula tracts and would have likely entered into the BLA or in an area between the central lateral and basolateral area thus ensuring that most of the 0.5 μl volume would have diffused into the basolateral amygdaloid complex with some entering the central lateral nucleus as well (see; Figure 35 and 36). One animal in the saline group lost its headcap during the infusion procedure and as a result had to be removed from the study. This left the saline group with N=10 and the SCH 23390 4.0 μg with N=11.
Figure 35. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the saline control group (N=10) of Experiment 1E that examined the effects of dopamine D₁ amygdaloid receptor antagonism on acoustic startle responding. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 36. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the SCH 23390 4.0 μg challenge group (N=11) of Experiment 1E that examined the effects of dopamine D₁ amygdaloid receptor antagonism on acoustic startle responding. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ±4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
11.2: Results and Discussion

11.2.1: Baselining

An independent t-test of the mean acoustic startle response scores obtained on the seventh baselining block that compared the saline group’s startle score to that of the 4.0 µg SCH 23390 drug challenge group revealed no significant group differences t(2, 19) = 0.0049, p = 0.9960 [n/s] {(saline mean = 141.9, SEM ± 26.5; SD= 83.8; N=10) vs. (SCH 23390 4.0 µg mean = 141.7, SEM ± 28.2; SD=93.4; N=11)}. This finding indicates that groups were properly equated on baseline startle responding prior to any drug manipulation.

11.2.2: Pre-Drug Startle vs. Post-Drug Startle

The main finding of this study was that intra-amygdaloid infusion of a 4.0 µg dose of SCH 23390 did not interfere with acoustic startle responding. ANOVA of the Pre-Drug vs. Post-Drug startle scores for the saline group versus the SCH 23390 drug challenge group yielded a non-significant result for Drug Group {F(1,19)=0.001, p = .9678 [n/s] and Test F(1,19)= 1.98, p=0.1753 [n/s], suggesting that SCH 23390 infusion into the basolateral nucleus of the amygdala did not interfere with acoustic startle performance or the sensorimotor functioning needed to perform this response. This result was further confirmed by a T-test of the difference scores obtained by subtracting the pre-drug acoustic startle score mean from the post-drug acoustic startle score mean for the saline and SCH 23390 drug groups. These results yielded a score of t(2,19) = 0.134, p = 0.8941, [n/s] {(Saline mean difference score=50.4; SEM ± 48.8; SD=154.4; N= 10) vs. (SCH 23390 4.0 µg mean difference score=61.1; SEM ± 61.0; SD=202.2; N= 11)} indicating that no significant between group differences in acoustic startle could be found between saline control animals and those administered intraamygdalar SCH 23390. Figure 37 depicts the pre-drug and post drug-startle scores and the difference scores obtained by subtracting the mean startle amplitude 20 noise-alone predrug startle score from the 40 noise-alone postdrug amplitudes for each drug group and then comparing them using an independent T-test.
The results of this experiment are important since they demonstrate that fairly high doses of SCH23990 infused into the amygdala do not impair startle responding and therefore do not cause any profound sensorimotor impairments. As a result, the blockade of fear-learning and retention observed in Experiment 1D following SCH23390 infusions into the BLA is likely more attributable to the specific action of this drug acting on DA D1 receptors to prevent the formation of CS-UCS associations and not on any drug induced performance deficits. These experimental results along with those in Experiment 1D supplement and support studies that previously examined the effects of DA D1 receptor antagonism on fear learning and expression (Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; Greba and Kokkinidis, 2000). Taken together, the results obtained from the acquisition studies carried out in this thesis suggest that mesoamygdaloid DA substrates and DA amygdaloid receptors are for the most part involved in neurochemical processes that contribute to fear-learning and expression (see Waddington-Lamont and Kokkinidis, 1998; Nader and LeDoux, 1999a; 1999b; Guarraci, et al., 1999; Greba and Kokkinidis, 2000).
The Effects of SCH 23390 on Baseline Startle Responding

Figure 37. Mean (S.E.M. ±) acoustic startle amplitudes on the pre-infusion and the post-infusion acoustic startle tests for rats infused with either saline (N=10) or the SCH 23390 4.0 µg challenge dose into the basolateral amygdala. The mean difference scores ([postinfusion startle]-[preinfusion startle]) (S.E.M. ±) demonstrates that infusion of a 4.0 µg challenge dose of SCH 23390 into the basolateral amygdala does not interfere with the capacity of animals to startle in response to a 95 decibel level white-noise burst, indicating normal sensorimotor responding to acoustic stimulus presentations.
12. EXPERIMENT 2
A BEHAVIOURAL STUDY: FIVE UN-SIGNALLED FOOTSHOCKS CAUSES THE REINSTATEMENT OF FEAR-POTENTIATED STARTLE IN RATS THAT HAVE EXPERIENCED FEAR EXTINCTION TRAINING.

12.10: Rationale

It is well documented that fear memories established through Pavlovian conditioning are quite enduring (Konorski, 1948; Brown, et al., 1951; Davis, 1992a, b, c; Kim, et al., 1993a,b, Falls and Davis, 1994; Lee, et al., 1996b; Maren, et al., 1996; Otto and Cousins, 1998; Maren, 1999; LeDoux, 1998; Davis, et al., 2003). Conditioned fear expression can be extinguished by the repeated presentation of a non-reinforced CS, but as stated previously, this does not mean that the fear memory established during Pavlovian fear conditioning is forgotten or no longer exists, since re-exposure to the UCS (i.e. footshock) generally leads to a rapid reinstatement of conditioned fear responding (Pavlov, 1927; Konorski, 1948; Boutin, 1979; Boutin and Bolles, 1979a; 1979b; 1980; Rescorla and Heth, 1975; Westbrook, et al., 2002; Walker and Davis, 1992 in Falls et al., 1992; Gewirtz, et al., 1997; Davis, et al., 2003). In terms of the FPS paradigm, Gewirtz, et al., (1997) revealed that conditioned fear expression was reinstated in fear-extinguished rats by administering a series of unsignalled footshocks (i.e. the UCS) to these animals. Since it is useful to develop testing techniques and a reliable paradigm that can eventually be employed to study the neurochemical factors that facilitate the reinstatement of FPS the first step was to carry out a behavioural experiment that demonstrates that FPS can be reinstated in rats that have received extinction training. This research effort is valuable in that it sets up the basic experimental protocol that will be followed by the remainder of the FPS reinstatement experiments in this thesis. It is hypothesised that administering unsignalled footshocks to fear-extinguished rats will result in a robust FPS reinstatement effect.
12.2: Method

12.21: Subjects

A total of 40 naïve, male Wistar rats bred and housed in the Psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 350 grams at the beginning of the experiment. A constant temperature of 20°Celsius (±1°Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulations occurred during the light portion of this cycle.

12.22: Apparatus

All apparatus used in this experiment were the same as those described in Experiment 1A.

12.23: Procedure

12.24: Baselining

The baselining startle protocol used in this experiment was designed to establish a stable noise-alone acoustic startle response (i.e. baseline startle) for each rat and to reduce between-subjects variability in acoustic startle responding. In doing so, baseline startle assessment helped reduce between-group variability in acoustic startle when rats were eventually assigned to different treatment conditions. Acoustic startle was assessed for each rat over 3 blocks of 20 white-noise trials per block. Thus, each rat received a total of 60 white-noise trials with an interstimulus interval (ISI) of 30 seconds. During the first 2 baselining blocks the noise intensity levels in decibels were adjusted (range = 86.0-103 dB) for each animal to establish a stable acoustic startle response (between 50 and 500 units). The mean acoustic startle score measured on the third block of 20 white-noise trials represented the animal’s baseline acoustic startle score. The noise intensity level recorded for each rat after the third baselining block was used for all subsequent acoustic startle
testing. Thus, a specific decibel value that produced stable acoustic startle responding was determined for each rat.

12.25: Fear Conditioning

Forty-eight hours after the baselining procedure, rats were placed into their designated test chambers, and after a 5 min habituation period they were exposed to 30 consecutive CS (i.e. light) plus footshock fear conditioning trials. The inter-trial interval was 60 s. After the fear conditioning session was completed, the rats were removed from the test chambers and returned to their home cage environment. Following fear conditioning, rats were given 48 hours to consolidate their memory of the CS + shock presentations. The fear conditioning techniques used in this experiment were designed to produce reliable levels of FPS in rats.

12.26: Pretesting, Extinction Training, and Unsignalled Shock Presentation

After the memory consolidation period elapsed, the specified noise level that produced stable baseline startle amplitudes were set for each rat’s test chamber. The rats were then placed into their designated test chambers and after a five minute habituation period were assessed for FPS. This pretest first involved presenting the rats with 20 noise-alone trials (30 s, ISI) followed two minutes later by 3 CS + noise trials (30 s, ISI). This pretest compared the mean startle amplitude of the last 3 noise-alone trials obtained from the 20 noise-alone trials to the mean startle amplitude of the 3 CS + noise trials. A difference score was also calculated (Difference Score = [3 CS + noise trials] - [last 3 noise-alone trials]) for each rat. Rats were assigned to one of four behavioural experimental groups (N=10 per group) with similar averaged startle scores, decibel levels, and levels of FPS as reflected through the difference scores calculation and analysis. Thus, animals across all experimental groups were matched on conditioned fear, baseline startle, and noise intensity level (measured in decibels).

Twenty-four hours later, half of the rats were placed in their designated test chambers and after a 5 min habituation period they were presented with 140 light-alone extinction trials (ISI 15 s). The remaining rats received no extinction training and served as experimental controls. Thus, a total of four behavioural experimental groups were created.
(N=10 each). These four behavioural experimental groups and the manipulations which followed can be summarised as; (Extinction + 5 Unsignalled Shock); (Extinction + No Shock); (No Extinction + 5 Unsignalled Shock); (No Extinction + No Shock).

Forty-eight hours after extinction training rats were placed into their designated test chambers and allowed 5 min to acclimatise to the test environment. Rats assigned to either the Extinction + 5 Unsignalled Shock group or the No Extinction + 5 Unsignalled Shock group were then exposed to 5 unsignalled footshocks (no light). The intershock interval used in this experimental manipulation was 60 s and the duration of the shock was 500 ms. Rats assigned to the No Shock groups did not receive any unsignalled foot shocks. After this experimental manipulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment.

12.27: Final Test

Twenty-four hours after exposure or no exposure to unsignalled footshock, rats were tested for the reinstatement of FPS. The final test employed a quasi-counterbalanced design. This procedure entailed presenting half of the rats in each experimental group with 10 noise-alone trials first followed by 10 light + noise trials, whilst the other half of the rats in each experimental group were exposed to 10 light + noise trials first followed by 10 noise-alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the white-noise burst was presented during the last 100 ms of the light interval. The final test was used to determine whether the presentation of 5 unsignalled footshocks would cause a reinstatement of FPS in rats.

12.28: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, these included; acoustic startle amplitude data obtained during baselining, shock reactivity data gathered during fear conditioning, and the level of FPS measured during the pretest and fear reinstatement tests. A 4 Group X 2 Stimulus Condition (light + noise vs. noise-alone) ANOVA was used to analyse the pretest and final test data. Newman-Keuls multiple comparisons (α = 0.05) were used to assess differences between experimental
group means. Within-group Newman-Keuls post hoc tests were used to assess differences between the two levels of the factor Stimulus Condition (i.e. CS + noise trials versus noise-alone trials) for each group when a main effect was found by the ANOVA. When Newman-Keuls post hoc tests were used to make between-group comparisons or when interactions between two or more factors occurred a pooled error term (i.e. a larger mean square error) was used to calculate the critical ranges required for statistical significance (Winer, 1962; Winer, Brown and Michels, 1991). This statistical practice of pooling individual error terms for each factor that contributes to an interaction establishes a more conservative critical range for the Newman-Keuls post hoc tests. This statistical technique is scientifically and mathematically valid because it recognizes that each factor that contributes to a significant interaction on an ANOVA also brings along its own level of error and degrees of freedom. Thus, when interactions occurred and between group comparisons needed to be made, the Newman-Keuls tests in this thesis employed a pooled error term to establish the critical ranges that are used to denote statistical significance (see Winer, 1962; Winer, Brown and Michels, 1991).

Shock reactivity was evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 light + UCS trials. The mean shock movement amplitude for each rat was entered into the formula used to calculate the ANOVA that was designed to determine if the four experimental groups differed on the dependent measure of shock reactivity. ANOVAs were also used to test whether or not all experimental groups were equated on noise intensity level, baseline startle amplitude, and FPS as measured by the pretest.

12.3: Results and Discussion

12.3i: Baselining, Noise Intensity Level and Shock Reactivity Results

ANOVA of baseline startle amplitude data revealed that there were no significant between-group differences on baseline startle F(3,36)=1.78, p =0.167 [n/s], indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 38). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result F(3,36)= 0.29, p=0.830 [n/s], demonstrating that
the groups were similarly matched on this measure (see Figure 39). ANOVA of the shock reactivity data obtained during fear conditioning revealed a non-significant result $F(3,36)=0.38, p=0.767 [n/s]$, suggesting that all experimental groups showed similar levels of movement amplitude during CS + footshock administration. The shock reactivity results during fear conditioning are shown in Figure 40. Thus, rats in all experimental groups exhibited similar baselining and shock reactivity scores before the pretest.
Baseline Startle Responses for Behavioural FPS Reinstatement Study

Figure 38. Mean (S.E.M. ± ) baseline startle amplitudes recorded on the third baselining block for the Extinction + Shock, Extinction + No Shock, No Extinction + Shock and No Extinction + No Shock experimental groups (N=10 each) in the behavioural study that examined the reinstatement of fear-potentiated startle.
White-noise Intensity Level Used to Induce Baseline Acoustic Startle

Figure 39. Mean white-noise intensity levels (S.E.M. ± ) in decibels that were used to induce stable acoustic startle responses in the Extinction + Shock, Extinction + No Shock, No Extinction + Shock and No Extinction + No Shock experimental groups (N=10 each) that were designed to study the reinstatement of fear-potentiated startle.
Shock Reactivity Scores During Fear Conditioning

Figure 40. Mean (S.E.M. ± ) movement amplitudes recorded during the presentation of 30 light + footshock fear conditioning trials to rats in the Extinction + Shock, Extinction + No Shock, No Extinction + Shock and No Extinction + No Shock groups (N=10 each).
**12.32: Pretest Results**

Statistical analysis of the pretest data revealed that all experimental groups displayed robust FPS 48 hours after being exposed to 30 light + footshock fear conditioning trials. ANOVA of the pretest data revealed a main effect of Stimulus Condition $F(1,36)=59.20$, $p<0.000001$, with no significant effect of Group, or Group X Stimulus Condition interaction. These results are depicted in Figure 41, and they clearly show that rats in all four experimental groups displayed robust FPS after fear conditioning. Also, all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. Newman-Keuls post hoc tests carried out on the main effect of Stimulus Condition (3 CS + noise trials vs. 3 noise-alone trials) revealed that rats in all four experimental groups exhibited significantly higher startle amplitudes on the 3 CS + noise trials than on the 3 noise-alone trials. Statistical analysis (ANOVA) of the difference score data (Difference Score = [3 light + noise trials]-[last 3 noise alone trials]) confirms that all four experimental groups had similar levels of fear $F(3,36)=0.05$, $p=0.982$ [n/s]. Figure 42 depicts the difference scores obtained from the pretest and clearly demonstrates that the four experimental groups were equated on fear prior to any experimental manipulation involving either extinction training or exposure to 5 unsignalled footshocks.
Figure 41. Mean (S.E.M. ±) acoustic startle amplitudes on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (★ P<0.05 relative to noise-alone trials) for the Extinction + Shock, Extinction + No Shock, No Extinction + Shock, and No Extinction + No Shock behavioural experimental groups (N=10 each). Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depicts the equally robust levels of fear-potentiated startle found in all groups 48 hours after 30 fear conditioning trials and before extinction training occurred.
Figure 42. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]- [3 noise-alone]) depict the equally robust levels of fear-potentiated startle expressed by the Extinction + Shock, Extinction + No Shock, No Extinction + Shock and No Extinction + No Shock (N=10 each) behavioural experimental groups 48 hours after 30 fear conditioning trials.
12.33: Final Test Fear-Potentiated Startle Reinstatement Results

The main result obtained from this experiment is that the presentation of 5 unsignalled footshocks 24 hours before final testing caused a reinstatement of FPS in fear-extinguished rats. A 4 Experimental Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition $F(1,36)=29.94$, $p<0.000005$; and a significant Experimental Group X Stimulus Condition interaction $F(3,36)=3.53$, $p<0.03$. Newman Keuls post hoc comparisons indicate that with the exception of the Extinction + No Shock experimental group, all other groups showed a CS-induced enhancement of acoustic startle. More specifically, Newman-Keuls analysis carried out on the simple main effect of Stimulus Condition demonstrated that the Extinction + Shock, No Extinction + Shock and the No Extinction + No Shock groups all had mean acoustic startle scores on the 10 CS + noise trials that were significantly higher than those obtained during the presentation of 10 noise-alone trials (see Figure 43). In marked contrast, the Extinction + No Shock control group’s mean startle score on the 10 noise-alone and 10 CS + noise trials were almost virtually identical and therefore did not differ significantly from each other. This indicates that fear-extinguished rats not exposed to 5 unsignalled footshocks displayed no conditioned fear whatsoever. In effect, this means that the Extinction + No Shock control group exhibited normal fear extinction.

Furthermore, Newman-Keuls post hoc tests carried out on the Group X Stimulus Condition interaction revealed that the mean startle score on the 10 CS + noise trials during the final test for the Extinction + Shock group was significantly higher than mean startle score obtained by the Extinction + No Shock controls (see Figure 43 double star symbol). Also, the No Extinction + No Shock and the No Extinction + Shock groups had higher mean startle amplitude on the 10 CS + noise trials than the mean startle score obtained by the Extinction + No Shock control group on this stimulus type (see Figure 43). Thus, a particularly important finding was that fear-extinguished rats showed a robust reinstatement of conditioned fear following exposure to five unsignalled footshocks, a finding that is consistent to that reported by Rescorla and Heth (1975) and similar to results obtained by other researchers who have examined the effects of UCS reexposure and context on fear reinstatement in fear-extinguished animals (Westbrook, et al., 2002; Walker and Davis,
The reinstatement of conditioned fear responding suggests that the aversive qualities associated with footshock exposure during classical conditioning may be stored in memory systems located in one or several brain nuclei that are known to be involved in classical conditioning when aversive stimuli are employed as the UCS. Thus, reexposure to footshock, even unsignalled footshock in the context where fear conditioning took place, causes the reinstatement of fear in fear-extinguished animals by activating or arousing the neural substrates involved in fear learning and thus, overcoming the inhibitory learning laid down during extinction training. In contrast to the Extinction + 5 Unsignalled Shock group, rats in the Extinction + No Shock group showed no significant levels of FPS on the final test. This indicates that 140 light-alone extinction trials were sufficient to produce complete inhibition of FPS. Similarly, 5 unsignalled footshocks in the experimental context were sufficient to overcome extinction training and cause a reinstatement of FPS responding. This result is supported by an ANOVA carried out on the difference scores which measure the magnitude of the FPS effect.

ANOVA of the difference score (Difference Score= [10 light + noise trials]-[10 noise-alone trials]) data yielded a significant Experimental Group effect $F(3,36)=3.53, p<0.03$. Newman Keuls post hoc comparisons revealed higher levels of FPS in the Extinction + 5 Unsignalled Shock, No Extinction + 5 Unsignalled Shock, and No Extinction + No Shock groups relative to the Extinction + No Shock group that showed no appreciable levels of fear (see Figure 44). Taken together, these behavioural results provide compelling evidence that fear memories are indelible, survive rigorous extinction training, and can readily be recruited or reinstated following reexposure to unconditioned stimuli that were used during Pavlovian fear conditioning. These behavioural results are quite important since they provide useful testing techniques and a reliable paradigm that can be employed to studying the neurochemical factors that facilitate the reinstatement of FPS.
Final Test: FPS Reinstatement Results

Figure 43. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of extinction training versus no extinction training and 5 unsignalled footshocks versus no unsignalled footshocks (★ P<0.05 relative to noise-alone trials). The mean difference scores ((10 CS + noise)-(10 noise-alone)) (S.E.M. ±) demonstrate the impact of 5 unsignalled footshocks on the reinstatement of fear-potentiated startle in rats that received fear extinction training (★ P<0.05 relative to the Extinction + Shock group and the two No Extinction groups. The behavioural experimental groups are: [Extinction + Shock], [Extinction + No Shock], [No Extinction + Shock] and [No Extinction + No Shock] N=10 per group. The double star symbol (★★) over the CS + Noise trial type of the Extinction + No Shock group denotes a statistically significant between-group difference in mean startle on CS + Noise trials relative to the Extinction + Shock and the No Extinction + Shock groups (★ P<0.05).
Final Test: Difference Score for FPS Behavioural Reinstatement Study

Figure 44. Mean difference scores (S.E.M. ±) ([10 CS + noise]-[10 noise-alone]) for the Extinction + Shock, Extinction + No Shock, No Extinction + Shock and No Extinction + No Shock groups (N=10 each) on the fear-potentiated startle reinstatement test conducted 24 hours after either 5 unsignalled footshock presentations or no footshock presentations (☆ P<0.05 relative to the Extinction + Shock group and the two No Extinction groups).
13. EXPERIMENTS 3A TO 3D
THE ROLE OF DOPAMINERGIC AND NON-DOPAMINERGIC RECEPTOR
SUBTYPES IN THE REINSTatement OF EXTINGUISHED FEAR
RESPONSES: INFUSION OF RACLOPRIDE,
SCH 23390, AP5, OR CNQX INTO THE BASOLATERAL AMYGDALA OF
RATS PRIOR TO 5 UNSIGNALLED FOOTSHOCKS AND THE EFFECT OF
THESE DRUGS ON THE REINSTatement OF FEAR-POTENTIATED
STARTLE

13.1: Rationale

It is now generally accepted that amygdaloidal DA receptors (i.e. D1 and D2 types) and
 glutamate receptors (i.e. NMDA and AMPA types) play a prominent role in conditioned
fear acquisition and expression. For instance, research obtained from several different
laboratories over the years has demonstrated that conditioned fear acquisition and
expression can be effectively blocked by either the infusion of NMDA or AMPA receptor
antagonists into the BLA (Miserendino, et al., 1990; Campeau, et al., 1992; Kim, et al.,
1993; Fanselow, and Kim, 1994; Maren, et al., 1996; Walker and Davis, 1997b; Walker and
Davis, 2000; Lee and Kim, 1998; Lee, et al., 2001; Goosens and Maren, 2003; also see
Walker and Davis, 2002; Davis, 2000). Research has also demonstrated that blockade of
DA D1 and D2 amygdaloid receptors disrupts conditioned fear learning and expression in
the defensive freezing and FPS paradigms (Nader and LeDoux, 1999b; Guarraci, et al.,
1999; 2000; Waddington-Lamont and Kokkinidis, 1998; Greba and Kokkinidis, 2000; also
see Experiments 1A and 1D). As mentioned earlier, glutamate receptors are involved in
LTP and synaptic transmission processes that make conditioned fear learning, fear memory
retrieval and fear expression possible (see Chapter 6 of this thesis).

Despite the research findings pointing towards the involvement of amygdaloid
dopaminergic and glutamatergic receptors in conditioned fear learning and expression, it
must be stated that the neural mechanisms and biochemical events that facilitate fear
reinstatement after extinction training have not been fully examined. While it is true that
some studies have demonstrated that exposure to a UCS or sensitising drugs can cause a
restoration of conditioned fear responding in fear-extinguished animals (Konorski, 1948;
Rescorla and Heth, 1975; Gewirtz, et al., 1997; Westbrook, et al., 2002; Borowski and Kokkinidis, 1998), research efforts to date have not made it a priority to explore the neurobiochemical processes that produce this behavioural manifestation. This is quite unfortunate as maladaptive fears, phobias and PTSD tend to reappear in humans if they are exposed to stressful situations or stimuli that remind them of what they were afraid of in the past (Foa and Dancu, 1994 in Zinbarg and Mineka, 2001; Fyer, 1998; Zinbarg and Mineka, 2001). Thus, there is a tangible reason to examine the neurophysiological and biochemical processes within the central nervous system that may be contributing to the fear-reinstatement effect.

Another reason to pursue this research is to more precisely evaluate neural systems that are involved in retrieving and reconstructing conditioned fear memories that are masked by inhibitory learning. In some experimental paradigms it is unclear whether a given drug blocks fear expression by interfering with fear retrieval mechanisms or by interfering with the ability of animals to perform a conditioned response. One advantage of the FPS reinstatement paradigm is that pharmacological manipulations of the amygdala can be made prior to the presentation of unpaired footshocks. In this paradigm, unpaired footshock function as an aversive reminder stimulus that displaces inhibitory learning and promotes the retrieval (i.e. reconsolidation) of the CS-UCS fear associations established during Pavlovian fear conditioning. Thus introducing dopaminergic or glutamatergic antagonist drugs into the amygdala of fear-extinguished rats around the time when fear memories are being retrieved (i.e. during and after the unpaired footshock trials) and then testing these animals for conditioned fear 24 hours later in a non-drug state, can to some degree circumvent the performance versus memory retrieval problem mentioned above.

Recent studies by Johnson, Baker and Azorlosa (2000) have demonstrated that the reinstatement of classically conditioned fear can be blocked by peripheral administration of the NMDA receptor antagonist (+)-5-methyl-10,11-dihydro-5H-dibenzo[a,d]cyclo-hepten-5,10-imine maleate (MK-801) that binds to a receptor site located within the opening of the channel pore just above the Mg$^{2+}$ binding site of the NMDA receptor (Kandel and Siegelbaum, 2000 in Kandel, Schwartz and Jessell 2000). Also, it is well documented that dopaminergic receptors (i.e. D$_1$ and D$_2$) and glutamatergic receptors (i.e. NMDA and
AMPA) are involved in Pavlovian fear learning and expression, LTP induction and/or maintenance, and synaptic transmission processes that contribute to memory retrieval (Miserendino, et al., 1990; Campeau, et al., 1992; Kim, et al., 1993; Fanselow and Kim, 1994; Walker and Davis, 1997b; 2000; 2002; Maren, 1999; Reyman, et al., 1989; McKernan and Shinnick-Gallagher, 1997; Nader and LeDoux, 1999b; Guarraci, et al., 1999; Guarraci, et al., 2000; Waddington-Lamont and Kokkinidis, 1998; Greba and Kokkinidis, 2000; Schafe, et al., 2001; Lee, et al., 2001; Bissière, et al., 2003; also see Chapter 6 of this thesis). Since it has been clearly established that the amygdala is essential for Pavlovian fear learning and expression, it is natural that this limbic region be chosen for research that explores the biochemical factors that facilitate the reinstatement of FPS in rats that have experienced fear-extinction training.

As will be recalled, the results from Experiment 2 clearly demonstrated that unsignalled footshock exposure caused FPS reinstatement in fear-extinguished rats. However, the mechanism by which this occurred is unclear. It is possible that unsignalled footshock may have restored FPS in these rats by increasing DA turnover in the amygdala and various limbic regions. This idea is supported by the fact that DA metabolites are increased in the amygdala, nucleus accumbens and prefrontal cortex in response to footshock (see Chapter 6). It is also possible that footshock may have increased glutamatergic receptor-mediated EPSPs and/or enhanced excitatory neurosynaptic transmission in the amygdala and nearby limbic regions. Thus, the purpose of this series of experiments is to assess the contribution of amygdaloidal DA (i.e. D1 and D2) and glutamate (i.e. NMDA and AMPA) receptors in mediating the reinstatement of FPS in rats. Specifically, the aim of Experiment 3A is to determine if infusion of the DA D2 receptor antagonist raclopride into the BLA before unsignalled footshock administration would prevent FPS reinstatement when animals were tested 24 hours later in a non-drug state. The goal of Experiment 3B is similar except that it is designed to evaluate the impact on FPS reinstatement caused by introducing the DA D1 receptor antagonist SCH 23390 into the BLA before the unsignalled footshocks. Hence, the main aim of Experiments 3A and 3B is to ascertain if dopaminergic amygdaloid receptors contribute to FPS reinstatement.

Experiments 3C and 3D are designed to evaluate the role of amygdala NMDA and AMPA glutamatergic receptors in facilitating conditioned fear reinstatement. The purpose
of Experiment 3C is to determine whether blockade of NMDA amygdaloid receptors with AP5 before presenting fear-extinguished rats with 5 unsignalled footshocks will prevent the reinstatement of FPS during final testing. The goal of Experiment 3D is to see if antagonism of amygdalar AMPA receptors with CNQX before unsignalled footshock presentation would disrupt the reinstatement of conditioned fear when rats are tested for FPS a day later. This research represents the first pharmacological assessment of the amygdala's role in FPS reinstatement. The methods and results of each of these experiments are described below.

13.2: Method

13.21: Subjects

A total of 108 naive, male Wistar rats bred and housed in the Psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 340 grams at the beginning of the experiment. A constant temperature of 20° Celsius (±1° Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulations occurred during the light portion of this cycle.

13.22: Testing Apparatus and Stereotaxic Surgery

The apparatus used in Experiment 3A-3D was identical to that employed in Experiment 1A. In addition, all details regarding surgical procedure and cannula implantation are the same as those described in Experiment 1A with the exception that animals were given a 16 day recovery period before any behavioural training or testing took place. This extra two day recovery period was implemented in order to ensure that any tenderness around the surgical wound area were the head cap was made had subsided.
13.23: Procedure

The behavioural training and testing procedure is generally the same as that described in Experiment 2. However, rats were first bilaterally implanted with guide cannulae 1.0 mm above the medial portions of the basolateral nucleus of the amygdala before baseline acoustic startle screening or fear conditioning was carried out. Any new protocols in the experimental methodology used in Experiments 3A to 3D are described below.

13.24: Baselining, Fear Conditioning and Pretesting

All details regarding baselining, fear conditioning, and pretesting are identical to those described in Experiment 2. The noise intensity level that was recorded for each rat after the third baselining block was used for all subsequent acoustic startle testing. As previously mentioned, acoustic startle screening (i.e. baselining) was used to acclimatise rats to the startle testing apparatus and reduce variability in the acoustic startle response.

13.25: Group Assignment and Extinction Training

The 108 rats were assigned to one of 9 experimental drug groups (N=12 per group) with similar averaged startle scores, noise intensity levels, and levels of FPS as reflected through the difference scores calculation and analysis. Thus, animals across all drug treatment groups were matched on conditioned fear, baseline startle, and noise intensity level. Twenty-four hours later, rats were placed in their designated test chambers and after a 5 min habituation period exposed to 140 light-alone extinction trials. The ISI for the light presentation was 15 s and the light remained on for 3.5 s. Animals from all 9 experimental drug groups experienced extinction training as describe above and were given 48 hours to consolidate the extinction training experience.

The nine experimental groups were further divided to create four distinct experiments (3A to 3D), each examining the role of different amygdaloid receptors in the reinstatement of FPS. Experiment 3A investigated the effects of raclopride infusion into the BLA on the reinstatement of FPS and contained three raclopride groups (2.0 µg, 4.0 µg and 8.0 µg doses; N=12 each). Experiment 3B examined the effects of SCH 23390 (4.0 µg) intra-amygdalar infusion on the reinstatement of FPS. Experiment 3C investigated the impact of AP5 (1.25 µg and 2.5 µg; N=12 each) BLA infusions on fear reinstatement whilst,
Experiment 3D studied the effect CNQX (2.5 μg and 5.0 μg; N=12) intra-amygdalar infusion on fear reinstatement as measured by the FPS paradigm. A group of rats (N=12) received phosphate buffered saline (PBS) amygdaloid infusion and served as the control group for each experiment since all experiments were run concurrently.

13.26: Drug Infusion Followed by 5 Unsignalled Reminder Shocks

Forty-eight hours after extinction training rats received bilateral intraamygdalar infusions with one of the following drugs, S(-)-raclopride-L-tartrate {2.0 μg, 4.0 μg, or 8.0 μg dose}, SCH 23390 R(+) {4.0 μg dose}, (±)-2-Amino-5-phosphonopentanoic acid (AP5){2.5 μg or 1.25 μg dose}, 6-Cyno-7-nitroquinoxaline-2,3-dione disodium (CNQX) {5.0 μg or 2.5 μg dose} or phosphate buffered saline (0.5μl/side; pH= 7.4). Drugs were dissolved in a phosphate buffered saline solution (pH= 7.4) and all drugs were purchase from Sigma-Aldrich (New South Wales, Australia). Prior to the drug infusion, the infusion pumps were primed to ensure that the appropriate drug or vehicle solution was being properly released from the infusion cannulae. During the infusion, rats were wrapped in a soft towel and hand held, the dummy stylets (C313DC; Plastics-One) were removed and a preloaded 28-gauge stainless steel internal infusion cannula (C313I; Plastics-One) was inserted into each guide cannula. The infusion cannula extended 1.0 mm below the tip of the guide cannula and were attached to a drug-filled polyethylene tube (PE 20) connected to a 2.0-μl Hamilton syringe.

Bilateral infusions into the basolateral amygdaloid nuclei were made concurrently over a 60 second interval using two Stoelting infusion pumps (model 310; Wood Dale, IL). The infusion cannulae were left in place for an additional 2.5 min to allow ample time for the drug solution (0.5 μl/side) to diffuse into the surrounding brain tissue. The infusion cannulae were then slowly removed and the dummy cannulae reinserted. Following the infusion procedure, rats were placed into their prescribed test chambers and allowed 5 min to acclimatise to the test environment. Rats were then administered 5 unsignalled footshocks. The inter-trial interval was 60 s and the shock duration was 500 ms. After this experimental manipulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment.
13.27: Final Test

Twenty-four hours after drug infusion and exposure to the 5 unsignalled footshocks, rats were tested for the reinstatement of FPS. The final test employed a quasi-counterbalanced design. This procedure entailed presenting half of the rats in each drug treatment group with 10 noise-alone trials first followed by 10 light + noise trials. This order of stimulus presentation was reversed for the remainder of the rats in each drug group, with rats being exposed to 10 light + noise trials first then 10 noise-alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the white-noise burst was presented during the last 100 ms of the light interval. This test was specifically designed to assess the impact on FPS reinstatement that would be created by blocking amygdaloid DA and glutamatergic receptors before 5 unsignalled reminder footshocks were administered to rats that had received fear-extinction training.

13.28: Histology

Rats were injected with a lethal dose of sodium pentobarbitone and perfused intracardially with saline followed by a solution of formaldehyde (10%), glacial acetic acid (10%) methanol (80%) (FAM). The brains were removed from each animal’s skull and then stored in the FAM mixture for four days. The brains were then removed from the FAM solution and placed in a 70% sucrose solution for several weeks prior to sectioning. Coronal sections (50 μm) thick were sliced and mounted on gel coated slides. The slides were stained with Cresyl violet and the sections were evaluated under a microscope to determine cannula placements. The location of guide cannula placements and approximate site in the amygdala where infusion needles would have been located during the drug infusion were plotted on figures taken from the rat brain atlas of Paxinos and Watson (1986; 1998). This was done to make sure that only rats with guide cannulae located near the BLA would be retained in the experiment and used for any subsequent statistical analysis.
13.29: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, these included; acoustic startle amplitude data obtained during baselining, shock reactivity data gathered during fear conditioning, and the level of FPS measured during the pretest and fear reinstatement tests. Depending on the drug type and dose used, a 2, 3, or 4 Drug Group X 2 Stimulus Condition (light + noise vs. noise-alone) ANOVA was used to analyse the pretest and final test data. Newman-Keuls multiple comparisons ($\alpha = 0.05$) were used to assess differences between experimental group means and are the same as those described in Experiment 2. Shock reactivity was evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 CS-UCS trials. ANOVAs were also used to demonstrate that all experimental groups were equated on noise intensity level, baseline startle amplitude, and FPS as measured by the pretest. In addition and where applicable, independent T-tests were used to statistically analyse some of the experimental data.

13.3: Results and Discussion (Experiment 3A):
Raclopride Infusion into the Basolateral Amygdala of Fear Extinguished Rats Prior to 5 Unsignalled Reminder Footshocks and Its Effect on the Reinstatement of Fear-Potentiated Startle.

13.31: Subjects

A total of forty-eight rats were used to evaluate the effect intraamygdalar raclopride infusion had on the reinstatement of FPS. Twelve of the rats were infused with phosphate buffered saline (0.5 µl/side; pH= 7.4) while the 36 remaining rats equally divided across three different drug dose treatment conditions (2.0 µg, 4.0 µg, and 8.0 µg doses; N=12 each) and were infused with raclopride. The infusions were administered to these fear-extinguished rats prior to being exposed to the 5 unsignalled footshocks. All particulars concerning apparatus, surgery, baselining, fear conditioning, pretesting, group assignment, extinction training, drug infusion, 5 unsignalled shock presentation, final testing, histology and data analysis are described in the methods section listed at the beginning of this series.
of experiments. With reference to some of the particulars regarding the startle apparatus, stereotaxic surgical techniques and behavioural testing methods used Experiments 1 and 2 should also be consulted.

13.32: Histological Results

In general, the surgical placement of guide cannulae was very accurate and based on microscopic evaluation of these cannula placements it was decided that no animals should be excluded from the study. This meant that all experimental groups in this study were left with N=12. Figures 45 to 48 depict the location of guide cannula and the approximate area the infusion needle would end up during the intra-amygdaloid raclopride or phosphate buffered saline infusion. As can be seen in Figures 46 to 48 rats in all three raclopride drug groups had the majority of guide cannulae located above the medial portions of the basolateral amygdaloid nucleus (also see the digital photographs displayed in Figure 48a). Some guide cannulae were located more medially in an area just above the border between the lateral capsular subdivision of the central amygdaloid nucleus and the basolateral complex. This would place the guide cannulae just dorsal to or in the amygdalostrial transition area. A few guide cannulae either strayed more medially just above the central amygdaloid nucleus or more laterally just above the dorsal endopiriform nucleus, but as a whole this was a very rare occurrence. Also, the guide cannula as a whole seemed to target the anterior one-half to two-thirds of the basolateral amygdaloid nuclear group (i.e. -1.80 mm to -2.80 mm posterior to bregma).
Figure 45. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the phosphate buffered saline (PBS) control group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 46. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 2.0 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ±4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 47. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 4.0 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 48. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 8.0 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 48(a) depicts some digital photographs of guide cannula placements above the basolateral amygadaloid complex. Digital photograph (A) displays guide cannulae tracks above the basolateral amygadaloid complex (approximately -2.30 mm to -2.56 mm posterior to bregma). Also included is photograph (B). The left-half of this photograph exhibits the guide cannula placement above the lateral and basolateral amygdala (approximately -2.30 mm to -2.56 mm posterior to bregma) and the right-half of this photograph displays a magnified close-up of this image. Notice the protruding nipple-shaped bump at the end of the guide cannula in the close-up. This is created by the dummy stylet that screws down onto the guide and protrudes 0.25 mm below the end of the guide cannula. During the infusion process, the internal cannula extended a full 1.0 mm beyond the end of the guide cannula, thus placing the infusion needle and the drug into the basolateral amygdala.
Based on these guide cannulae locations and the approximate position of the infusion cannula (i.e. 1.0 mm below the termination point of the guide cannula) it is likely that most raclopride was infused into the basolateral complex which included the lateral and basolateral amygdaloid nuclei and their respective subdivisions. However, since a 0.5 μl volume was infused in each hemisphere some raclopride could have spread to the lateral capsular subdivision of the central nucleus of the amygdala, the central nucleus of the amygdala, the basomedial amygdalar nucleus and the intercalated cell mass. Some drug also likely diffused up the infusion cannula toward the more dorsally situated amygdalostrial transition area and lateral amygdaloid nucleus. Finally, some raclopride may have reached parts of the dorsal endopiriform nucleus as well. Nevertheless, it is highly probable that most of the raclopride was taken up by the basolateral, lateral and central amygdaloid nuclei and parts of the amygdalostrial transition area.

13.33: Experimental Results

ANOVA of baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the phosphate buffered saline (PBS) control group (N=12) to the three raclopride infusion drug groups (N=12 each) $F(3,44)=0.149$, $p=0.929$ [n/s], indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 49). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result $F(3,44)=1.86$, $p=0.151$ [n/s], demonstrating that the groups were matched on dB levels (see Figure 50). ANOVA of the shock reactivity data obtained during classical fear conditioning revealed another non-significant result $F(3,44)=1.13$, $p=0.346$ [n/s], suggesting that all experimental groups showed similar levels of movement amplitude during CS + footshock administration (see Figure 51).
Baseline Acoustic Startle Responses

Figure 49. Mean (S.E.M. ±) baseline acoustic startle amplitudes recorded on the third baselining block for the phosphate buffered saline (PBS) and the three raclopride (2.0, 4.0 and 8.0 μg) groups (N=12 per group).

Drug Group

PBS control  Rac 2.0μg  Rac 4.0μg  Rac 8.0μg
White-noise Intensity Used to Induce Startle Responding

Figure 50. Mean white-noise intensity levels (S.E.M. ± ) recorded in decibels that were used to induce stable acoustic startle responding for the phosphate buffered saline (PBS) control group (N=12) and the three raclopride (2.0 μg, 4.0 μg and 8.0 μg) drug groups (N=12 per group).
Shock Reactivity During Fear Conditioning

Figure 51. Mean (S.E.M. ±) movement amplitudes recorded during the administration of 30 light + footshock fear conditioning trials to rats in the phosphate buffered saline group (PBS; N=12) and the three raclopride groups (2.0, 4.0 and 8.0 μg; N=12 per group).
13.34: Pretest Results

The results obtained from the pretest demonstrate that Pavlovian fear conditioning produced robust FPS in all experimental groups in this study. ANOVA (4 Drug X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1,44)= 37.42, p<0.000001$, with no significant effect of Group, or Group X Stimulus Condition interaction. These results are depicted in Figure 52, and they clearly show that rats in all four drug treatment groups displayed robust FPS after fear conditioning. Newman-Keuls analysis on the main effect of Stimulus Condition (3 CS + noise trials vs. 3 noise-alone trials) found that all groups had higher startle amplitudes on the 3 CS + noise trials than on the 3 noise-alone trials. Also, all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. Statistical analysis (i.e. ANOVA) of the difference score data (Difference Score = [3 light + noise trials] - [last 3 noise-alone trials]) confirms that all four experimental groups had similar levels of conditioned fear after exposure to 30 light + footshock fear conditioning trials $F(3,44)=0.498, p=0.985$ [n/s]. Figure 53 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the PBS and three raclopride drug groups were equated on fear prior to any experimental manipulation involving extinction training, drug infusion, or exposure to 5 unsignalled footshocks.
Pretest Results For FPS After Fear Conditioning

Figure 52. Mean (S.E.M. ±) acoustic startle amplitudes obtained on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (★ P<0.05 relative to noise-alone trials). Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]−[3 noise-alone]) display the equally robust magnitude of fear-potentiated startle found in all groups 48 hours after 30 fear conditioning trials and before extinction training and drug infusion occurred.
Figure 53. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise] - [3 noise-alone]) depict the equally robust levels of fear-potentiated startle found in the PBS (N=12) and raclopride (2.0, 4.0 and 8.0 μg; N=12 each) experimental groups 48 hours after 30 fear conditioning trials and before the extinction training and drug infusion occurred.
Intra-amygdaloid raclopride infusion did not diminish the amplitude of reflexive responses (i.e. jumping and flinching) measured during the presentation of 5 unsignalled footshocks. Statistical analysis (i.e. ANOVA) of the shock reactivity data obtained from the 5 unsignalled reminder shocks administered to rats after either PBS, 2.0 μg, 4.0 μg, or 8.0 μg raclopride infusion into the BLA yielded a non-significant result $F(3,44)=1.42$, $p=0.250$ [n/s]. This result indicates that the raclopride infusion had no effect on activity levels in response to footshock (see Figure 54). This finding is consistent with reports that have shown that raclopride infusion into the BLA does not cause a significant attenuation of reflexive movements in response to footshock presentation (see Experiment IA and ID; also see Greba, Gifkins and Kokkinidis, 2001).
Reactivity to 5 Unsignalled Footshocks Following Drug Infusion into the Amygdala

**Drug Group**

Figure 54. Mean (S.E.M. ±) movement amplitudes recorded during the presentation of 5 unsignalled footshocks to fear extinguished rats infused with either phosphate buffered saline (PBS) or raclopride (2.0, 4.0 or 8.0 μg) into the basolateral amygdaloid nucleus (N=12 each).
13.36: Final Test Results and Discussion

The final test results demonstrate that intra-amygdaloid raclopride infusion dose-dependently blocked the reinstatement of FPS in rats. A 4 Drug Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition F(1,44)=14.36, p<0.0004; and a significant Drug Group X Stimulus Condition interaction F(3,44)=5.04, p<0.004. Newman-Keuls post hoc comparisons revealed that the PBS and the 2.0 μg raclopride infusion group had significantly higher startle amplitudes on the CS + noise trials than on the noise-alone trials. This suggests that the 2.0 μg raclopride and the PBS infused rats exhibited a FPS effect. In stark contrast to the PBS control rats, the 4.0 μg and 8.0 μg infused rats failed to show a CS-induced startle effect when administered the final test (see Figure 55). This was supported by the fact that Newman-Keuls post hoc tests did not detect a significant difference between the startle mean obtained from the 10 CS + noise trials and the startle mean obtained from the 10 noise-alone trials for the 4.0 and 8.0 μg raclopride groups.

In terms of the between-group comparisons that pertained to the Drug Group X Stimulus Condition interaction, the Newman-Keuls post hoc comparisons revealed that the mean startle score on the 10 CS + noise trials for the PBS group was significantly higher than mean startle scores on the 10 CS + noise trials obtained by the 8.0 μg raclopride drug group (see Figure 55 double star symbol). Although not statistically significant the PBS groups mean startle amplitude on the 10 CS + noise trials were still considerably higher than the mean startle scores exhibited by either the 4.0 μg or 2.0 μg groups on this particular stimulus condition. In a similar fashion, the raclopride 2.0 μg groups' mean startle score on the 10 CS + noise presentations was significantly higher than that obtained by the 8.0 μg raclopride group. Taken together, these results suggest that infusion of either the 4.0 μg or 8.0 μg dose into the BLA prior to the 5 unsignalled shock presentation had the greatest effect in blocking fear reinstatement, whereas the 2.0 μg dose only marginally inhibited fear reinstatement (see Figure 55). This finding was backed up by statistical analysis on the final test difference scores that assessed the magnitude of conditioned fear expressed.

Statistical analysis (ANOVA) of the difference scores [Difference Score = (10 light + noise)-(10 noise-alone)] yielded an effect of Drug Treatment F(3,44)=5.04, p< 0.004 and as
depicted in Figure 56, raclopride dose-dependently blocked the reinstatement of FPS when infused into the BLA prior to 5 unsignalled reminder shocks. Newman-Keuls post hoc tests of this data revealed that the magnitude of FPS exhibited by the PBS infusion group was significantly higher than the 8.0 µg raclopride infusion group and approached significance when compared to the 4.0 µg group (p=.081). The Newman-Keuls analysis did not detect any significant difference in fear magnitude between the PBS controls and the 2.0 µg raclopride group however, a significant difference between the 2.0 µg and 8.0 µg groups was apparent. The results of all the difference score statistical analysis when combined with post hoc analysis carried out on the numbers obtained from the 4 Drug Group X 2 Stimulus Condition ANOVA seem to suggest that raclopride blocks the reinstatement of FPS in a dose-dependent fashion and that the functional relationship between drug dose and the magnitude of fear reinstatement appears to follow a linear pattern (See Figure 55 and 56).
Final Test: FPS Reinstatement Results

Figure 55. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of infusion of PBS (N=12) or raclopride (2.0, 4.0 and 8.0 µg; N=12 each) into the basolateral amygdala 24 hours earlier and just prior to 5 unsignalled footshock presentation ( ★ P<0.05 relative to noise-alone trials). The mean difference scores ([10 CS + noise]-[10 noise-alone]) (S.E.M. ±) demonstrate the blocking effect of intra-basolateral amygdaloid raclopride infusion (2.0, 4.0 and 8.0 µg) on the reinstatement of fear-potentiated startle when administered prior to the 5 unsignalled reminder footshocks ( + P<0.05 relative to the PBS control group). The double star symbol (★★) over the 8.0 µg raclopride group indicates a statistically significant between-group difference in startle on the CS + Noise trial type relative to the PBS group ( ★★★ P<0.05 relative to the PBS group's CS + Noise mean startle score).
Figure 56. Mean difference scores (S.E.M. ±) ([10 CS + noise]-[10 noise-alone]) for the PBS control and raclopride (2.0, 4.0 and 8.0 μg) groups on the final test to assess the fear-potentiated startle reinstatement when bilateral basolateral amygdaloid infusions had been carried out 24 hours earlier and just prior to the presentation of 5 unsignalised footshocks (★ P<0.05 relative to the PBS control group; N=12 for each group).
Thus although, the 4.0 µg raclopride groups’ magnitude of fear reinstatement approached but did not reach statistical significance when it was compared to the PBS group via Newman-Keuls post hoc examination of the difference score analysis, it should not be taken to mean that the 4.0 µg dose was ineffective in blocking the reinstatement of FPS, rather it highlights the pattern of statistical results that can be generated when dose-dependent responses follow a systematically progressive and linear trend. For example, the 2.0 µg raclopride dose had a very small dampening effect on fear reinstatement, the 4.0 µg dose had a much greater impact in blocking fear-reinstatement, whilst the 8.0 µg group was the most effective in preventing the reinstatement of FPS (see Figures 55 and 56).

In order to assess the effectiveness of a 4.0 µg dose of raclopride on FPS reinstatement more independently, a T-test was used to compare the difference scores of the PBS and 4.0 µg raclopride treated animals. This T-test revealed that there indeed was a significant difference in the magnitude of fear between the PBS control group and 4.0 µg raclopride group on the final test \{t(2,22)=2.45, p< .03;\}. This result indicated that a 4.0 µg dose of raclopride is effective in disrupting the reinstatement of FPS. It is important to note that this T-test result is in agreement with the Newman-Keuls analysis carried out on the data computed by the 4 Drug Group X 2 Stimulus Condition ANOVA. These Newman-Keuls post hoc tests clearly bring to light the disruption caused to the reinstatement of FPS by the 4.0 µg raclopride dose when it was infused into the amygdala (see above; also see Figure 55). Figure 57 depicts the mean difference score of the PBS group relative to the 4.0 µg raclopride group as analysed by the T-test \{(PBS mean=165.9; SEM= ±32.6; SD=113.1; N = 12) vs. (Raclopride 4.0 µg mean=44.9; SEM= ±36.9; SD=127.8; N = 12)\}. From Figure 57, it can be clearly seen that the 4.0 µg dose of raclopride does cause quite a considerable blockade of fear reinstatement when compared only to the PBS control group.
Figure 57. Mean (S.E.M. ±) difference scores ([10 CS + noise]-[10 noise-alone]) for the phosphate buffered saline (PBS) and the 4.0 µg raclopride infusion groups (N=12 each) on the fear-potentiated startle reinstatement test. The star ( ★ ) represents the significantly different result between the PBS and raclopride 4.0 µg group when an independent T-test was carried out on the difference score data for these two groups ( ★ P< 0.05 relative to the PBS control group).
The preliminary results of this experiment suggest that the relative effectiveness of a 4.0 µg intra-amygdalar raclopride dose in blocking the reinstatement of FPS is slightly better than moderate. In contrast, higher doses of raclopride appear to be more effective in inhibiting fear reinstatement, as was the case with the 8.0 µg dose, while lower doses such as the 2.0 µg raclopride dose seem to be almost ineffective. Taken together, the result of this experiment indicate that blockade of amygdaloid DA D2 receptors with moderate to higher doses of raclopride (i.e. 4.0 µg and 8.0 µg) just prior to the administration five unsignalled reminder shocks prevents the reinstatement of FPS in rats that have received inhibitory fear-training. At any rate, the above findings are consistent with the supposition that intra-amygdalar raclopride application dose-dependently blocks FPS reinstatement.

It is important to note that the above results cannot be attributed to any abnormally high levels of conditioned fear expression in the PBS group during final testing. As a matter of fact, the magnitude of fear expressed by the phosphate buffered saline (PBS) control group during the final test of the present experiment was very similar to the fear levels exhibited by the Extinction + 5 Shock, No Extinction + 5 Shock, and No Extinction + No shock groups in the behavioural FPS reinstatement study (see Experiment 2). For example, in the current experiment the PBS infusion groups' magnitude of fear as measured by the mean difference score calculated from the final test data was equal to 165.9 (SEM= ± 32.6; SD=113.1; N=12) and this value was similar to the mean difference scores obtained from rats belonging to the Extinction + 5 unsignalled Shock group (Mean=206.9; SEM= ± 55.7; SD=176.1; N=10), the No Extinction + No Shock group (Mean= 171.9; SEM= ± 54.4; SD=172.1; N=10) and the No Extinction + 5 unsignalled Shock group (Mean=174.6; SEM= ± 51.5; SD=162.8; N=10) during final testing carried out in the behavioural FPS study (see Figure 44 in Experiment 2 and compare it to the PBS control group in Figure 56 of Experiment 3A). Thus, the magnitude of fear expressed by the PBS group during final testing in the current experiment seems to fall into a normal range. Hence, the fear expressed by the PBS control group in the present experiment cannot be attributed to any additional stress and excitation caused by the handling of animals during PBS infusion nor can it be attributed to the PBS directly increasing the excitation of basolateral amygdaloid neurons and creating abnormally high level of conditioned fear. The most likely explanation for the recurrence of fear in the PBS-infused rats is that amygdaloid
functioning was intact just prior to and during the administration of 5 reminder shocks. This meant that the UCS information would have probably been processed in the amygdala more readily. As a result, the memories associated with footshock coupled with the reexcitation of neurons and pathways of the amygdala-based fear system most likely reestablished (i.e. reconsolidated) CS-UCS associations and retrieved the fear memories that had become established during Pavlovian fear conditioning. Because no significant fear reinstatement occurred in the raclopride 4.0 and 8.0 µg drug groups, the most parsimonious explanation available at the present time is that raclopride dose-dependently blocks the reinstatement of FPS.

13.4: Results and Discussion (Experiment 3B):
SCH 23390 R+ infusion into the Basolateral Amygdala Prior to 5 Unsignalled Reminder Footshocks and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats that have Undergone Fear Extinction Training.

As was communicated earlier, DA D₁ receptors are involved in the acquisition and expression of FPS and conditioned defensive freezing behaviour in rats (Greba and Kokkinidis, 2000; Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; Nader and LeDoux, 1999b). Additionally, DA D₁ receptors are involved in mediating LTP in hippocampal brain slices (Huang and Kandel, 1995; Frey, et al., 1990; 1991; Otmakhova and Lisman, 1996) and it has been suggested that amygdaloid D₁ DA receptors are involved in conditioned fear memory retrieval (Waddington-Lamont and Kokkinidis, 1998; Guarraci, et al., 1999; Nader and LeDoux, 1999b). This means that DA D₁ receptors in the amygdala might also contribute to FPS reinstatement. Therefore, the purpose of this experiment was to determine whether intra-BLA infusion of the D₁ DA receptor antagonist SCH 23390 would block FPS reinstatement in fear-extinguished rats.

13.41: Subjects
A total of twenty-four rats were used to evaluate the effect of intraamygdalar SCH 23390 infusion on the reinstatement of FPS. Twelve of the rats were infused with
phosphate buffered saline (0.5 µl/side; pH = 7.4) and were the same phosphate buffered saline group used in Experiment 3A. The 12 remaining rats were placed into the 4.0 µg SCH 23390 drug treatment condition. The infusions were administered to these fear-extinguished rats prior to being exposed to the 5 unsignalled footshocks. It is important to point out that some rats in the SCH 23390 group had difficulties during the infusion process. This occurred when the dummy cannulae (stylets) in some animals were difficult to remove because they were occluded with dried blood and cerebral spinal fluid that must have flowed into the guide cannula during surgery. Thus, although some of the guide cannula were cleaned out with saline and by the repeated insertion and removal of the dummy cannulae, there is the possibility that some of the drug infusions in this particular group may have been compromised by having the infusion needle blocked by foreign materials as it was introduced into the guide cannula. This difficulty was confined to some animals and only to this particular experimental drug group, so in general, the majority of the drug infusions went ahead smoothly and did not seem to be negatively affected.

All particulars concerning the design of the startle testing apparatus and the surgical procedures used to implant cannulae 1.0 mm above the BLA were the same as those described in Experiment 1A. Also, baselining, fear conditioning, and pretesting protocols were identical to those described in Experiment 2. Group assignment, extinction training, drug infusion, 5 unsignalled shock presentation, final testing, histology and data analysis are described in the methods section listed in Experiment 3A and were used in this experiment. Finally, because this particular experiment involved a comparison between two experimental groups independent T-tests in addition to ANOVAs were also used to statistically evaluate the experimental data in some instances. Any statistically significant ANOVA results were then evaluated by using the same Newman-Keuls post hoc techniques that were described earlier in Experiment 2.

13.42: Histological Results

Figures 58 and 59 depict guide cannulae locations and the approximate position of infusion cannulae during SCH 23390 or PBS infusions made into the amygdala. Both Figure 58 and 59 are based on coronal sections taken from the rat brain atlas of Paxinos and Watson (1986; 1998). Microscopic examination of the guide cannulae locations revealed
that most guide cannulae were located above the medial portions of the basolateral amygdaloid nucleus and the more medially situated lateral subdivision of the central amygdaloid nucleus. A few of the guide cannula were located at the very rostral pole of the amygdala where the BLA begins (-1.60 mm to -1.80 mm posterior to bregma). The rest of the guide cannulae were situated at the anterior one third of the basolateral amygdaloid complex between -1.80 mm to -2.30 mm posterior to bregma. In relation to the raclopride drug treatment groups, the SCH 23390 rat’s guide cannulae were on the whole, found to be slightly more rostrally positioned. Generally speaking the guide cannulae placements were sufficiently accurate and positioned above the basolateral and central amygdaloid nuclei, as such no animals were discarded from the study, except for animals that lost acrylic head caps or chewed off their dummy cannulae before drug infusion.

The placement of the cannulae in this experiment would have meant that the SCH 23390 would have likely reached the basolateral complex and some near by amygdaloid nuclei. As was mentioned above, the major concern in this particular experiment was not the cannulae placements but rather was the number of rats that had guide cannulae that had become occluded with dried blood and cerebral spinal fluid. Despite the efforts made to open and clean out the blocked guide cannulae, some debris may have still been present when infusion needles were inserted into the guide cannulae. As a result, it is possible that some debris may have affected the infusion processes and prevented the drug from reaching the amygdala altogether.
Figure 58. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the phosphate buffered saline (PBS) control group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 59. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the SCH 23390 R+ 4.0 μg group (N=10) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
13.43: Experimental Results

One SCH 23390 rat had its dummy stylets chewed off, and one other SCH 23390 rat lost its headcap before the infusion phase of the experiment was completed, leaving the SCH 23390 group with \( N = 10 \) subjects. An independent T-test of the baseline startle amplitude data revealed that there was no significant between group differences on baseline startle amplitudes when comparing the PBS control group \( (N=12) \) to the 4.0 \( \mu g \) SCH 23390 infusion group \( (N=10) \) \( t(2,20)=0.757, p=0.459, [n/s] \), indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 60). The T-test of the noise intensity levels also yielded a non-significant result \( t(2,20)=0.852, p=0.403, [n/s] \), demonstrating that the groups were matched on this measure (see Figure 61). An independent T-test of the shock reactivity data obtained during classical fear conditioning revealed a non-significant result \( t(2,20)=0.412, p=0.684, [n/s] \), suggesting that the experimental groups showed similar levels of movement amplitude during CS + footshock administration (see Figure 62). This indicates that rats in both experimental groups exhibited similar reflexive reactions (i.e. jumping and flinching) in response to the aversive footshocks.
Figure 60. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the phosphate buffered (PBS) saline group (N=12) and the SCH 23390 4.0 µg group (N=10).
White-noise Intensity Level Used to Induce Baseline Startle

![Bar chart showing mean white noise intensity levels (S.E.M. ±) recorded in decibels that was used to induce stable acoustic startle responses for the phosphate buffered saline (PBS) group (N=12) and the SCH 23390 4.0 µg group (N=10).]
Figure 62. Mean (S.E.M. ±) movement amplitudes recorded during the presentation of 30 light + footshock fear conditioning trials to rats in the phosphate buffered saline (PBS) control group (N=12) and the SCH 23390 4.0 µg group (N=10).
The pretest results revealed that both experimental groups acquired conditioned fear 48 hours after being administered 30 light + shock training trials. ANOVA (2 Drug Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1,20)=14.95, p<0.001$, with no significant effect of Drug Group, or Drug Group X Stimulus Condition interaction. These results are depicted in Figure 63 and clearly show that rats in both drug treatment groups displayed robust FPS responding after fear conditioning. Newman-Keuls post hoc comparisons demonstrated that both PBS and SCH 23390 rats exhibited higher mean acoustic startle scores on the 3 CS + noise trials than on the 3 noise-alone trials of the pretest. Furthermore, both experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction.

Statistical analysis (Independent T-test) of the difference score data (Difference Score = [3 light + noise trials] - [last 3 noise alone trials]) confirms that both experimental groups had similar levels of fear $t(2,20)=0.591, p=0.560 \text{ [n/s]}$. Figure 64 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the PBS group and the 4.0 μg SCH 23390 drug group were equated on fear prior to any experimental manipulation involving extinction training, drug infusion, or exposure to 5 unsignalled footshocks.
Figure 63. Mean (S.E.M. ±) acoustic startle amplitudes for the phosphate buffered saline (PBS) control group (N=12) and the SCH 23390 4.0 µg group (N=10) on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (* P<0.05 relative to noise-alone trials). The mean difference scores of the pretest ([CS + noise] - [noise-alone]) display the equally robust levels of fear-potentiated startle expressed by both experimental groups 48 hours after 30 fear conditioning trials and before extinction training occurred.
Figure 64. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) for the phosphate buffered saline (PBS) control group (N=12) and the SCH 23390 4.0 µg group (N=10) depict the near equivalent magnitude of fear-potentiated startle exhibited by the experimental groups 48 hours after fear conditioning.
Intra-amygdaloid SCH 23390 (4.0 μg) did not diminish the capacity of rats to perform reflexive responses (i.e., defensive jumping and flinching) in reaction to unsignalled footshock presentation. Statistical analysis (independent T-test) of the shock reactivity data obtained from the 5 unsignalled reminder shocks administered to rats after either PBS or 4.0 μg SCH 23390 infusion into the BLA yielded a non-significant result $t(2,20)=0.257$, $p=0.799$[n/s]. This result indicates that the SCH 23390 infusion had no effect on the rat’s reactivity to unsignalled footshocks (see Figure 65). Thus, SCH 23390 did not appear to cause any measurable impairment in shock perception or defensive reflexes (i.e., jumping and flinching) when rats were exposed to the 5 unsignalled reminder footshocks as both the PBS and SCH 23390 experimental groups exhibited similar levels of movement amplitude during shock administration.
Shock Reactivity During 5 Unsignalled Footshocks After Intra-Amygdalar Drug Infusion

Figure 65. Mean (S.E.M. ±) movement amplitudes recorded during the presentation of 5 unsignalled footshocks to fear extinguished rats infused with either phosphate buffered saline (PBS; N=12) or SCH 23390 (4.0 μg; N=10) into the basolateral amygdaloid complex.
13.46: Final Test Results and Discussion

The main finding of this experiment was that infusion of the DA D₁ receptor antagonist SCH 23390 (4.0 μg) into the BLA did not block FPS reinstatement. A 2 Drug Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA only revealed a significant main effect of Stimulus Condition F(1,20)=35.64, p<0.00001. The Drug Group X Stimulus Condition interaction was not significant F(1,20)=2.09, p=0.163 [n/s]. Newman-Keuls post hoc comparisons carried out on the main effect of Stimulus Condition revealed that both the PBS and the 4.0 μg SCH 23390 infusion groups had significantly higher startle amplitudes on the CS + noise trials than on the noise-alone trials (see Figure 66). This result suggests that infusion of a 4.0 μg dose of SCH 23390 into the BLA prior to the 5 unsignalled shock presentation had no effect in blocking fear reinstatement.

Statistical analysis (i.e. an independent T-test) of the difference score data failed to find any significant differences between the PBS and SCH 23390 group \( t(2,20)=1.44, p=0.163 \) [n/s]. As depicted in Figure 67, infusion of SCH 23390 into the BLA failed to block the reinstatement of FPS. If we examine the difference score means for the PBS group (mean=165.9, SEM ± 32.6) and compare it to the 4.0 μg SCH 23390 group’s difference score mean (mean=101.2, SEM ± 29.4) we can see only a slight dampening in the magnitude of fear, however this difference is not significant. Based on the failure of SCH 23390 to block fear reinstatement, it appears that amygdaloid DA D₁ receptors may not be involved or contribute to the neural processes involved in mediating fear reinstatement. On the basis of the experimental findings reported thus far, it appears that DA D₂ amygdaloid receptors are more involved in processes mediating the reinstatement of FPS than are DA D₁ amygdaloid receptors.

Despite the apparent dissociation that seems to exist between amygdaloid DA receptor subtypes as far as fear reinstatement is concerned, some caution needs to be taken in interpreting these results in this way, especially since the occluded guide cannula in some of the animals may have contributed to the results that were obtained. In other words, it is conceivable that SCH 23390 may have been prevented from reaching the basolateral complex and lateral subdivisions of the central nucleus where numerous DA D₁ receptors
are present simply because debris may have blocked the infusion needles. Consequently, this would have meant that little to no blockade of DA D1 amygdaloid receptors would have occurred during the presentation of the 5 unsignalled reminder footshocks. This would have had the effect of increasing the probability that animals would show fear reinstatement during the final test 24 hours later. As a result, further research needs to be carried out on DA D1 receptor systems in the amygdala and other brain areas to more fully understand their effect on the reinstatement of FPS. Also, it is possible that a more potent DA D1 receptor antagonist such as R(-) SCH-12679 maleate that can be mixed at higher concentrations may be more effective in blocking conditioned fear reinstatement if it were tested using this paradigm. However, this is a question that future research must address.
Figure 66. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of infusion of either phosphate buffered saline (PBS; N=12) or 4.0 μg of SCH 23390 (N=10) into the basolateral amygdala 24 hours earlier and just prior to the presentation of 5 unsignalled footshocks ( * P<0.05 relative to the noise-alone trials). The mean difference scores ([10 CS + noise]-[10 noise-alone]) and the corresponding S.E.M. ± values highlight the apparent inability of intra-amygdalar SCH 23390 infusions to block the reinstatement of fear-potentiated startle.
Final Test: FPS Reinstatement Difference Scores

![Bar chart](image)

**PBS control**  
**SCH 23390 4.0 µg**

**Drug Group**

Figure 67. Mean difference scores (S.E.M. ± ) ([10 CS + noise]-[10 noise-alone]) for the phosphate buffered saline (PBS) control group (N=12) and the SCH 23390 4.0 µg drug group (N=10) on the final test used to assess fear-potentiated startle reinstatement when bilateral amygdaloid infusions had been carried out 24 hours earlier and just prior to the presentation of the 5 unsignalled footshocks.
13.5: Results and Discussion (Experiment 3C):

AP5 infusion into the Basolateral Amygdala Prior to 5 Unsignalled Reminder Footshocks and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats that have Undergone Fear Extinction Training.

13.5.1: Purpose of Experiment

The purpose of the present investigation was to determine whether amygdaloidal NMDA receptors are involved in facilitating the reinstatement of FPS. As was previously discussed, research has shown that blockade of NMDA receptors in the amygdala with AP5 prevents the acquisition of FPS in rats (Miserendino, et al., 1990; Campeau, et al., 1992; Walker and Davis, 2000; 2002; also see Chapter 6). Furthermore, electrophysiological studies in conjunction with pharmacologically-based experimentation have highlighted the importance of NMDA receptors in LTP induction, synaptic transmission, and conditioned fear learning and expression (Gean, et al., 1993; Li, et al., 1995; Maren and Fanselow, 1995; McKernan and Shinnick-Gallagher, 1997; Zinebi, Mckernan and Shinnick-Gallagher, 2002; Malenka and Nicoll, 1993; Malenka, Kauer, Perkel, and Nicoll, 1989; Reymann, et al., 1989; Miserendino, et al., 1990; Campeau, et al., 1992; Walker and Davis, 2000; Lee and Kim, 1998; Fanselow and Kim, 1994; Lee, et al., 2001). Recent experimental work conducted by Johnson and associates (2000) demonstrated that intraperitoneal injections of the NMDA receptor antagonist MK-801 (0.1 mg/kg, or 0.05 mg/kg) just prior to a series of 10 unsignalled footshocks dose-dependently blocked the reinstatement of the conditioned suppression of drinking response when rats were tested for conditioned fear 24 hours later in a non-drug state. Since antagonism of amygdaloid NMDA receptors block conditioned fear acquisition and expression (Miserendino, et al., 1990; Campeau, et al., 1992; Walker and Davis, 2000; Fanselow and Kim, 1994; Lee, et al., 2001) and peripheral administration of MK-801 disrupts the reinstatement of Pavlovian conditioned fear in fear-extinguished rats (Johnson, et al., 2000), the present experiment’s goal will be to determine whether intra-BLA infusion of AP5 (an NMDA receptor antagonist) prevents the reinstatement of FPS in fear-extinguished rats.
13.52: Subjects

A total of thirty-six rats were used to evaluate the effect of intraamygdalar AP5 infusion on the reinstatement of FPS. Twelve of the rats were infused with phosphate buffered saline (0.5 μl/side; pH= 7.4) and were the same phosphate buffered saline group use in Experiment 3A. The 24 remaining rats were divided equally into two different drug dose treatment conditions (2.5 μg, and 1.25 μg doses; N=12 each) and were infused with AP5. The infusions were administered to these fear-extinguished rats prior to being exposed to the 5 unsignalled footshocks. All particulars concerning the experimental design and protocols were the same as those described in the methods section for Experiment 2 and 3A. The design of the startle testing/fear conditioning apparatus were the same as described in Experiment 1A as were many of the surgical protocols used to implant guide cannulae above the basolateral nucleus of the amygdala. The only exception was that rats were given a sixteen day post-operative recovery period before any behavioural training or testing took place (see Experiment 3A).

13.53: Histological Results

Figures 68 to 70 display the location of guide cannulae placements along with the approximate position of the infusion cannulae in the amygdala during the AP5 infusions. Microscopic examination of the coronal brain sections (i.e. 50 μm in thickness) revealed that guide cannulae for rats belonging to the AP5 2.5 μg drug group were mainly situated in an area just dorsal to the rostral one third to one half of the basolateral amygdaloid complex (i.e. -1.60 mm to -2.30 mm posterior to bregma). In contrast, the guide cannulae location for the 1.25 μg AP5 group were more evenly spread out along the rostral to caudal extent of the basolateral complex (i.e. -1.80 mm to -3.14 mm posterior to bregma). It was found that in both AP5 drug groups, the guide cannula locations were clustered approximately 0.75 to 1.30 mm dorsal to the medial portions of the basolateral nucleus of the amygdala. As was the case in other experiments, the guide cannulae for rats belonging to this particular experiment seemed to be very accurately placed during surgery which meant that no animals were removed from the present study. This left the drug infusion groups in this experiment with N=12 each.
As can be seen from the schematic reconstruction of guide cannulae placements and infusion needle locations depicted in Figures 69 and 70, most AP5 would have diffused into the basolateral, lateral, and central amygdaloid nuclei, with some drug spreading to the dorsally situated amygdalostratal transition area and dorsal subdivision of the lateral nucleus. Additionally, some drug would have reached ventral portions of the basolateral complex and parts of the basomedial amygdaloid nucleus as well. It is also possible that some AP5 may have reached the main intercalated amygdaloid nucleus located slightly medial to the basolateral nucleus and ventral to central and lateral capsular subdivisions of the central amygdaloid nucleus (see Watson and Paxinos, 1998). Based on these histological results and the volume of drug infused (0.5 μl), it is highly probable that AP5 blocked numerous NMDA receptors residing in the basolateral complex before the 5 unsignalled reminder footshocks were administered (see Figures 68 to 70).
Figure 68. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the phosphate buffered saline (PBS) control group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 69. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the AP5 1.25 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 70. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the AP5 2.5 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
13.54: Experimental Results

ANOVA of baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the phosphate buffered saline (PBS) control group (N=12) to the two AP5 infusion drug groups (N=12 each) F(2,33)=0.54, p= 0.584 [n/s], indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 71). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result F(2,33)= 0.89, p=0.417 [n/s], demonstrating that the groups were matched on this variable (see Figure 72). ANOVA of the shock reactivity data obtained during fear conditioning revealed another non-significant result F(2,33)=0.86, p=0.432 [n/s], suggesting that all experimental groups showed similar levels of movement amplitude during CS + footshock administration (see Figure 73). Together, these findings suggest that rats in all three experimental groups exhibited similar levels of acoustic startle and defensive reflexive reactions (i.e. jumping and flinching) in response to white-noise bursts and footshocks respectively.
Baseline Acoustic Startle Responses

Figure 71. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the phosphate buffered saline (PBS) and the AP5 (1.25 µg and 2.5 µg) experimental groups (N=12; each).
White-Noise Intensity Level Used to Induce Baseline Startle

![Bar chart showing mean white-noise intensity levels (S.E.M. ±) recorded in decibels for PBS control, AP5 1.25 µg, and AP5 2.5 µg groups.](chart)

Figure 7.2. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that were used to induce stable acoustic startle responses for the phosphate buffered saline (PBS) group (N=12) and the two AP5 (1.25 and 2.5 µg) groups (N=12 each).
Shock Reactivity During Fear Conditioning

Figure 73. Mean (S.E.M. ±) movement amplitudes recorded during the administration of 30 light + footshock fear conditioning trials to rats in the phosphate buffered saline (PBS) and AP5 (1.25 μg and 2.5 μg) experimental groups (N=12; each).
13.55: Pretest Results

Results obtained from the pretest indicate that all three experimental groups exhibited FPS 48 hours after being administered 30 light + footshock fear conditioning trials. ANOVA (3 Drug X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1,33)=35.45, p<0.000002$, with no significant effect of Group, or Group X Stimulus Condition interaction. Newman-Keuls post hoc tests revealed that all 3 experimental groups had significantly higher mean acoustic startle scores on the 3 CS + noise trials than on the 3 noise-alone trials. These results are depicted in Figure 74, and clearly show that rats in all three drug treatment groups exhibited robust FPS responding after fear conditioning. Also, all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. Statistical analysis (i.e. ANOVA) of the difference score data (Difference Score= [3 light + noise trials]-[last 3 noise alone trials]) confirms that the three experimental groups had similar levels of fear $F(2,33)=0.15, p=0.853$ [n/s]. Figure 75 portrays the mean difference scores obtained from the pretest and clearly demonstrates that the PBS and two AP5 drug groups were closely matched on fear prior to any experimental manipulation involving extinction training, drug infusion, or exposure to 5 unsignalled footshocks.
Figure 74. Mean (S.E.M. ±) acoustic startle amplitudes obtained on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (★ P<0.05 relative to noise-alone trials). Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) highlight the equally robust levels of fear-potentiated startle found in all groups 48 hours after 30 fear conditioning trials and before extinction training and drug infusion occurred.

The experimental groups listed above include, the phosphate buffered saline group (N=12) and two AP5 groups (1.25 µg and 2.5 µg; N=12 each).
Pretest Fear-Potentiated Startle Difference Scores

![Graph showing Pretest Fear-Potentiated Startle Difference Scores]

Figure 75. Mean difference scores (S.E.M. ±) of the pretest ([CS + noise] - [noise-alone]) depict the equally robust levels of fear-potentiated startle found in the PBS (N=12) and AP5 (1.25 μg and 2.5 μg; N=12 each) drug groups 48 hours after 30 fear conditioning trials and before extinction training was initiated.
Infusion of the NMDA receptor antagonist AP5 (1.25 µg and 2.5 µg doses) into the BLA did not cause any disruptions in the ability of rats to exhibit reflexive responses to unsignalled footshocks. Statistical analysis (i.e. ANOVA) of the shock reactivity data obtained from the 5 unsignalled reminder shocks administered to rats after either PBS, 2.5 µg, or 1.25 µg AP5 infusion into the BLA yielded a non-significant result F(2,33)=0.51, p=0.603 [n/s]. Figure 76 displays the mean shock reactivity scores for each experimental group during the presentation of the 5 unsignalled footshocks. As depicted in Figure 76, there were no statistically significant differences between the PBS-infused and the AP5-infused rats in mean movement amplitudes in response to the unsignalled footshocks. These results seem to indicate that the AP5 infusion into the BLA had no effect on either the rat’s perception of or reactivity to footshock. Thus, AP5 did not appear to create any impairment in shock perception or defensive reflexes (i.e. jumping and flinching) when AP5-treated rats were exposed to the 5 unsignalled footshocks.
Reactivity to 5 Unsignalled Footshocks After Drug Infusion into the Amygdala

Figure 76. Mean (S.E.M. ±) movement amplitudes recorded during the presentation of 5 unsignalled footshocks to fear extinguished rats infused with either phosphate buffered saline (PBS) or 1.25 and 2.5 μg of AP5 into the basolateral amygdaloid complex (N=12 each).
The principal finding of this experiment was that AP5 infusion into the BLA blocked the reinstatement of FPS. A 3 Drug Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition $F(1,33)=23.48, p<0.00003$; and a significant Drug Group X Stimulus Condition interaction $F(2,33)=8.24, p<0.002$.

Newman-Keuls post hoc comparisons revealed that the PBS-infusion rats had significantly higher startle amplitudes on the 10 CS + noise trials than on the 10 noise-alone trials. In stark contrast to the PBS-control rats, the 2.5 µg and 1.25 µg AP5-infused rats failed to show a CS-induced startle effect on the final test. This was reflected by the Newman-Keuls analysis that showed there was no significant difference in startle amplitudes between the CS + noise trials and the noise-alone trials for the two AP5 drug groups (see Figure 77).

Furthermore, Newman-Keuls post hoc tests that examined the between-group differences of the Drug Group X Stimulus Condition interaction revealed that the PBS groups’ startle amplitudes on the CS + noise trials were significantly higher than those obtained by either the AP5 1.25 µg or AP5 2.5 µg group (see Figure 77 double star symbols). The results of this experiment are depicted in Figure 77 and they suggest that both doses of AP5 were equally effective in blocking the reinstatement of FPS when infused into the BLA prior to the 5 unsignalled shock presentations. This experimental effect is confirmed by statistical analysis carried out on the difference scores (Difference Score = $[10 \text{ CS + noise}] - [10 \text{ Noise-alone}]$).

Statistical analysis (ANOVA) of the difference scores yielded an effect of Drug Treatment $F(2,33)=8.24, p<0.002$ and as depicted in Figure 78 both doses of AP5 blocked the reinstatement of FPS on the final test when infused into the BLA prior to 5 unsignalled reminder shocks. Newman-Keuls post hoc tests of this data revealed that the magnitude of FPS exhibited by the PBS infusion group was significantly more robust than that expressed by either the 2.5 µg or 1.25 µg AP5 infusion groups. Thus, blockade of amygdaloid NMDA receptors with AP5 before exposure to unsignalled reminder footshocks prevents the restoration of Pavlovian conditioned fear responding in fear-extinguished rats. This indicates that NMDA amygdaloid receptors may be essential for initiating biochemical cascades that enhance fear-memory retrieval during times of extreme stress. In this way,
NMDA receptors may help reconsolidate or reconstruct CS-UCS fear associations that are inhibited or masked by extinction training. The blockade of NMDA receptors in the amygdala of fear-extinguished rats during and after the unsignalled footshock presentations may have disrupted excitatory synaptic transmission and intracellular cascades and effectively shut-down processes that normally would activate the neurons and pathways of the amygdala-based fear system (see General Discussion). This of course would mean that the amygdala was not able to conjure up the CS-UCS fear memories established during Pavlovian conditioning in the face of inhibitory learning. Consequently, FPS reinstatement was blocked in the AP5-treated rats, but present in the phosphate buffered saline group.
Figure 77. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear potentiated startle reinstatement as a function of infusion of PBS (N=12) and AP5 (1.25 μg and 2.5 μg; N=12 each) into the basolateral amygdala 24 hours earlier and prior to 5 unsignalled footshock presentation (★ P<0.05 relative to noise-alone trials). The mean difference scores ([10 CS + noise]-[10 noise-alone]) (S.E.M.±) demonstrate the impact of amygdaloidal AP5 infusion on the reinstatement of fear-potentiated startle († P<0.05 relative to the PBS control group). The double star symbols (‡) over the two AP5 groups denote a statistically significant between-group difference in startle on the CS + Noise trial type relative to the PBS group (§ P<0.05 relative to the PBS group's CS + Noise mean startle score).
Final Test Difference Scores for the Reinstatement of FPS

Figure 78. Mean difference scores (S.E.M. ± ) ([10 CS + noise]-[10 noise-alone]) for the PBS control and AP5 (1.25 and 2.5 µg) groups on the final test to assess the fear-potentiated startle reinstatement when bilateral basolateral amygdaloid infusions had been carried out 24 hours earlier and just prior to the presentation of 5 unsignalled footshocks ( ★ P<0.05 relative to the PBS control group; N=12 for each group).
13.6: Results and Discussion (Experiment 3D):

CNQX infusion into the Basolateral Amygdala Prior to 5 Unsignalled Footshocks and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats that Have Undergone Extinction Training

13.6.1: Purpose of Experiment

The purpose of the present investigation was to determine whether amygdaloidal AMPA receptors are involved in facilitating the reinstatement of FPS. As was previously discussed, research has shown that blockade of AMPA receptors in the amygdala with CNQX prevents the expression of FPS in rats (Kim, et al., 1993). Similarly, antagonism of AMPA receptors in the BLA via NBQX infusion blocks both FPS and light-enhanced startle (Walker and Davis, 1997b). Furthermore, electrophysiological studies in conjunction with pharmacologically based experimentation have demonstrated the importance of amygdaloidal AMPA receptors in LTP induction, synaptic transmission and fear learning, and fear memory retrieval (Gean and Chang, 1992; Li, et al., 1995; Rogan, et al., 1997b; McKernan and Shinnick-Gallagher, 1997; Mahanty and Sah, 1998; Walker and Davis, 1997b).

Despite these findings, the contribution of AMPA receptors to fear reinstatement has not been explored scientifically. This is somewhat perplexing as it is quite possible that AMPA-mediated synaptic transmission may set off biochemical cascades and events that make it possible for organisms to reconstruct labile CS-UCS associations that in turn make fear reinstatement and expression possible. Because AMPA amygdaloid receptor-mediated events have been shown to contribute to conditioned fear learning and expression, it is a worthy research endeavour to evaluate the role of AMPA amygdaloid receptors in the reinstatement of FPS. Hence the aim of this experiment is to determine if the blockade of AMPA receptors located in the amygdala would prevent the restoration of FPS in fear-extinguished rats. This research is valuable in that it may provide some insight into how glutamatergic synaptic transmission processes in the human brain may be involved in the retrieval of traumatic fear memories that have been displaced either by the passage of time or by cognitive behavioural therapies that contain elements of extinction training built in to specially designed coping strategies.
13.62: Subjects

A total of thirty-six rats were used to assess the effects of bilateral intraamygdaloid CNQX infusion on the reinstatement of FPS. Twelve of the rats were infused with phosphate buffered saline (0.5 µl/side; pH= 7.4) and were the same phosphate buffered saline group used in Experiment 3A, 3B, and 3C. The 24 remaining rats were divided equally into two different drug dose treatment conditions (5.0 µg, and 2.5 µg doses; N=12 each) and were infused with CNQX. The infusions were administered to these fear-extinguished rats prior to being exposed to the 5 unsignalled footshocks. All particulars concerning the experimental design and protocols were the same as those described in the methods section for Experiment 2 and 3A. The design of the startle testing/fear conditioning apparatus were the same as described in Experiment 1A as were many of the surgical protocols used to implant guide cannulae above the basolateral nucleus of the amygdala. The only exception was that rats were given a sixteen day post-operative recovery period before any behavioural training or testing took place (see Experiments 1A, 2 and 3A for a review).

13.63: Histological Results

Microscopic evaluation of coronal sections taken from the brains of rats belonging to the two CNQX drug groups revealed that the location of guide cannulae and infusion needles was very similar to that observed in the previous experiments (3A to 3C). In general, most guide cannulae were located just dorsal to the medial portions of the basolateral nucleus of the amygdala with a few located more medially over the lateral subdivision of the central nucleus of the amygdala. These histological results are depicted in Figures 79 to 81 and are schematic reconstructions of the guide cannulae and infusion needle placements of the PBS and two CNQX drug groups. Figures 79 to 81 were constructed with the aid of coronal figure sections taken from the rat brain atlas of Paxinos and Watson (1986). As can be seen guide cannulae were implanted accurately during stereotaxic surgery and most cannulae are located between 1.80 mm to 3.14 mm posterior to bregma in a region just dorsal to the basolateral and lateral portions of the central amygdaloid nuclei. Based on the histological results, it was decided that no animals should be excluded from the experiment. This
meant that all drug treatment groups were left with an equal number of subjects (N=12) for
the series of statistical analyses that were to follow.

Figures 80 and 81 depict the location of the guide cannulae and approximate position
where the infusion cannulae would have been situated for the CNQX intra-amygdaloid
infusion groups. Based on the location of the infusion needles it would be highly probable
that the volume of CNQX used in this study (i.e. 0.5μl/hemisphere) would have diffused
into the basolateral, lateral, and central amygdaloid nuclei, with some drug spreading to the
amygdalostrial transition area. The aqueous CNQX solution would have also reached
ventral parts of the basolateral amygdaloid complex and regions of the basomedial
amygdaloid nucleus. Also, some of the CNQX may have reached the main intercalated
amygdaloid nucleus that lies medial and ventral to the basolateral and central amygdaloid
nuclei respectively. At any rate, it is quite probable that the CNQX infusion (5.0 μg and 2.5
μg) blocked large quantities of AMPA receptors residing in the basolateral amygdaloid
complex before rats were administered the 5 unsignalled footshocks. This would mean that
any attenuation of FPS reinstatement on the final test could be attributed to the antagonistic
action of CNQX on amygdaloid AMPA receptors.
Figure 79. Schematic depictions of guide cannula locations represented as filled circles ( ● ) on the left and the approximate location of infusion needles represented as open circles ( ○ ) on the right for the phosphate buffered saline (PBS) control group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 80. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the CNQX 2.5 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 81. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the CNQX 5.0 μg group (N=12) of the fear-potentiated startle reinstatement study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
13.64: Experimental Results

ANOVA of baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the phosphate buffered saline (PBS) control group (N=12) to the two CNQX infusion drug groups (N=12 each) F(2,33)=0.527, p= 0.595 [n/s], indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 82). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result F(2,33)= 0.188, p=0.828 [n/s], indicating that the groups were matched on this measure (see Figure 83). ANOVA of the shock reactivity data obtained during Pavlovian fear conditioning revealed another non-significant result F(2,33)=2.04, p=0.145 [n/s], suggesting that all experimental groups showed similar levels of movement amplitude during CS + footshock administration (see Figure 84).
Baseline Acoustic Startle Responses

Figure 82. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the phosphate buffered saline (PBS; N=12) and the CNQX groups (2.5 and 5.0 μg; N=12 each).
White-noise Intensity Used to Induce Baseline Startle Responding

Figure 83. Mean white-noise levels (S.E.M. ± ) recorded in decibels that were used to induce stable acoustic startle responding for the phosphate buffered saline (PBS) group (N=12) and the two CNQX (2.5 and 5.0 µg) groups (N=12 each).
Shock Reactivity During Fear Conditioning

Figure 84. Mean (S.E.M. ±) movement amplitudes recorded during the administration of 30 light + footshock fear conditioning trials to rats in the phosphate buffered saline (PBS; N=12) and the CNQX groups (2.5 and 5.0 µg; N=12 each).
The pretest results confirm that all three experimental groups displayed robust FPS 48 hours after 30 light + footshock pairings. ANOVA (3 Drug Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1,33)=33.04$, $p<0.000003$, with no significant effect of Group, or Group X Stimulus Condition interaction. Newman-Keuls post hoc tests revealed that all groups showed higher mean acoustic startle scores on the 3 CS + noise trials than on the 3 noise-alone trials. These results are depicted in Figure 85 and clearly show that rats in all three drug treatment groups displayed robust FPS after Pavlovian fear conditioning. Also, all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction.

ANOVA of the difference score data (Difference Score= [3 light + noise trials]-[last 3 noise-alone trials]) further confirms that the three experimental groups had similar levels of fear $F(2,33)=0.064$, $p=0.938$ [n/s]. This nonsignificant finding based on the difference score analysis indicates that all three groups had similar levels of conditioned fear after exposure to the 30 light + shock trials. Figure 86 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the PBS and two CNQX drug groups were closely matched on fear prior to any experimental manipulation involving extinction training, drug infusion, or exposure to 5 unsignalled footshocks.
Pretest Results of FPS After Fear Conditioning

Figure 85. Mean (S.E.M. ±) acoustic startle amplitudes obtained on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (★ P<0.05 relative to noise-alone trials). Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) display the equally robust levels of fear-potentiated startle found in all groups 48 hours after 30 fear conditioning trials and before extinction training occurred. The experimental groups listed above include, the phosphate buffered saline group (N=12) and two CNQX groups (2.5 μg and 5.0 μg; N=12 each).
Figure 86. Mean difference scores (S.E.M. ±) of the pretest ([CS + noise] - [noise-alone]) depict the equally robust levels of fear-potentiated startle found in the phosphate buffered saline (PBS; N=12) and CNQX (2.5 µg and 5.0 µg; N=12 each) experimental groups 48 hours after 30 fear conditioning trials and before extinction training commenced.
13.66: Reflexive Responding to Unsignalled Footshocks

Infusion of the AMPA receptor antagonist CNQX (5.0 μg and 2.5 μg doses) into the BLA did not cause any disruptions in the ability of rats to exhibit reflexive responses to unsignalled footshocks. Statistical analysis (ANOVA) of the shock reactivity data obtained from the 5 unsignalled reminder shocks administered to rats after either PBS, 5.0 μg, or 2.5 μg CNQX infusion into the BLA yielded a non-significant result F(2,33)=0.570, p=0.571 [n/s]. This result indicates that the CNQX infusion into the basolateral complex had no effect on either the perception of or the reactivity to footshock (see Figure 87). As depicted in Figure 87, there were no statistically significant differences between the PBS-infused and the CNQX-infused rats in mean movement amplitudes in response to the unsignalled footshocks. Thus, CNQX application to the amygdala did not appear to create any impairment in shock perception or defensive reflexes (i.e. jumping and flinching) when CNQX-treated rats were exposed to the 5 unsignalled footshocks.

Interestingly, however, CNQX-infused rats seemed to urinate and defecate less in response to the 5 unsignalled footshocks than did the PBS and raclopride-treated rats. Although bolus and urine amounts were not measured and quantified for any of the experiments listed above, the limited amount of urination and defecation exhibited by the CNQX-treated animals may indicate that AMPA or kainate amygdaloid receptors help regulate certain sympathetic and parasympathetic nervous system responses that occur during highly charged and emotionally arousing situations. Whether the lack of urination and the low bolus count reflects reduced fear levels during unsignalled shock exposure is difficult to determine with any degree of certainty especially since the CNQX-rats did exhibit shock-induced movement amplitudes that were not significantly different to those displayed by the PBS control group (see Figure 87).
Reactivity to 5 Unsignalled Footshocks After Intra-Amygdalar Drug Infusion

Figure 87. Mean (S.E.M. ±) movement amplitudes recorded during the presentation of 5 unsignalled footshocks to fear extinguished rats infused with either phosphate buffered saline (PBS) or CNQX (2.5 and 5.0 µg) into the basolateral amygdaloid complex (N=12 for each group).
13.67: Final Test Results and Discussion

The main finding of this experiment was that CNQX infusion into the basolateral region of the amygdala blocked the reinstatement of FPS in fear-extinguished rats. A 3 Drug Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition $F(1,33)=30.28$, $p<0.000005$; and a significant Drug Group X Stimulus Condition interaction $F(2,33)=7.95$, $p<0.002$. Newman-Keuls post hoc comparisons revealed that the PBS-infusion rats had significantly higher startle amplitudes on the CS + noise trials than on the noise-alone trials. However, this was not the case for the CNQX 5.0 μg and 2.5 μg drug groups as the Newman-Keuls analysis failed to find any significant differences between the CS + noise and the noise-alone trials for these two groups. Thus, in contrast to the PBS control rats, the 5.0 μg and 2.5 μg CNQX-infused rats failed to show a CS-induced startle effect on the final test (see Figure 88 and 89).

Nevertheless, it is important to note that Newman-Keuls analysis used to compare the CS + noise to the noise-alone trials for the 2.5 μg CNQX group approached significance ($p=0.0529$), indicating that the lower dose of CNQX just blocked the reinstatement of FPS. However, post hoc analysis also revealed that the noise-alone mean startle score for the 2.5 μg CNQX group (Mean= 151.1; SEM= ± 31.8; SD=110.1; N=12) was significantly lower than the noise-alone mean startle score for the PBS group (Mean= 285.7; SEM= ± 82.0; SD= 284.1; N=12) on the final test. As can be seen in Figure 88, overall acoustic startle responding on the final test for both CNQX drug groups does seem to be depressed relative to the PBS control group. As a result, it is plausible that CNQX administration 24 hours before final testing may have lowered startle reflexive responding (i.e. lowering of the startle ceiling). This may have allowed more latitude for the CNQX 2.5 μg group to exhibit an upward increase on the CS + noise trials since the noise-alone responses were a bit lower than usual. The only difficulty with this interpretation is that it is difficult to imagine how CNQX would only affect noise-alone startle and not CS + noise startle on the final test. One possible explanation is that the CNQX 2.5 μg group's noise-alone startle response was lower than usual even during the pretest before any drug had been administered (see Figure 85). Thus, this particular group may have naturally had low noise-alone startle responsiveness to begin with but whether this had any effect on the
outcome during final testing for FPS is difficult to answer without running further experiments.

With respect to the significant Drug Group X Stimulus Condition interaction, Newman-Keuls post hoc tests that compared between-group differences revealed that the mean startle response on the CS + noise trials for the PBS group were significantly higher than the startle scores attained by both the CNQX 5.0 µg and the CNQX 2.5 µg groups on this particular stimulus type (see Figure 88 double star symbols). This finding suggests that the PBS group showed appreciably higher levels of fear reinstatement than either CNQX drug groups. As a whole, these results demonstrate that both doses of CNQX were effective in blocking the reinstatement of FPS when they were infused into the BLA prior to the 5 unsignalled shock presentations however, the 2.5 µg dose was generally less effective than the 5.0 µg dose. The data comparing the PBS control group to the two CNQX drug doses are displayed in Figure 88 and clearly demonstrate that bilateral CNQX infusion into the BLA prior to 5 unsignalled reminder shocks prevents the reinstatement of FPS in rats that experienced extinction training.

13.68: Final Test Difference Score Results

Statistical analysis (i.e. ANOVA) of the difference scores yielded an effect of Drug Treatment F(2,33)=7.95, p<0.002 and as depicted in Figure 89 both doses of CNQX blocked the reinstatement of FPS on the final test when infused into the BLA prior to 5 unsignalled reminder shocks. Newman-Keuls post hoc tests of this data revealed that the level of FPS exhibited by the saline infusion group was significantly more robust than either the 5.0 µg or 2.5 µg CNQX drug infusion groups. Taken together, these results strongly suggest that blockade of AMPA and kainate amygdaloid receptors by CNQX infusion prior to 5 unsignalled reminder footshocks prevented the reinstatement of FPS in rats that previously received fear-extinction training.
Figure 88. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and the 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of infusion of PBS (N=12) or CNQX (2.5 and 5.0 μg; N=12 each) into the basolateral amygdala 24 hours earlier and just prior to the presentation of 5 unsignalled footshocks (☆ P<0.05 relative to noise-alone trials). The mean difference scores ([10 CS + noise]-[10 noise-alone]) (S.E.M. ±) demonstrate the impact of amygdaloidal CNQX infusion on the reinstatement of fear-potentiated startle (☆ P<0.05 relative to the PBS control group). The double star symbols (★★) over the two CNQX drug groups denote a statistically significant between-group difference in startle on the CS + Noise trial type relative to the PBS group (★★ P<0.05 relative to the PBS group's CS + Noise mean startle score).
Figure 89. Mean difference scores (S.E.M. ±) ([10 CS + noise]-[10 noise-alone]) for the PBS control and CNQX (2.5 and 5.0 μg) groups on the final test to assess fear-potentiated startle reinstatement when bilateral basolateral amygdaloid infusions had been carried out 24 hours earlier and just prior to the presentation of 5 unsignalled footshocks (★ P<0.05 relative to the PBS control group: N=12 for each experimental group).
14. Experiment 4: 
Expression Control Experiments for Fear-Potentiated Startle Reinstatement Study: 
Testing for Drug Effects on Fear Expression

14.1: Rationale

Although raclopride, AP5, and CNQX appeared to have blocked the reinstatement of FPS, it is conceivable that the drug infused into the BLA before the 5 unsignalled shocks may not have been completely metabolized and may have still been present in sufficient quantities 24 hours later when the final testing occurred. It is therefore possible, that these drugs may have blocked the capacity for fear expression rather than blocking fear reinstatement. To test this possibility, it was necessary to run a series of control groups to examine whether an intraamygdalar drug infusion 24 hours prior to final testing would block fear expression in rats that did not receive extinction training but did still receive the 5 unsignalled footshocks. Thus, Experiment 4 was designed to determine whether intraamygdaloid infusions of PBS, raclopride (8.0 µg), AP5 (1.25 µg), or CNQX (5.0 µg and 2.5 µg) twenty-four hours before final testing in non-fear-extinguished rats would prevent the expression of FPS.

14.2: Method

14.21: Subjects

A total of 48 naive, male Wistar rats bred and housed in the Psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 340 grams at the beginning of the experiment (range 265-368). A constant temperature of 20° Celsius (±1° Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulations occurred during the light portion of this cycle.
14.22: Procedure

All particulars concerning startle apparatus and stereotaxic surgery are the same as those described in Experiment 1A. The baselining, fear conditioning, pretesting, group assignment, drug infusion, 5 unsignalled footshock presentation, and fear testing were identical to the procedures described in Experiments 2, 3A, 3B, and 3C. The only key difference in experimental procedure for this particular experiment was that the rats did not receive extinction training. Thus, following pretesting rats were assigned to one of four drug treatment groups (N= 8; each) such that all groups had similar baseline startle responding and levels of FPS. Seventy-two hours after the pretest, rats received bilateral infusions into the BLA of either phosphate buffered saline (0.5 μl/side; pH= 7.4), raclopride (8.0 μg, dose), AP5 (1.25 μg, dose), CNQX (2.5 μg, dose) or CNQX (5.0 μg, dose).

These particular drug doses were chosen because they all appeared to be effective in blocking the reinstatement of FPS in rats tested in Experiments 3A, 3C, and 3D. Also it was decided that both CNQX doses (i.e. 2.5 μg and 5.0 μg) should be run in this experiment, since it was shown that the CNQX 2.5 μg dose seemed to dampen noise-alone startle and only just block the reinstatement of FPS. Hence, the CNQX 5.0 μg dose was used as a challenge dose that was designed to evaluate its effects on fear expression and acoustic startle responding when it was infused 24 hours prior to final testing. Essentially this experimental protocol made it possible to not only evaluate the effective life of CNQX, but it also helped to determine whether the infusions of CNQX described in experiment 3D actually blocked fear-reinstatement or simply blocked fear expression. The inclusion of the CNQX 5.0 μg group also made it possible to see if blockade of AMPA/kainate amygdaloid receptors 24 hours before final testing would produce an overall dampening of acoustic startle responding. This of course would be of some interest since noise-alone startle responses in the CNQX groups of Experiment 3D seemed a bit depressed on the final test relative to the PBS controls.

Following drug or PBS infusion, rats were administered the 5 unsignalled footshocks with a 60 s intershock interval. Twenty-four hours after the infusion rats were administered the final test to determine whether or not the drug infusion 24 hours earlier would block the expression of FPS. Rats that are fear conditioned and receive no extinction training normally exhibit robust levels of FPS (see Experiment 2; also see Davis, 1992a; Walker
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and Davis, 2002; Davis, et al., 2003). Thus, any reductions in FPS on the final test may indicate that the drugs were still present and affecting neurochemical events in the amygdala that are involved in memory retrieval and fear expression.

14.23: Histology Results

One animal in the PBS control group developed a loose head cap and was excluded from the study. One animal belonging to the CNQX 2.5 µg drug group had cannula placements which missed the desired target area above the basolateral complex and was excluded from the study. Also, one rat that belonged to the raclopride 8.0 µg group was found dead in its home cage before perfusions were carried out. This left the PBS control group, the CNQX 2.5 µg group, and the raclopride 8.0 µg group all with an N= 7 animals each. The CNQX 5.0 µg group and the AP5 1.25 µg group were both left with N= 8 rats. It was also noticed that the cannulae placements in the CNQX drug groups were slightly more ventral which would mean that some drug may have diffused to ventral portions of the BLA and to dorsal portions of the basal medial amygdaloid nucleus. Some guide cannulae were also found a little more medial in an area just above the amygdalo striatal transition area. This would mean that the infusion cannulae would be located between the lateral subdivision of the central nucleus of the amygdala and the basolateral complex. Thus, the drug may have spread not only to the basolateral complex but also to portions of the central amygdaloid nucleus and intercalated amygdalar nuclei. Some drug may have also diffused upward to the amygdalo striatal transition area. Nevertheless, most guide cannula were positioned such that infusion needles and drug infusions would have targeted the amygdaloid nuclei that have been shown to be involved in fear acquisition and expression, these being the lateral, basolateral and central amygdaloid nuclei and their corresponding subdivisions. Figures 90 to 94 depict the location of guide cannulae placements in a region above the basolateral complex and the approximate location of the infusion needles 1.0 mm below the guides during PBS or drug (raclopride 8.0 µg; AP5 1.25 µg; CNQX 2.5 µg; and CNQX 5.0 µg) infusions.
Figure 90. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the phosphate buffered saline (PBS) control group (N=7) of the expression control study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom: - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 91. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the raclopride 8.0 μg group (N=7) of the expression control study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 92. Schematic depictions of guide cannula locations represented as filled circles (○) on the left and the approximate location of infusion needles represented as open circles (□) on the right for the AP5 1.25 μg group (N=8) of the expression control study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ±4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 93. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the CNQX 2.5 µg group (N=7) of the expression control study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 94. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the CNQX 5.0 µg group (N=8) of the expression control study. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Experimental Results

ANOVA of baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the phosphate buffered saline (PBS) control group (N=7) to the four distinct drug infusion groups (raclopride 8.0 μg, N=7; AP5 1.25 μg, N=8; CNQX 5.0 μg, N=8; and CNQX 2.5 μg, N=7) F(4,32)=0.348, p=0.8433 [n/s]. This indicates that the groups had been properly matched and had similar baseline acoustic startle responding (see Figure 95). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result F(4,32)=1.65, p=0.1844 [n/s], demonstrating that the groups were properly matched on this variable (see Figure 96). ANOVA of the shock reactivity data obtained during fear conditioning yielded another non-significant result F(4,32)=1.59, p=0.1988 [n/s], suggesting that all experimental groups showed similar levels of movement amplitude during CS + footshock administration. These shock reactivity results are presented in Figure 97.
Baseline Startle for Expression Control Experiment

Figure 95. Mean (S.E.M. ± ) baseline startle amplitudes recorded on the third baselining block for the phosphate buffered saline (PBS; N=7), raclopride 8.0 μg (N=7), AP5 1.25 μg (N=8), CNQX 2.5 μg (N=7) and CNQX 5.0 μg (N=8) drug groups of the expression control experiment.
White-noise Intensity Used to Induce Startle Responding

Figure 96. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that were used to induce stable acoustic startle responses for the phosphate buffered saline (PBS; N=7), raclopride 8.0 μg (N=7), AP5 1.25 μg (N=8), CNQX 2.5 μg (N=7) and the CNQX 5.0 μg (N=8) drug groups of the expression control experiment.
Shock Reactivity During Fear Conditioning: Expression Control Experiment

Figure 97. Mean (S.E.M. ±) movement amplitudes recorded in response to footshocks presented during the administration of 30 light + footshock fear conditioning trials to rats belonging to the phosphate buffered saline (PBS); N=7, raclopride 8.0 μg (N=7), AP5 1.25 μg (N=8), CNQX 2.5 μg (N=7) and CNQX 5.0 μg (N=8) drug groups of the expression control experiment.
14.25: Pretest Results

The pretest results revealed that high levels of FPS was present in all experimental groups 48 hours after light + footshock fear conditioning had been administered. ANOVA (5 Drug Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition F(1,32)= 79.69, p<0.000001, with no significant effect of Group, or Group X Stimulus Condition interaction. These results are depicted in Figure 98, and clearly show that rats in all five drug treatment groups displayed robust FPS after fear conditioning. Newman-Keuls post hoc comparisons of these data revealed that all groups exhibited higher mean startle amplitudes on the 3 CS + noise trials than on the 3 noise-alone trials of the pretest, indicating that 30 light + shock training trials were sufficient to produce conditioned fear. This finding is supported by the fact that all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. ANOVA of the difference score data (Difference Score=[3 light + noise trials]-[last 3 noise alone trials]) further confirms that the five experimental groups had similar levels of fear F(4,32)=0.275, p=0.8914 [n/s]. Figure 99 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the PBS and other drug groups were closely matched on fear prior to any experimental manipulation involving either drug infusion, or exposure to 5 unsignalled footshocks.
Pretest FPS Scores After Fear Conditioning

Figure 98. Mean (S.E.M. ±) acoustic startle amplitudes for the phosphate buffered saline (PBS; N=7), raclopride 8.0 µg (N=7), AP5 1.25 µg (N=8), CNQX 2.5 µg (N=7) and CNQX 5.0 µg (N=8) drug groups on 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only ( * P<0.05 relative to noise-alone trials). The mean difference scores (S.E.M. ± ) of the pretest ([3CS + noise]-[3 noise-alone]) depict the nearly equivalent and robust magnitude of fear-potentiated startle expressed by the drug groups of the expression control study 48 hours after Pavlovian fear conditioning.
Figure 99. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise] - [3 noise-alone]) depict the nearly equivalent and robust levels of fear exhibited by the phosphate buffered saline (PBS; N=7), raclopride 8.0 μg (N=7), AP5 1.25 μg (N=8), CNQX 2.5 μg (N=7) and CNQX 5.0 μg (N=8) drug groups of the expression control study 48 hours after Pavlovian fear conditioning.
14.26: Reflexive Responding to Unsignalled Footshocks

Statistical analysis (i.e. ANOVA) of the shock reactivity data obtained from the 5 unsignalled reminder shocks administered to rats after either PBS, raclopride, AP5 or CNQX infusion into the BLA yielded a non-significant result $F(4,32)=0.775, p=0.549$ [n/s]. This result indicates that infusion with any of these drugs had no effect on either the perception of or the reactivity to footshock as the 5 drug groups did not differ from each other in the level of shock-induced movement amplitude recorded during shock administration. One interesting behavioural observation was that CNQX-infused rats once again did not seem to urinate or defecate as much as the PBS or other drug groups in response to unsignalled footshock administration. As will be remembered, CNQX infusion into the BLA produced a similar effect on urination and defecation levels in Experiment 3D.

Each experimental group's reactivity to the 5 unsignalled footshocks is represented graphically in Figure 100 and as can be seen all groups displayed equally high levels of responsiveness (i.e. jumping and flinching) while being exposed to the aversive footshocks. This indicates that intra-amygdaloid application of raclopride, AP5, or CNQX did not alter reflexive responses induced by footshock. This is supported by the fact that the PBS control group's shock-induced movement amplitude is not statistically different from the other 4 experimental drug groups.
Expression Control Study: Reactivity to 5 Unsignalled Footshocks After Intra-Amygdalar Drug Infusion

Figure 100. Mean (S.E.M. ±) movement amplitudes recorded in response to the presentation of 5 unsignalled footshocks to non-extinguished rats of the expression control study after the bilateral infusion of phosphate buffered saline (PBS; N=7), raclopride (8.0 µg; N=7), AP5 (1.25 µg; N=8) and CNQX (2.5 µg and 5.0 µg; N=7 and N=8 respectively) into the basolateral amygdaloid complex.
14.27: Final Test Results and Discussion

A 5 Drug Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition $F(1,32)=34.61, p<0.000003$. Newman Keuls post hoc comparisons used to evaluate the main effect of Stimulus Condition revealed that only the PBS, AP5 1.25 μg, and Raclopride 8.0 μg amygdaloid-infused rats had significantly higher startle amplitudes on the CS + noise trials than on the noise-alone trials on the final test. This finding indicates that all 3 of these drug groups did not differ significantly and as such exhibited similar levels of fear expression on the final test.

However, the same cannot be said about the CNQX drug groups, since their levels of noise-alone and CS + noise startle seemed dampened on the final test (see Figure 101). Although both CNQX drug groups (i.e. 2.5 μg and 5.0 μg) did show substantial increases on the CS + noise trials over the noise-alone trials on the final test, the Newman Keuls analysis failed to find a significant difference between these trial types for these two drug treatment groups. At a glance, this result indicates that CNQX infusion just before the 5 unsignalled shocks and 24 hours prior to final testing leads to impairments in the expression of FPS in non-extinguished rats. As a consequence, the CNQX infusions made during Experiment 3D may have blocked fear expression rather than fear reinstatement. Nevertheless, caution must be taken before making a hasty interpretation since statistical analysis (i.e. ANOVA) of the difference scores that measure the magnitude of the fear effect (i.e. [10 CS + noise trials minus 10 noise-alone trials]) yielded a non-significant effect of Drug Treatment $F(4,32)=1.08, p=.381$ [n/s]. Newman-Keuls analysis of the difference scores indicate that the drug treatment groups are not significantly different from each other on the magnitude of FPS expressed during the final test. The Newman-Keuls post hoc test found no significant between group differences when the mean difference scores were compared for all five infusion groups. These results are depicted in Figure 102 and indicate that raclopride and AP5 infusion into the amygdala definitely do not interfere with fear expression when made 24 hours prior to final testing.

The CNQX results are more difficult to interpret but they seem to suggest blockade of AMPA/kainate amygdaloid receptors 24 hours prior to final testing produces a robust dampening of FPS expression, but it does not totally block the expression of this fear
response. Moreover, the noise-alone startle responses for the CNQX drug infusion groups were reduced on the final test, suggesting that rats may have had a drug induced performance deficit that reduced their capacity to startle. The problem with this interpretation is that an ANOVA of the noise-alone startle responses failed to find any significant between group differences {F(4,32)=1.04, p=0.3977, [n/s]}. Taking this into account it appears as though CNQX may inhibit AMPA receptors and disrupt other biochemical fear-retrieval mechanisms for a prolonged period of time. In this way CNQX may function to suppress FPS at time points long after the drug infusion has taken place.
Final Test Scores for the Expression of FPS

Figure 101. Mean (S.E.M. ±) acoustic startle amplitudes for non-fear-extinguished rats of the expression control study on the 10 noise-alone and 10 CS + noise trials of the final fear-potentiated startle test as a function of infusion of phosphate buffered saline (PBS; N=7), raclopride (8.0 μg; N=7), AP5 (1.25 μg; N=8) and CNQX (2.5 μg; N=7 and 5.0 μg; N=8) into the basolateral amygdaloid complex 24 hours earlier and just prior to 5 unsignalled footshock presentations ( * P<0.05 relative to the noise-alone trials). With the exception of CNQX, the mean (S.E.M. ±) difference scores ([10 CS +noise]-[10 noise-alone]) of the final test demonstrate that intra-amygdaloid infusions of PBS, raclopride and AP5 24 hours before final testing does not diminish the capacity of non-fear-extinguished rats to express fear to a specific CS.
Expression Control Study: Final Test FPS Difference Scores

![Bar graph showing difference scores for various drug groups.](image)

Figure 102. Mean (S.E.M. ± ) difference scores ([10 CS + noise] - [10 noise-alone]) on the final test for non-fear-extinguished expression control study rats that received bilateral intra-amygdaloid infusions of phosphate buffered saline (N=7), raclopride (8.0 µg; N=7), AP5 (1.25 µg; N=8) and CNQX (2.5 µg and 5.0 µg; N=7 and N=8 respectively) and 5 unsignalled footshocks 24 hours before testing for the expression of fear potentiated startle responding.
Thus although, the raclopride and AP5 drug groups showed levels of fear expression similar to that exhibited by the PBS group, it is important to note that the level of fear expression was significantly reduced in the 2.5μg and 5.0 μg CNQX infusion groups relative to the other drug treatment groups, with the 5.0 μg dose being more effective in blocking fear expression than the 2.5 μg dose. This indicates that CNQX may have the capacity to interfere with synaptic transmission processes at AMPA/ kainate receptors within the amygdala for longer periods of time, and in doing so, may block fear reinstatement and/or fear expression. This seems plausible since CNQX infusion into the BLA has been shown to block fear expression (Kim, Campeau, Falls, and Davis, 1993) and AMPA receptors are known to influence synaptic transmission and LTP processes in the amygdala and hippocampus (Wang, et al., 2002; Davis, Rainnie and Cassell, 1994; Rainnie, Asprodini, and Shimnick-Gallagher, 1991b; Li, et al., 1995; Maren and Fanselow, 1995; Gean and Chang, 1992; Wang, Wilson and Moore, 2001; Mahanty and Sah, 1998). Alternatively, CNQX infusions into the basolateral amygdaloid complex may have had the effect of lowering startle responsiveness in general since the two CNQX drug groups did have lower noise-alone startle responses. However, it must be emphasised that an ANOVA of the 10 noise-alone startle responses on the final test and the Newman-Keuls post hoc tests found no significant between group differences on this measure, indicating that CNQX infusion did not significantly decrease noise-alone startle but may have only dampened this response \(F(4,32)=1.04\ p=0.3977\). Another plausible alternative is that CNQX infusions prior to the administration of 5 unsignal1ed footshocks in non-extinguished rats could have interfered with the consolidation of contextual fear memories and nociceptive memories associated with footshock. This drug effect could have altered or interfered with the animals' ability to direct their attention towards biologically significant contextual and conditioned cues during the final test, thus making it difficult for animals to reconsolidate and retrieve stable CS-UCS associative memories. This is particularly important since it has been recently shown that fear-memories are labile during the period of reconsolidation and retrieval (LeDoux, 2002). It is therefore quite conceivable that the presence of CNQX in the BLA of non-extinguished animals during 5 unsignal1ed footshock administrations may have had an interfering effect on memory reconsolidation that carried over to the final test, thus making
the retrieval of a stable CS-UCS memory-trace difficult. This process could have made it difficult for CNQX-infused animals to discriminate between contextual memories and cues and CS specific memories and cues. This deficit may have contributed to the reduced magnitude of fear expression observed in the two CNQX drug treatment groups on the final test. In essence, the presentation of 5 unsignalled footshocks could have combined with the interfering effects of CNQX to destabilise CS-UCS fear memories and as a result block fear expression during the final test.

The role of AMPA/kainate receptors and their involvement in synaptic transmission processes and fear retrieval and expression will be examined in more detail in the general discussion section, but for now, it is sufficient to state that the results of this expression control experiment demonstrate that intra-amygdalar infusions before 5 unsignalled footshocks and 24 hours prior to final testing did not interfere with fear expression when raclopride and AP5 were infused into the BLA of non-extinguished rats. The CNQX results are more difficult to interpret for a couple of reasons. First they suggest that CNQX infusions made 24 hours prior to final testing blocks the expression of FPS by causing a drug induced performance deficit. Second, the results could also suggest that CNQX plus 5 unsignalled shocks somehow combined to interfere with the accuracy of reconsolidation and fear-memory retrieval thus impairing the level of fear expressed during final testing. To more accurately examine the effects of CNQX on fear expression, and to determine whether CNQX infusion produces performance deficits or consolidation/retrieval difficulties a control group using rats that did not undergo fear-extinction training was carried out. The only difference in the experimental protocol used this time was that rats were infused only with a high dose of CNQX (i.e. 5.0 μg) and they received no unsignalled footshocks after the intra-amygdalar drug treatment. This experimental designs’ goal was to remove the variable of 5 unsignalled footshocks out of the equation so as to better assess the effects of CNQX independently. The experimental protocol and results of this control study are presented below as Experiment 5.
15. Experiment 5:

CNQX Control Experiment for the Expression Control Study: Ruling out the Effects of Unsignalled Footshocks

15.1: Method

15.1.1: Subjects

Eight naïve male Wistar rats bred and housed in the Psychology Departments’ animal facility at the University of Canterbury served as subjects in this experiment. Rats weighed 350 grams at the beginning of the experiment. All details regarding the housing conditions, temperature, and food and water availability were the same as those described in previous experiments.

15.1.2: Surgery, Apparatus, and Experimental Procedure

The testing apparatus and surgical procedures and protocols are identical to those reported in Experiment 1A and 3A to D, and those mentioned in the expression control study referred to as Experiment 4. Sixteen days following surgery rats were assessed for baseline startle using 3 blocks of 20 white-noise bursts (30 s, ISI). Noise intensity levels (range 83.0 to 104.5 dB) that produced stable baseline startle were used in this experiment. Each rat was assigned a dB score based on white-noise intensity measurements taken after the last baselining block and this sound intensity level was used for all subsequent testing that followed. This procedure ensured that the variability in the noise-alone startle was effectively reduced.

Forty-eight hours after baselining all rats (N= 8) received 30 light + shock fear conditioning trials (60 s ISI) employing the same techniques described previously. Two days after fear conditioning rats were placed in their designated startle chambers and allowed 5 minutes to acclimatise to the testing apparatus. Rats were then exposed to 20 noise-alone trials followed by 3 CS (i.e. light) + noise trials. Means for the last 3 noise-alone startle responses were compared to the 3 CS + noise trials to determine the levels of FPS responding on the pretest.
Rat in this control study received no fear extinction training and were given a 72 hour rest period during which time no manipulation occurred. This was done in order to keep the amount of time normally used for experimentation similar across all experiments. After this 72 hour period elapsed, the eight rats for this control experiment received bilateral infusions of CNQX (i.e. a 5.0 μg dose) into the basolateral nucleus of the amygdala. This time rats were not administered the customary five unsignalled footshocks. Twenty-four hours after drug infusions, the rats (N= 8) were administered the final test to evaluate the effects of CNQX infusions on the level of fear expression. The final test was exactly the same as described in Experiments 2, 3A to D, and Experiment 4.

15.13: Results

Histological results indicate that guide cannulae in all eight rats were accurately implanted above the basolateral and central amygdaloid nuclei. Schematic representations of the guide cannula and approximate location of infusion needles for this particular CNQX control group are shown in Figure 103.

This experimental control group had baseline acoustic startle responding levels that were similar to those observed in Experiment 4. Also, the levels of movement or reactivity recorded during light + shock fear conditioning trials were not significantly different from the levels observed in Experiment 4. More importantly, the FPS on the pretest is not significantly different from that observed in the experimental groups found in Experiment 4. A dependent T-test comparing the 3 CS + noise trials to the 3 noise-alone trials for this particular control group (N= 8) indicates that mean startle responding was significantly higher on the 3 CS + noise trials than on the 3 noise-alone trials t(1,7)= 4.69, p < 0.003; ((Mean CS + noise= 721.9, SEM= ± 129.5, SD= 366.3) vs. (Mean noise-alone= 146.7, SEM= ± 40.4, SD= 114.3). This result indicates that rats in the CNQX 5.0 μg No Shock group expressed robust FPS on the pretest (see Figure 104). The mean difference score (i.e. Difference Score= 3 CS + noise trials – last 3 noise-alone trials) was 575.2 (SEM= ± 122.5, SD=346.6, N=8) which is comparable to the difference scores obtained by rats in Experiment 4 (see Figure 99).
Figure 103. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for No Extinction + No Unsignalled footshock CNQX 5.0 μg (N=8) control group that was to further evaluate the effects of CNQX basolateral amygdala infusions on the expression of fear potentiated startle in the absence of unsignalled footshocks. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Pretest FPS Score For CNQX 5.0 ug No Extinction No Unsignalled Shock Expression Control Group

![Graph showing mean startle amplitudes for CNQX 5.0 ug (N=8) No Extinction + No Unsignalled Shock experimental control group on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (* P<0.05 relative to the noise alone trials). The mean (S.E.M. ±) difference scores ([3 CS + noise] - [3 noise-alone]) of the pretest demonstrates the robust level of fear exhibited by the CNQX 5.0 ug No Extinction + No unsignalled Shock experimental control group 48 hours after fear conditioning.](image-url)
Final Test Results and Discussion

A dependent T-test carried out on the final test data that compared the 10 CS + noise trials to the 10 noise-alone trials revealed a significant result t(1,7)=3.07, p < .02, indicating that CNQX infusions made 24 hours earlier did not block the expression of FPS when rats received no unsignalled footshocks after the drug infusion. The mean acoustic startle score on the 10 CS + noise trials was found to be significantly different to the mean startle responses obtained from the 10 noise-alone trials {(Mean 10 CS + noise= 295.4, SEM= ±62.3, SD=176.3) vs. (Mean 10 noise-alone= 105.2, SEM= ± 18.8, SD=53.2)}. In addition, the mean difference score (i.e. 10 CS + noise trials – 10 noise-alone trials) was equal to 190.2 (SEM= ± 61.8, SD=174.7, N= 8) which is similar to the mean difference score obtained by the PBS control, raclopride 8.0 µg, and AP5 5 µg groups in Experiment 4 (see Figure 102). The FPS results for the CNQX 5.0 µg No Extinction + No Shock group is shown in Figure 105.
Final Test: FPS Expression Test For CNQX
5.0 ug No Extinction No Unsignalled Shock
Group

Figure 105. Mean (S.E.M. ±) startle amplitudes for the CNQX 5.0 µg No Extinction + No unsignalled Shock experimental control group (N=8) on the 10 noise-alone and 10 CS + noise trials of the final test conducted 24 hours after intra-amygdaloid infusions of CNQX (5.0 µg) only (★ P<0.05 relative to noise-alone trials). The mean (S.E.M. ±) of the difference score ([10 CS + noise]-[10 noise-alone]) depicts the robust magnitude of fear exhibited by the CNQX 5.0 µg control group (N=8) on the final test when no unsignalled footshocks were administered after amygdaloidal CNQX infusions had been made.
To ensure that the mean difference scores were similar, an ANOVA of the difference scores which contained data from the PBS control, raclopride 8.0 μg and AP5 1.25 μg groups of Experiment 4 and the data from the CNQX 5.0 μg no unsignalled shock control rats of Experiment 5 was carried out. The 4 Drug Group ANOVA of the difference score revealed no significant between group differences in the magnitude of fear between these drug groups \( F(3,26)=0.086, p=0.9668 \) [n/s]. These results are depicted in Figure 106 and clearly show that the CNQX 5.0 μg No Shock group of rats in Experiment 5 did not differ significantly in their level of FPS on the final test from the PBS, raclopride and AP5 infused animals of Experiment 4. This was confirmed by a 4 Drug Group X 2 Stimulus Condition ANOVA and Newman-Keuls post hoc statistical analysis which revealed that all 4 groups just mentioned in the previous difference score analysis above exhibited higher mean startle scores on the 10 CS + noise trials than on the 10 noise-alone trials of the final test (see Figure 107).

The results of the difference score analysis are displayed in Figure 106 and they clearly demonstrate that CNQX 5.0 μg no unsignalled shock rats belonging to Experiment 5 exhibited significant CS-potentiated startle on the final test that was similar to the fear levels expressed by the 3 other treatment groups of Experiment 4 (i.e. the PBS, raclopride and AP5-infused rats). Nevertheless, it is important to point out that Newman Keuls analysis revealed that the PBS, raclopride (8.0 μg), and AP5 (1.25 μg) No Extinction + 5 unsignalled footshock groups of Experiment 4 did have significantly higher acoustic startle amplitude during noise-alone stimulus presentation than did the CNQX 5.0 μg No Extinction + No Shock group of Experiment 5 (see Figure 107). It is therefore likely that CNQX infusion 24 hours prior to final testing does cause an overall depression of acoustic startle responding but not enough to prevent the expression of FPS since the CNQX 5.0 μg No Extinction + No unsignalled footshock group still exhibited a significant CS induced enhancement of startle when 10 CS + noise trials were compared to the 10 noise-alone trials using Newman-Keuls within-group multiple comparisons (see Figure 107).

The larger noise-alone startle scores obtained by the PBS, raclopride and AP5 groups of Experiment 4 may represent elements of contextual fear since rats belonging to these three experimental groups received 5 unsignalled footshocks after drug infusion, whereas the CNQX 5.0 μg group of Experiment 5 did not. However, this explanation does not seem
plausible because the PBS, raclopride and AP5 groups of Experiment 4 exhibited higher mean startle amplitudes on the 10 CS + noise trials of the final test than did the CNQX 5.0 μg group of Experiment 5. Thus, the most likely explanation is that intra-amygdalar CNQX infusions made 24 hours before testing caused an overall depression in acoustic startle across both stimulus conditions (i.e. noise-alone and light + noise trials), but it was not enough to interfere with the capacity of rats to express FPS. This indicates that acoustic startle responding is dampened by amygdaloidal AMPA receptor blockade but the ability to express fear is still left intact, albeit to a lesser extent. Thus, future research should be directed at examining the effect of intra-amygdalar CNQX infusion on baseline acoustic startle responding and FPS.
Final Test Difference Scores

![Graph showing final test difference scores](image)

**Figure 106.** Mean (S.E.M. ±) difference scores ([10 CS + noise] - [10 noise-alone]) on the final test for the non-extinguished + 5 unsignalled footshock groups from Experiment 4 (PBS N=7; raclopride 8.0 µg N=7; AP5 1.25 µg; N=8) versus the mean difference score of the CNQX 5.0 µg No Extinction + No 5 Shock group (N=8) of Experiment 5. It is important to note that ANOVA of the difference data yielded a non-significant result, indicating that all groups represented in the above figure exhibited roughly equivalent levels of conditioned fear as measured through the potentiation of acoustic startle responding.
Final Test of Fear-Potentiated Startle Expression

Figure 107. Mean (S.E.M. ±) acoustic startle amplitudes on 10 noise-alone and 10 CS + noise trials of the final test for the non-extinguished + 5 unsignalled shock drug groups of Experiment 4 that expressed fear-potentiated startle 24 hours after intra-amygdalar infusion of PBS (N=7), raclopride (8.0 µg; N=7) or AP5 (1.25 µg; N=8) are shown on the left under the 5 Unsignalled Shocks title. On the extreme right are the final test results for the CNQX 5.0 µg No Unsignalled Shock group of Experiment 5 along with the mean (S.E.M. ±) acoustic startle amplitude on 10 noise-alone and 10 CS + noise trials. The mean (S.E.M. ±) difference scores ([10 CS + noise]-[10 noise-alone]) of the final test indicate that the CNQX rats of Experiment 5 exhibited a magnitude of fear similar to that expressed by certain groups in Experiment 4. It is noteworthy that all groups depicted in the above figure exhibited robust FPS ( * P<0.05 relative to noise-alone trials). However, the CNQX 5.0 µg group did show a general depression in acoustic startle responding to both types of stimulus presentation.
Taken together, these results seem to indicate that the CNQX infusion made into the BLA during Experiment 4 did not induce performance deficits when FPS was blocked 24 hours later, but rather interfered with the accurate reconsolidation and memory retrieval of CS-UCS associations. In other words, the presence of CNQX in the amygdala in combination with the 5 unsignalled footshocks administered during Experiment 4 may have somehow disrupted, degraded, or interfered with the integrity of the memory trace of the CS-UCS association that was established during Pavlovian fear conditioning. As a result, the CNQX-infused rats in Experiment 4 most likely found it difficult to discriminate between contextual cues and conditioned cues and to make appropriate conditioned fear responses to the CS. The lack of FPS exhibited by the CNQX-infused rats in Experiment 4 almost seems to indicate that contextual fears were actively competing with sensory specific conditioned fear to gain behavioural expression. When the interfering effects of 5 unsignalled footshocks were removed in the present control study (i.e. Experiment 5), CNQX infusion made 24 hours prior to testing did not disrupt the expression of FPS even though overall acoustic startle responding was significantly reduced. This finding tends to support the notion that CNQX infusions made into the amygdala likely block both the expression of FPS and the reinstatement of FPS by interfering with fear-memory reconsolidation and retrieval and not by causing performance deficits. Hence, the combined results of the expression control experiment, which includes Experiments 4 and 5, provide additional insight into how or why CNQX is effective in blocking both the expression of FPS (Kim, et al., 1993) and the reinstatement of FPS (See experiment 3D) when it is infused into the amygdala prior to final testing.

Alternatively, the combination of depressed startle responding caused by CNQX infusion into the amygdala may have worked in conjunction with the ability of CNQX to interfere with fear-memory reconsolidation and retrieval to block the reinstatement of FPS in rats that were part of Experiment 3D. Although the exact neurochemical mechanism by which the blockade of AMPA/kainate amygdaloid receptors by CNQX application prevents fear-memory retrieval and FPS reinstatement is not clearly understood, some revelations regarding AMPA receptor-mediated functions as they pertain to synaptic transmission and fear learning and expression have been made (Wang, et al., 2002; Wang, et al., 2001; Mahanty and Sah, 1998; Rainnie, Asprodini, and Shinnick-Gallagher, 1991; Farb, Aoki and
LeDoux, 1995; Davis, Rainnie and Cassell, 1994; Fendt and Fanselow, 1999; Koch, 1999) and the general discussion section will incorporate many of these research findings in order to explain the CNQX results obtained in Experiment 3D.
16. Experiment 6:

Infusion of AP5 into the Basolateral Amygdala of Rats and its Effects on Acoustic Startle Responding

16.1: Rationale

Experiment 3C demonstrated that AP5 infusion (1.25 µg and 2.5 µg) into the BLA prior to 5 unsignalled reminder footshocks prevented the reinstatement of FPS in rats (see Figure 77 and 78 for a quick review). As can be seen in Figure 77 both AP5 groups seemed to exhibit reduced levels of noise-alone startle responding on the final test relative to the PBS control group.

It has been reported that high doses of AP5 infused into the BLA may cause ataxia (Miserendino, et al., 1990), and as such, could cause impairments in baseline startle responding. Although an ANOVA carried out on the final test noise-alone startle responses that compared the PBS control group to the two AP5 infusion groups (1.25 µg and 2.5 µg) was not statistically significant ($F(1,33)= 1.12, P = 0.335 [ns]$) it still is conceivable that AP5 intra-amygdalar infusions made 24 hours before final testing could have had an impact on the capacity of animals to perform a startle response. Since one of the reinstatement experiments infused a high dose (2.5 µg) of AP5 into the BLA, it is plausible that this high dose could have caused some sensorimotor impairment that may have prevented this group from expressing fear. To rule out this possibility it was necessary to assess the effects of AP5 infusion on baseline startle responding.

16.2: Method

16.21: Subjects

Eight naive, male Wistar rats bred and housed in the Psychology Department animal facility at the University of Canterbury served as subjects in this experiment. Rats weighed 340 grams at the beginning of the experiment. All other particulars concerning housing conditions, temperature, and food and water are the same as those reported in the previous experiments.
16.22: Surgery, Apparatus, and Experimental Procedure

The testing apparatus, surgical procedures and drug infusion protocols are identical to those reported in Experiment 1A, 3A and 3C. Sixteen days following surgery rats were assessed for baseline startle using 3 blocks of 20 white-noise bursts (30 s, ISI). Noise-alone levels (range 90.0 to 96.5 dB) that produced stable baseline startle were used in this experiment. Each rat’s dB score (i.e. white-noise intensity) was used for all subsequent testing that followed. Two days later rats were placed into their designated startle chambers and after a 5 min habituation period were administered 20 predrug noise-alone trials (30 s ISI). Rats were then removed from the startle boxes and infused bilaterally with a 2.5 μg dose of AP5 into the BLA. Rats were returned to their test boxes and after a 5 min acclimatisation period, were exposed to 40 post-drug noise-alone trials (30 s ISI). This experimental protocol made it possible to make comparisons between predrug acoustic startle responding and postdrug (AP5 2.5 μg) acoustic startle responding.

16.23: Histology

Subjects were over dosed with a lethal dose of sodium pentobarbitone (300mg/kg) and intracardially perfused with saline followed by the (FAM) solution. The brains were removed from each animal’s skull and placed into individual bottles filled with (FAM) solution. Three days later the brains were transferred to a 70% sucrose solution for several weeks before sectioning. Brains were sliced at a thickness of 50 μm and mounted onto gel-coated slides. Sections were then stained with Cresyl violet and cannulae placements were evaluated microscopically in conjunction with a rat brain atlas (Paxinos and Watson, 1986; 1998). Figure 108 depicts the guide cannulae placements and approximate location of infusion needles for this control group. Most guide cannulae were located above the medial portion of the basolateral complex but some cannulae were located a bit more medially in an area just above the amygdalostriatal transition area and central lateral subdivision of the central amygdaloid nucleus. This would have meant that in addition to reaching the basolateral amygdaloid complex, some AP5 may have diffused into the central amygdala and the amygdalostriatal transition area as well. One animal lost its head cap and was not included in the study, leaving this control group with N= 7.
Figure 108. Schematic depictions of guide cannula locations represented as filled circles (●) on the left and the approximate location of infusion needles represented as open circles (○) on the right for the 2.5 μg AP5 Ataxia Control group (N=7) of Experiment 6. Guide cannulae were implanted 1.0 mm above the medial portion of the basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. -7.40 mm from the skull surface). The infusion needles extended 1.0 mm ventrally beyond the locations of the guide cannula. Representative sections (top to bottom: -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
16.24: Results and Discussion

The main finding of this control experiment was that AP5 infusion into the amygdala did not alter acoustic startle responding in laboratory rats. A dependent T-test was used to statistically analyse the pre-drug and post-drug baseline startle amplitude data. The dependent T-test yielded a non-significant result $t(1,6) = 0.377, p = 0.719$, [n/s] indicating that the 20 pre-drug mean startle amplitude scores were not significantly different from the 40 post-drug mean startle amplitude scores (see Figure 109). The mean startle score pre-drug was 171.3 (SEM = ±44.8, SD=118.4, N=7) and the mean startle score post-drug was 187.8 (SEM = ±30.2, SD=80.0, N=7). The results obtained from this experiment demonstrate that the 2.5 µg dose of AP5 infused into the amygdala did not cause sensorimotor deficits or affect baseline acoustic startle responding. It is important to note that no behaviours indicative of ataxia were observed in the rats infused with this dose of AP5. It is therefore unlikely, that the high dose of AP5 caused impairments that would have prevented rats from expressing conditional fear. Thus, it is more likely that the infusion of AP5 prior to 5 unsignalled shocks in Experiment 3C blocked the reinstatement of FPS by antagonizing amygdaloidal NMDA receptors that are known to be involved in synaptic transmission and fear memory consolidation processes (for a review see Koch, 1999; Fendt and Fanselow, 1999; Wang, et al., 2002; Wang, et al., 2001; Davis, Rainnie and Cassell, 1994; LeDoux, 2000; Walker and Davis, 2002).
Effect of AP5 on Baseline Startle

![Bar graph showing the effect of AP5 on baseline startle amplitudes. The graph compares pre-drug and post-drug startle amplitudes with and without AP5 infusion. The difference score is depicted by the third bar of the graph and clearly indicates that there were no significant differences between predrug and postdrug acoustic startle amplitudes.

Figure 109. Mean (S.E.M. ±) baseline startle amplitudes before (Pre-Drug) and after (Post-Drug) the infusion of 2.5 μg of AP5 into the basolateral amygdaloid complex. The mean (S.E.M. ±) difference score ([Post-drug startle]-[Pre-drug startle]) is depicted by the third bar of the graph and clearly indicates that there were no significant differences between predrug and postdrug acoustic startle amplitudes.
17. EXPERIMENT 7A:
ELECTRICAL STIMULATION OF THE BASOLATERAL AMYGDALA AND ITS EFFECTS ON REINSTATING FEAR-POTENTIATED STARTLE RESPONDING IN RATS THAT HAVE RECEIVED EXTINCTION TRAINING

17.1: Rationale

The results of Experiment 3A to 3D were the first to demonstrate that both DAergic and glutamatergic receptor antagonist block the reinstatement of FPS when these compounds are infused into the BLA complex prior to unsignalled footshock are administered. These experiments and Experiment 2 used unsignalled footshock presentation in an attempt to reinstate conditioned fear responding in laboratory rats. Footshock is a gross arousal mechanism that most likely activated the amygdala and the entire amygdala-based fear system. In order to expand our knowledge of the mechanisms that contribute to the FPS reinstatement effect the next series of experiments sought to use electrical stimulation of discrete brain regions in place of footshock to reinstate conditioned fear. Using this method, different areas in the amygdala-based fear system could be electrically stimulated in order to evaluate their contribution to the FPS reinstatement effect. Employing such a technique makes it possible to quickly and efficiently identify the neural substrates and circuitry that triggers FPS reinstatement in fear-extinguished rats. Of course, one obvious place to start such an electrophysiological investigation is with the amygdala.

Either electrical stimulation of the human amygdala and adjacent temporal lobe or epileptic discharge localized in these brain regions has been shown to provoke fearful thoughts, behaviours, and memory flashbacks (Gloor, et al., 1981; Gloor, 1990; 1992; Halgren, et al., 1978; Depaulis, et al., 1997; Piazzini and Canger, 2001; Dodrill and Batzel, 1986). In some of these cases epileptic discharge or stimulation of the amygdala can resurrect many of the fearful memories that a person has actually experienced at some point in their life (Gloor, 1990; 1992; Halgren, et al., 1978; Depaulis, et al., 1997; Piazzini and Canger, 2001; Dodrill and Batzel, 1986). Interestingly, electrical stimulation of the amygdala in rats enhances acoustic startle responding and produces fear-like behaviours (Rosen, 1988a, b; Koch and Ebert, 1993). Furthermore, kindling of the rat amygdala induced by repeated electrical stimulation has been shown to enhance emotionality and

Thus, the emotionality and fearfulness observed in humans suffering from temporal lobe epilepsy does seem to resemble the fear-like responding and emotional profile exhibited by amygdala-kindled rats.

Electrical stimulation of the thalamus, a brain region that provides the lateral and basal amygdaloid nuclei with diverse forms of sensory input, has been shown to induce LTP in the lateral amygdala and be an effective UCS during Pavlovian fear conditioning (Clugnet and LeDoux, 1990; Cruikshank, Edeline, and Weinberger, 1992). This research once again provides more evidence that thalamo-amygdaloid neurocircuitry is crucial for conditioned fear learning and expression. In addition, electrophysiological experiments have demonstrated that the lateral and basolateral amygdaloid nuclei undergo LTP during fear conditioning (Rogan, et al., 1997a; Quirk, et al., 1995) and there is some indication that these amygdaloid nuclei initiate electrophysiological change at a much faster rate than the Te1 and Te3 areas of the temporal cortex (Quirk, et al., 1997). This suggests that neurons in the lateral and basolateral region of the amygdala respond faster to stimuli paired with aversive events and as a consequence most likely develop CS-UCS fear associations much more quickly than the temporal cortex (see Quirk, et al., 1995; Quirk, et al., 1997; Rogan, et al., 1997a). Since electrical stimulation of the amygdala has been shown to elevate acoustic startle responsiveness, exaggerate FPS, and increase many autonomic and behavioural responses that are commonly associated with fear or anxiety (Rosen and Davis, 1988a, b, 1990; Koch and Ebert, 1993; Anand and Dua, 1956; Hilton and Zbrozyna, 1963; Kapp, et al., 1982; Applegate, et al., 1983; 1985; Gelsema, et al., 1987; Iwata, et al., 1987; LeDoux, 1987; Gloor, 1992; Rosen, et al., 1996; Depaulis, et al., 1997; Wintink, et al., 2003; Helfer, et al., 1996), it is possible that electrical stimulation of the amygdala will cause a reinstatement of FPS in rats that have been exposed to fear-extinction training and in the process lower after-discharge (AD) current threshold levels measured from the amygdala.
Hence, the purpose of the present experiment is to determine whether electrical stimulation of the amygdala will trigger a FPS reinstatement effect in fear-extinguished rats. This work represents the first experimental attempt to reinstate FPS responding via electrical stimulation of the amygdaloid complex. It is hypothesised that electrical stimulation of the amygdala will reinstate conditioned fear responding by re-exciting and reactivating amygdaloid neurons and pathways that contain a fear-memory trace (i.e. CS-UCS association) of the conditioning experience established during Pavlovian fear conditioning. In contrast, it is expected that non-stimulated fear-extinguished rats will display no FPS reinstatement and no decrements in AD current threshold levels. The method and result sections of Experiments 7A and 7B are presented below.

17.2: Method

17.21: Subjects

A total of sixty naive, male Wistar rats bred and housed in the psychology animal facility at the University of Canterbury served as subjects in this series of experiments. On average, rats weighed 340 grams at the beginning of the experiment (range 265-370 grams). A constant temperature of 20°Celsius (±1°Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulation took place during the light portion of this cycle.

17.22: Apparatus

Five identical cages (15 cm X 9.5 cm X 10 cm), constructed from stainless steel wire mesh mounted on a Plexiglas frame, served as fear conditioning, startle testing and electrical brain stimulation administration apparatus. Each individual cage was suspended 4 cm above the centre of a strip of piezo-electric film (29 cm X 23 cm) and housed inside a sound-attenuating Styrofoam chamber (33 cm X 26 cm X 27 cm, interior dimensions). The piezo-electric strip was surrounded with insulating foam and covered with cardboard and a Mylar sheath. Fluctuations in voltage amplitude resulting from kinetic energy or movement on the piezo were transferred into a signal that was filtered, amplified, and then measured by a specially designed sample-and-hold circuit interfaced to a 386
microcomputer (5 mV peak voltage amplitude equals 100 units). A 6.3 volt light (neutral stimulus), located 4 cm above the centre of each stainless steel wire mesh cage served as the conditioned stimulus (CS). A high frequency speaker (10 cm in diameter), used to administer white noise bursts, was embedded in the side-wall of each Styrofoam test chamber. The acoustic stimulus consisted of a 100-ms white noise burst with a rise-decay time of 5-ms and was produced by a Med Associates white-noise generator (Fairfield, VT). The ambient noise level in the chambers was 55 dB as measured by a Simpson (model 860; Elgin, IL) sound level meter (A scale).

Electrical footshock was administered through a second Mylar sheath and served as the unconditioned stimulus (UCS) in the fear acquisition experiment. Each half of the Mylar sheath was aluminium-taped (Manco Inc., Avon, OH) and separated by a glass rod embedded in a silicone barrier. The glass rod embedded in silicone served as an insulated border between the positive and negatively charged portions of aluminium tape. This barrier divided the surface of the Mylar sheath along its central length so as to prevent rat urine and faeces from short circuiting the stimulator. The stainless steel wire mesh walls and Plexiglas frame of the cages were nickel-coated (CG Electronics, Rockford, IL) and connected to the aluminium taped Mylar. A constant current stimulator (Schnabel Electronics, Saskatoon, SK, Canada) delivered an 800 μA current (monopolar square wave, 200 Hz frequency, 0.8 ms pulse duration) for 500 ms through the taped aluminium Mylar sheath, thus electrifying the floor and the nickel coated Plexiglas and stainless steel mesh walls of the cage. This design ensured that the footshock delivered to the subjects during fear conditioning was inescapable.

17.23: In Context Stimulation Apparatus

In addition to fear conditioning and startle testing, the five startle cages housed in Styrofoam described earlier also served as the In Context stimulation environment where rats received electrical brain stimulation. Electrical brain stimulation to induce fear-reinstatement was administered using a programmable constant current stimulator (Schnabel Electronics, Saskatoon, SK, Canada). The stimulator was connected to a System Accessory Terminal (SAT 16) with electronic relay switches built into the circuitry. The SAT 16 was connected to the 386 PC which had programmable software designed to
trigger the constant current stimulator via the electronic relays. This allowed the brain stimulation and any other stimuli presentation to be computer controlled and delivered accurately.

17.24: Out of Context Stimulation Apparatus

The Out of Context brain stimulation apparatus consisted of two Styrofoam chambers (30 cm X 39 cm X 34 cm, exterior dimensions) enclosing a circular stainless steel cylinder (27.5 cm diameter). The floor was made of stainless steel bars (0.5 cm diameter) spaced about 1 cm apart. The Styrofoam lid was located 24 cm above the stainless steel bars. A small hole was located in the middle of the lid to allow the bipolar electrode cable to descend into the chamber so that it could be connected to the rat’s bipolar electrode. This apparatus was chosen as the “out of context” stimulation environment because it was distinct to the startle testing apparatus used for “in context” brain stimulation. The differences in physical make up made it possible to determine if out of context amygdaloid stimulation would reinstate FPS in rats that have received extinction training. All particulars concerning the type of stimulator used, the connections to the SAT 16, and the connections to the 386 computer are the same as those described in the In Context Stimulation Apparatus section.

17.25: After Discharge Testing Apparatus

The after-discharge testing chamber was manufactured from clear Plexiglas and measured 46 cm in length, 26 cm in width, and 34 cm in height. The test chamber had an electrically grounded floor made of stainless steel rods (0.7 cm diameter) spaced 1.1 cm apart. Stainless steel rods were wired to each other and the rods were connected to the ground plug on the electroencephalographic (EEG) machine to ensure that animals were properly grounded when in the test chamber. This grounding ensured that all external electrical noise was filtered out making it possible to record focal EEG activity. All focal EEG activity and after-discharge thresholds were recorded on a model 5D Grass model 79E EEG machine.

The electrical stimulus used to induce AD activity in the amygdala originated from a constant current stimulator (Schnabel Electronics, Saskatoon, SK, Canada) which was
connected to the rat’s bipolar electrode via a bipolar electrode cable {305-S304-CCT 80CM TT2 (CS); Plastics-One, Roanoke V.A.}. A specially designed relay switch was connected to both the EEG machine and the constant current stimulator. This switch had two positional settings, one was a stimulate option and the other was an EEG record option. When set in the stimulate position, a brief 500 ms brain stimulation could be delivered directly to the rat in the desired brain region under investigation. When set to the EEG record position, focal EEG activity could be recorded from a desired brain region with one of the bipolar electrode wires acting as a recording electrode while the other served as an internal ground. This switching system made it possible to stimulate the desired brain area and then quickly change positions to record focal EEG or any AD activity through the single bipolar electrode.

17.26: Stéréotaxic Surgery

Rats were anesthetised with sodium pentobarbitone (95.0 to 110 mg/kg), mounted in a Stoelting stereotaxic instrument (Wood Dale, IL), and then surgically implanted with a single bipolar stainless steel twisted electrode (MS-303/1; Plastics-One, Roanoke, VA) aimed at the medial portion of the BLA. Prior to surgery each electrode was prepared by first cutting the twisted wires to a length of 12 mm below the plastic pedestal. The two insulated twisted wires were separated at the tip and then gapped so that the distance between the two wires was 0.5 mm. To ensure good electrical conductivity the insulation was scraped from the tip of each of the electrode wires leaving 0.4 mm of bare wire exposed. The electrode implant was perpendicular to the horizontal plane and the incisor bar was carefully adjusted for each rat so that the horizontal surface was level for the anterior (bregma) and posterior (lambda) portions of the skull.

The stereotaxic coordinates used in this experiment were taken from the rat brain atlas of Paxinos and Watson (1986) and were A.P. -2.80 mm from bregma, M.L. ± 4.80 mm from the sagittal suture, and V -8.60 mm from the skull surface. Electrodes were unilaterally implanted with half of the animals receiving an electrode in the right hemisphere and the remaining animals implanted in the left hemisphere. The electrode was secured to the skull surface via a head cap constructed from dental acrylic. This head cap was attached to five stainless steel self-tapping jeweller screws (3.20 mm in length, Lomat
Precision Tools, Montreal Quebec, Canada) embedded in the skull surface. Specially designed dummy dust caps (303DC and 303DCA; Plastics-One, Roanoke, VA) were screwed onto the secured electrode to prevent rats from chewing the plastic portion of the electrode and making it inoperative. Following surgery, rats were placed into a recovery cage until the anaesthetic wore off and then were returned to normal group housing conditions. Rats were allowed a 14 day recovery period to ensure their wounds were adequately healed before any behavioural testing or experimental manipulation commenced.

17.27: Procedure

17.28: Baselining

Acoustic startle was assessed for each rat over 3 blocks of 20 white noise trials per block. Thus, each rat received a total of 60 white-noise trials with an interstimulus interval (ISI) of 30 seconds. During the first 2 baselining blocks the noise intensity levels were adjusted (range 83.0 to 103.5 dB) for each animal to establish a stable acoustic startle response (between 50 and 500 units). The mean acoustic startle score measured on the third block of 20 white-noise trials represents the baseline acoustic startle score. The noise intensity level that was recorded for each rat after the third baselining block was used for all subsequent acoustic startle testing. This baselining protocol was used to establish stable baseline acoustic startle responses for each rat and to reduce between-subjects and between-groups variability in acoustic startle responding. This method ensured that experimental groups were equated on critical variables that could potentially have an impact other dependent measures of interest (i.e. the reinstatement of FPS).

17.29: Fear Conditioning

Forty-eight hours after the baselining procedure, rats were placed into their designated test chambers, and after a 5 min habituation period were exposed to 30 consecutive CS (light) plus footshock fear conditioning trials. The inter-trial interval was 60 s and the duration of each footshock (UCS) that occurred during the last 0.5 s of each CS (light) presentation was 500 ms. After the fear conditioning session was completed, the rats were
removed from the test chambers and then returned to their home cage environment. Following fear conditioning, rats were given 48 hours to consolidate their memory of the CS-shock presentations.

17.30: Pretesting, Extinction Training, and Unsignalled Electrical Stimulation of the Basolateral Amygdala

After the 48 hour memory consolidation period elapsed, the specified white-noise level that produced stable baseline acoustic startle amplitudes were set for each rat’s test chamber. The rats were then placed into their designated test chambers and after a five minute habituation period were assessed for FPS. This pretest first involved presenting the rats with 20 noise-alone trials (30 s, ISI) followed two minutes later by 3 CS + noise trials (30 s, ISI). This pretest compared the mean startle amplitude of the last 3 noise-alone trials obtained from the 20 noise-alone trials to the mean startle amplitude of the 3 CS + noise trials. A difference score was also calculated (Difference Score = [3 CS + noise trials] - [last 3 noise-alone trials]) for each rat. Rats were assigned to one of four In Context Experimental groups (N= 12 per group) with similar averaged startle scores, noise intensity levels, and levels of FPS as reflected through the difference scores calculation and analysis.

A fifth experimental group (Out of Context Stimulation Group) containing N=12 rats was also formed at the same time as the In Context experimental groups. This Out of Context Stimulation Group received extinction training and was designed to examine the effects of out of context electrical amygdaloid stimulation on fear reinstatement. Thus, animals across all experimental groups were matched on noise intensity, baseline startle and pretest FPS. This technique ensured that all experimental groups were matched on variables that could potentially influence the key dependent measure which is the level of fear reinstatement exhibited by fear extinguished rats exposed to electrical stimulation of the amygdala.

Twenty-four hours after the pretest, half of the rats assigned to the In Context Stimulation Groups were placed in their designated test chambers and after a 5 min habituation period were exposed to 140 light-alone extinction trials. The interstimulus interval for the light presentation was 15 seconds and the entire extinction training procedure took 35 minutes. The remaining In Context Stimulation rats received no
extinction training and were used to determine if amygdaloid stimulation would interfere with fear expression. As stated above rats assigned to the Out of Context Stimulation group all received 140 light-alone extinction trials. Thus, the four In Context experimental groups and the one Out of Context experimental group and the manipulations which followed can be summarised as; (Extinction + In Context Basolateral Amygdala Stimulation); (Extinction + No Stimulation); (No Extinction + In Context Basolateral Amygdala Stimulation); (No Extinction + No Stimulation) and (Extinction + Out of Context Basolateral Amygdaloid Stimulation).

Forty-eight hours after extinction or no extinction training, rats were connected to a programmable constant current stimulator via an insulated bipolar electrode cable (305-S304-CCT 80CM TT2 CS, Plastics One, Roanoke VA) which connected to the MS-303/1 bipolar electrode. The wire cable was protected by a stainless steel coil spring to prevent rats from damaging the wire. After rats were connected to the stimulator, they were then placed into their designated chambers (In Context or Out of Context) and allowed 5 min to acclimatise to the test environment. Rats assigned to either the Extinction + In Context Basolateral Amygdala Stimulation group or the No Extinction + In Context Basolateral Amygdala Stimulation group were then administered 100 unsignalled electrical brain stimulations. The current intensity used to stimulate the BLA was 400 μA (base to peak current) with a pulse-duration of 0.1 ms, and a frequency of 100 Hz. The interstimulus interval used in this experimental manipulation was 3 s and the duration of the electrical stimulation was 500 ms.

The programmable constant current stimulator was connected to the 386 computer that ran the startle testing and fear conditioning programs in previous experiments. In this experiment, a specially designed computer program was used to administer the electrical brain stimulation by activating the constant current stimulator at the selected intervals. Rats in the Extinction + Out of Context Basolateral Amygdala Stimulation Group received electrical stimulation in the out of context stimulation apparatus that was physically distinct from the in context apparatus. After the electrical stimulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment. Rats assigned to the In Context No Stimulation groups did not receive any electrical brain stimulation.
17.31: Final Test

Twenty-four hours after stimulation or no stimulation administration, rats were tested for the reinstatement of FPS. The final test employed a quasi-counterbalanced design. This procedure entailed presenting half of the rats in each experimental group with 10 noise-alone trials first followed by 10 light + noise trials, whilst the other half of the rats in each experimental group were exposed to 10 light + noise trials first followed by 10 noise-alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the white-noise burst was presented during the last 100 ms of the light interval. This final test was specifically designed to determine whether electrical stimulation of the amygdala would facilitate a reinstatement of FPS responding in fear-extinguished rats.

17.32: After Discharge Thresholds and Seizure Classification

Ninety-six hours after the final test all rats were assessed for after-discharge (AD) current threshold levels and classification of seizure type produced when the first AD was recorded. The purpose for assessing AD current threshold levels was to determine if the 100 electrical pulses used to stimulate the amygdala and induce fear-reinstatement also caused neural sensitisation and/or long-term changes in cellular functioning within the amygdala.

In brief, rats were placed into the Plexiglas after-discharge testing chamber and had their electrode connected to the insulated bipolar electrode cable. The bipolar electrode cable was connected to a programmable constant current stimulator that was in turn connected to a model 5D Grass EEG machine. After a 2 minute habituation period elapsed, an EEG recording period began so as to establish a record of baseline EEG activity in the amygdala. The baseline recording lasted for a period of 2 minutes. Rats then received the first electrical amygdaloid stimulation to try to initiate AD activity in the amygdala. The first current intensity used to initiate AD activity in the amygdala was 400 μA (base to peak current; 0.1 ms pulse duration; 100 Hz frequency; 500 ms duration) which was the same current used to reinstate FPS in fear extinguished rats. EEG activity in the amygdala was then recorded for a period of 1 minute after amygdaloid stimulation. If no AD activity was recorded and confirmed, the current was raised by 50 μA and the rat’s amygdala was again
stimulated. Following the electrical stimulation EEG activity was again recorded in the amygdala for a 1 min period. The current was raised in increments of 50 μA followed by 1 minute of EEG recordings until an AD was recorded in the amygdala. Once an AD was recorded and confirmed, recording of EEG activity continued for 2 minutes after the AD occurred. The after-discharge and seizure classification results will be disclosed after all the fear-potentiated reinstatement results in Experiments 7A and 7B have been presented.

The level of seizure associated with the AD was classified on a scale of 1 to 5 for each rat (Racine, 1972a,b). These seizures are categorized from Type 1 to Type 5 and each type generally produces a different behavioural manifestation that makes it possible to distinguish the intensity of the seizure. The classifications are as follows; 1) Type 1 Seizures produce simple behavioural arrest and AD activity. 2) Type 2 Seizures produce behavioural arrest, oral movements and head bobbing. 3) Type 3 Seizures produce unilateral lifting of forelimbs and rapid shaking. 4) Type 4 Seizures produce clonus of both forelimbs and rearing behaviour. Type 5 Seizures generally produce clonus of both forelimbs, rearing behaviour, and then complete falling down where the animal falls backwards as if it has lost balance. During some of these seizure types rapid and repeated eye-blinking and mastication is also present.

17.33: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, these included; acoustic startle amplitude data obtained during baselining, shock reactivity data gathered during fear conditioning, and the level of FPS measured during the pretest and fear reinstatement tests. A 4 Stimulation Group X 2 Stimulus Condition (light + noise vs. noise-alone) ANOVA was employed to analyse the pretest and final test data. Newman-Keuls multiple comparisons (α = 0.05) were used to assess differences within and between experimental group means. Statistically, the between-group Newman-Keuls post hoc comparison involves the evaluation of interactions between two factors, in this case Group X Stimulus Condition. As a result, a pooled error term was used to calculate the critical ranges required for statistical significance when Newman-Keuls post hoc tests were used to make between-group comparisons when interactions between factors occurred (Winer, 1962; Winer, Brown and Michels, 1991). This statistical practise of pooling
individual error terms for each factor that contributes to an interaction sets a more conservative and stringent critical range for the Newman-Keuls post hoc tests. This technique is valid because it recognizes that each factor that contributes to a significant interaction on an ANOVA also brings along its own level of error and degrees of freedom. Thus, when interactions occurred and between-group comparisons needed to be made, the Newman-Keuls tests used a pooled error term to establish the critical ranges required for statistical significance (see Winer, Brown and Michels, 1991).

Shock reactivity was evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 light + shock trials. Results obtained from ANOVAs were used to confirm that all experimental groups were equated on noise intensity level, baseline startle amplitude scores, and pretest FPS levels. Hence, ANOVA statistical analysis were used to demonstrate that experimental groups were matched on certain key variables that could possibly influence other important dependent measures of interest such as the reinstatement of FPS triggered by electrical stimulation of the amygdala.

17.34: Histology

At the end of experimentation rats were injected with a lethal dose of sodium pentobarbitone and perfused intracardially with saline followed by a solution of formalin (10%), glacial acetic acid (10%) and methanol (80%) (FAM). The brains were removed from each animal's skull and then stored in the FAM mixture for 4 days. The brains were then removed from the FAM solution and placed in a 70% sucrose solution for several weeks prior to sectioning. Coronal sections (50 μm) thick were sliced and mounted on gel-coated slides. The slides were stained with Cresyl violet and the sections were evaluated under a microscope to determine bipolar electrode placements. This histological practice made it possible to determine the final composition of each experimental group in this particular study.
17.4: Results

17.41: Histology Results

One rat in the No Extinction + Stimulation group had its dust cap removed and its electrode chewed by its cage mates, leaving this group with an N=11. Microscopic analysis of electrode placement revealed that all electrodes were located in the amygdala. Most electrode tips were found in the dorsal half of the basolateral complex. Some electrodes were located in the lateral nucleus of the amygdala and could be seen just touching the dorsal portion of the basolateral amygdaloid nucleus. At more anterior levels some electrode tips were located just ventral to the amygdalostrial transition area in a region where the basolateral and central amygdaloid nuclei come in contact with each other.

Figures 110 to 112 depict the electrode placements for each experimental group and as can be seen from these figures, most electrodes were on target with the selected stereotaxic coordinates. The location of these electrodes meant that the lateral and basolateral nuclei along with portions of the central amygdaloid nucleus and amygdalostrial transition region were activated during electrical stimulation. The accurate placement of the electrodes ensured that the basolateral complex and parts of the central nucleus were thoroughly activated by the 400 μA current that was used to attempt to reinstate FPS. Figures 113(a) to 113(e) display some digital photographs of electrode placements in the lateral and basolateral amygdaloid nuclei for rats of Experiment 7A. Based on the histological results, all animals were kept in the study. The only exception was the one rat from the No Extinction + Stimulation group that had its electrode chewed off. This meant that all experimental groups had an N=12, whereas the No Extinction + Stimulation group had an N=11.
Figure 110. The above figure schematically depicts the location of bipolar electrodes in the basolateral and central lateral amygdaloid nuclear groups and is represented by the solid filled circles (•). On the left are the approximate location of implanted electrodes for the Extinction + Stimulation group (N=12) and on the right are the electrode placements for the Extinction + No Stimulation group (N=12) of Experiment 7A. Bipolar electrodes were unilaterally implanted into either the left or right basolateral amygdaloid nucleus (coordinates A.P. -2.80 mm from bregma, M.L. ±4.80 mm from the midline sagittal suture, D.V. -8.60 mm from the skull surface). Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 111 schematically depicts the location of bipolar electrodes in the basolateral and central lateral amygdaloid nuclear groups. The electrode placements are represented by the solid filled circles (●). On the left are the approximate location of implanted electrodes for the No Extinction + Stimulation group (N=11) and on the right are the electrode placements for the No Extinction + No Stimulation group (N=12) of Experiment 7A. Bipolar electrodes were unilaterally implanted into either the left or right basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 8.60 mm from the skull surface). Representative sections (top to bottom; -1.80 mm, -2.12 mm, -2.30 mm, -2.56 mm, -2.80 mm, and -3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 112 schematically depicts the location of bipolar electrodes in the basolateral and central lateral amygdaloid nuclear groups. The electrode placements are represented by the solid filled circles (●). On the left are the approximate location of implanted electrodes for the Extinction + In Context Stimulation group (N=12) and on the right are the electrode placements for the Extinction + Out of Context Stimulation group (N=12) of Experiment 7B. Bipolar electrodes were unilaterally implanted into either the left or right basolateral amygdaloid nucleus (coordinates A.P. - 2.80 mm from bregma, M.L. ± 4.80 mm from the midline sagittal suture, D.V. - 8.60 mm from the skull surface). Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm, - 2.80 mm, and - 3.14 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 113(a). These two digital photographs of a rat brain clearly depict the location of a bipolar electrode in the basolateral amygdala (coronal sections were taken approximately -1.80 mm to -2.12 mm posterior to bregma). Notice the presence of the electrode tip near the middle of the basolateral amygdaloid complex (photo slides A and B) as denoted by the small dark arrows.
Figure 113(b). These two digital photographs of a rat brain also depict the placement of a bipolar electrode in the basolateral amygdala (coronal sections were taken approximately -2.12 mm to -2.30 mm posterior to bregma). Again, notice the presence of the electrode tip near the middle of the basolateral amygdaloid complex (photo slides C and D) as denoted by the small dark arrows.
Figure 113(c). These two digital photographs are magnified close-ups of photo-slides A and B from Figure 113 (a). These close-up photographs clearly demonstrate that the non-insulated portion of the bipolar electrode was located in the basolateral amygdala.
Figure 113(d). These two digital photographs are magnified close-ups of photo-slides C and D from Figure 113 (b). Again notice that these close-up photographs clearly demonstrate that the non-insulated portion of the bipolar electrode tips were located in the basolateral amygdala.
Figure 113(e). The digital photograph (A) of another rat brain clearly shows the location of a bipolar electrode in the basolateral amygdala (coronal sections taken at -2.30 mm to -2.56 mm posterior to bregma). The small dark arrow in the photograph points to the location of the electrode tip (photo A). Digital photograph (B) is a magnified version of photograph A. As can be seen the bipolar electrode tip in this rat was clearly in the middle of the basolateral amygdaloid complex.
17.42: Experimental Results

ANOVA of baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the 4 In Context experimental groups to each other $F(3,43)=1.22$, $p=0.313$ [n/s], indicating that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 114). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result $F(3,43)=0.42$, $p=0.737$ [n/s], demonstrating that the groups were properly matched on this particular scale (see Figure 115). ANOVA of the shock reactivity data obtained during fear conditioning revealed a non-significant result $F(3,43)=0.35$, $p=0.787$ [n/s], suggesting that all experimental groups showed similar levels of movement amplitude during light + footshock administration (see Figure 116).
Figure 114 Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) groups.
White-noise Intensity used to Generate Acoustic Startle Responding

Figure 115. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that was used to induce stable acoustic startle responses in the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) groups.
Shock Reactivity During Fear Conditioning

Figure 116. Mean (S.E.M. ± ) movement amplitudes recorded during the presentation of 30 light + footshock fear conditioning trials to rats in the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) experimental groups.
17.43: Pretest Results

The pretest results demonstrate that all four In Context experimental groups expressed robust levels of FPS 48 hours after exposure to 30 light + footshock fear conditioning trials. ANOVA (4 Stimulation Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition F(1,43)= 71.97, p<0.000001, with no significant effect of Group, or Group X Stimulus Condition interaction. Newman-Keuls post hoc comparisons carried out on the main effect of Stimulus Condition (3 CS + noise trials vs. 3 noise-alone trials) revealed that mean acoustic startle responses on the 3 CS + noise trials were significantly higher than startle responses on the last 3 noise-alone trials for all experimental groups on the pretest. These results are depicted in Figure 117, and clearly show that rats in all four In Context experimental groups displayed robust FPS after Pavlovian fear conditioning. Also, all experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction.

(ANOVA) of the pretest difference score data (Difference Score= [3 light + noise trials]-[last 3 noise alone trials]) confirms that the four experimental groups had similar levels of fear F(1,43)=0.43, p=0.733 [n/s]. Figure 118 depicts the mean difference scores obtained from the pretest and clearly demonstrates that all experimental groups were closely matched on fear prior to any experimental manipulation involving either extinction training or administration of 100 electrical stimulations of the BLA. These results indicate that unilateral implantation of bipolar electrodes in the basolateral or nearby nuclei did not produce any serious damage to the amygdala since rats were still able to express a conditioned fear response that was acquired during the presentation of 30 light + footshock trials.
Figure 117. Mean (S.E.M. ±) acoustic startle amplitudes on the 3 noise-alone and 3 CS + noise trials of the pretest for the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) groups as a function of fear conditioning only ( ★ P<0.05 relative to noise-alone trials). Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depicts the equivalent levels of fear-potentiated startle exhibited by all groups 48 hours after 30 fear conditioning trials.
Pretest FPS Difference Scores After Fear Conditioning

Figure 118. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the equally robust magnitude of fear-potentiated startle responding expressed by the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) experimental groups 48 hours after exposure to 30 fear conditioning trials.
17.44: Final Test Results

The principal finding of this experiment was that electrical stimulation of the amygdala caused a reinstatement of FPS responding in fear-extinguished rats. A 4 Stimulation Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA revealed a significant main effect of Stimulus Condition F(1,43)=31.10, p<0.000003; and a significant Stimulation Group X Stimulus Condition interaction F(3,43)=3.14, P<0.04. Newman-Keuls post hoc within group comparisons revealed that the Extinction + Stimulation group, the No Extinction + Stimulation group, and the No Extinction + No Stimulation group showed significantly higher acoustic startle amplitudes on the 10 light + noise trials than on the 10 noise-alone trials (see Figure 119). On the other hand, post hoc analysis for the Extinction + No Stimulation group (i.e. control group) failed to find any statistically significant difference between the 10 light + noise trials and the 10 noise-alone trials on the final test, indicating that 140 CS only trials produces a robust extinction of FPS responding.

Furthermore, Newman-Keuls post hoc tests used to assess the Stimulation Group X Stimulus Condition interaction demonstrated that the No Extinction + Stimulation group had a significantly higher mean startle amplitude score on the 10 CS + noise trials than did the Extinction + No Stimulation control group. Moreover, the Extinction + Stimulation and No Extinction + No Stimulation groups also clearly had higher startle amplitudes on the 10 CS + noise trials than the Extinction + No Stimulation control group (see Figure 119) however these only approached significance when Newman-Keuls between-group comparisons were made. Nevertheless, the overall results demonstrated that fear-extinguished and non-stimulated rats exhibited no appreciable levels of FPS, while fear-extinguished and amygdaloid-stimulated rats showed robust levels of FPS. This finding is confirmed by the statistical analysis carried out on the difference scores (Difference Score=[10 light + noise trials]−[10 noise-alone trials]).

ANOVA of the difference scores yielded an effect of Stimulation F(3,43)=3.15, p<0.04. Newman-Keuls post hoc tests of this data revealed that the level of FPS exhibited by the in context Extinction + Stimulation, No Extinction + Stimulation, and the No Extinction + No Stimulation groups were significantly more robust than the Extinction + No Stimulation group. As can be seen by Figure 120, these results indicate that 100 electrical stimulations
of the BLA in the same context where fear conditioning took place caused a reinstatement of FPS in rats that received extinction training. Also, electrical stimulation of the amygdala did not appear to interfere with the expression of FPS in rats that received no extinction training. For example, Newman-Keuls post-hoc tests revealed that rats in the No Extinction + In Context Stimulation group (N=11) did not differ significantly in the magnitude of FPS when compared to rats in the No Extinction + No Stimulation group. Taken together, the in-context stimulation experimental results suggest that repeated electrical stimulation of the amygdala, like unsignalled shock, effectively reinstates FPS in rats that have received extinction training. The underlying mechanisms that contribute to this stimulation-induced FPS reinstatement effect will be discussed more fully in the general discussion.
Figure 119. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of extinction or no extinction training and electrical stimulation of the basolateral amygdala or no stimulation (★ P<0.05 relative to noise-alone trials). The mean (S.E.M. ± ) difference scores ([10 CS + noise]-[10 noise-alone]) for the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + Stimulation (N=11) and No Extinction + No Stimulation (N=12) groups demonstrate the impact of electrical stimulation of the basolateral amygdala 24 hours earlier on the reinstatement of fear-potentiated startle during the final test (➕ P<0.05 relative to the Extinction + BLA Stimulation and the two No Extinction groups).
Final Test: FPS Reinstatement Difference Score Results

Figure 120. Mean (S.E.M. ± ) difference scores ([10 CS + noise]-[10 noise-alone]) trials for the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12) No Extinction + No BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) groups on the final test to assess the reinstatement of fear-potentiated startle 24 hours after rats either received 100 stimulations of the basolateral amygdaloid complex (BLA) or no BLA stimulations ( * P<0.05 relative to the Extinction + BLA Stimulation group and the two No Extinction experimental groups).
18. Experiment 7B:

In Context vs. Out of Context Electrical Stimulation of the Amygdala and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats: The Importance of Contextual Factors and Cues in Fear Reinstatement

18.1: Rationale

To determine the role experimental context plays in the amygdaloid stimulation induced reinstatement of FPS, the Extinction + In Context Basolateral Amygdala Stimulation group (N=12) was compared statistically to the Extinction + Out of Context Basolateral Amygdala Stimulation group (N=12). Hence, the purpose of this smaller experiment was to determine whether out of context stimulation of the amygdala would cause FPS reinstatement in fear-extinguished rats. Independent T-tests carried out on the baseline startle amplitudes {t(1,22)=1.72, p=0.10}, noise intensity levels {t(1,22)=1.12, p=0.27}, shock reactivity scores {t(1,22)=1.04, p=0.31}, and the pretest difference scores {t(1,22)=0.22, p=0.83}, revealed no significant group differences indicating that both groups were matched on these variables prior to extinction training and amygdaloid stimulation (see Figures 121, 122, 123 and 125 respectively).
Baseline Acoustic Startle Responses

Figure 121. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the Extinction + In Context BLA Stimulation and the Extinction + Out of Context BLA Stimulation experimental groups (N=12; each).
White-noise Intensity Levels Used to Induce Acoustic Startle Responding

Figure 122. Mean white-noise intensity levels (S.E.M. ± ) recorded in decibels that was used to induce stable acoustic startle responses in the Extinction + In Context BLA Stimulation (N=12) and the Extinction + Out of Context BLA Stimulation (N=12) groups.
Shock Reactivity During Fear Conditioning

Figure 123. Mean (S.E.M. ± ) movement amplitude recorded during the presentation of 30 light + footshock fear conditioning trials to rats in the Extinction + In Context BLA Stimulation and Extinction + Out of Context BLA Stimulation experimental groups (N=12; each).
18.2: Results

18.21: Pretest Results

Consistent with previous experiments, the pretest results indicated that both experimental groups exhibited FPS 48 hours after being administered 30 light + footshock fear conditioning trials. ANOVA of the pretest yielded an effect of Stimulus Condition (3 CS + noise trials vs. 3 noise-alone trials) only $F(1,22)=39.04, p<0.000004$ with no Stimulation Group X Stimulation Context interaction. Newman-Keuls post hoc tests revealed that rats in both experimental groups exhibited higher startle scores on the 3 CS + noise trials relative to the 3 noise-alone trials, indicating that conditioned fear responding had been acquired after exposure to 30 light + footshock training trials. The lack of a significant Stimulation Group X Stimulation Context interaction combined with the failure of the T-test to find any significant between group differences on the pretest difference scores $([3 \text{ CS} + \text{ noise}]-[3 \text{ noise-alone}])$ demonstrates that the two experimental groups in question were equated on FPS before any extinction training or amygdaloid stimulation was initiated (see Figure 124 and 125).
Figure 124. Mean (S.E.M.) acoustic startle amplitudes on the 3 noise-alone and 3 CS + noise trials of the pretest for the Extinction + In Context BLA (N=12) and the Extinction + Out of Context BLA (N=12) stimulation groups as a function of fear conditioning only (★ P<0.05 relative to noise-alone trials). Mean difference scores (S.E.M. ± ) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the equivalent levels of fear-potentiated startle exhibited by both experimental groups 48 hours after 30 fear conditioning trials were administered.
Figure 125. Mean difference scores (S.E.M. ± ) of the pretest ([3 CS + noise]-[3 noise-alone]) depicts the equally robust magnitude of fear-potentiated startle expressed by both the Extinction + In Context BLA Stimulation and the Extinction + Out of Context BLA Stimulation groups (N=12; each) 48 hours after exposure to 30 light + footshock fear conditioning trials.
18.22: Fear-Potentiated Startle Reinstatement Test Results

The principal result of this experiment is that both in context and out of context stimulation of the amygdala causes a reinstatement of FPS in fear-extinguished rats. ANOVA (2 Stimulation Group X 2 Stimulus Condition) on the final test revealed an effect of Stimulus Condition F(1,22)=36.42, p<0.000005, as well as a significant Stimulation Group X Stimulus Condition interaction F(1,22)=5.29, p<0.04. Newman-Keuls post hoc comparisons revealed that both the Extinction + In Context Basolateral Amygdala Stimulation group and the Extinction + Out of Context Basolateral Amygdala Stimulation group showed significantly higher levels of acoustic startle responding on the CS + noise trials than on the noise-alone trials on the final test (see Figure 126). These results demonstrate that electrical stimulation of the amygdala that occurs either in the training/testing context or out of the testing/training context causes a reinstatement of FPS in rats that have received extinction training. A more interesting observation was that the Extinction + In Context Basolateral Amygdala Stimulation group seemed to have higher acoustic startle scores on the 10 CS + noise trials on the final test than the Extinction + Out of Context Basolateral Amygdala Stimulation Group (see Figure 126). Newman-Keuls post hoc tests that used a pooled error term to calculate critical ranges failed to find a significant difference between the In Context and Out of Context stimulation groups on this particular stimulus condition (i.e. 10 CS + noise trials) when the significant Group X Stimulus Condition interaction was compared.

Nevertheless, the result still seemed to indicate that the In Context Amygdala Stimulation group exhibited a greater magnitude of fear than the Out of Context Amygdala Stimulation group. This was confirmed by an independent T-test carried out on the Difference Score data {Difference Score= (10 light + noise trials) - (10 noise-alone trials)} which yielded a significant effect t(1,22)=2.30, p<0.04. Figure 127 depicts the mean difference score results of the final test. Figure 127 clearly shows that the In Context Amygdala Stimulation group had a greater magnitude of fear than the Out of Context Amygdala Stimulation group. Examination of the difference scores demonstrates that the In Context Amygdala Stimulation group’s difference score (mean = 133.7, SEM ± 26.8) is significantly greater than the Out of Context Amygdala Stimulation group’s difference score (mean= 59.9, SEM ± 17.6). In terms of percentage increases, the In Context
Amygdala Stimulation group showed an 82.2% mean increase in CS-enhanced acoustic startle over the noise-alone condition, whereas the Out of Context Amygdala Stimulation group showed only a 38.3% mean increase in CS-enhanced acoustic startle over the noise-alone condition.

These final test results are interesting since they suggest that experimental context and contextual cues may be important factors in facilitating the reinstatement of FPS in rats that have received extinction training. It is plausible that both neural excitation and fear reinstatement are significantly more enhanced when amygdaloid stimulation is administered in a context where fear conditioning occurred as opposed to a distinct context where fear conditioning did not occur. Thus although both stimulation groups show fear-reinstatement, the magnitude of fear was smaller in the out of context amygdaloid stimulation group, raising the interesting possibility that rats in this group did not show the same level of fear reinstatement as they had no access to the fearful environmental and contextual cues that would facilitate robust fear reinstatement. As a result, the rats in the out of context stimulation group could not as easily overcome extinction training simply because they did not have access to the fear-eliciting contextual cues in the experimental apparatus and as such they could not as easily overcome the inhibitory learning laid down by extinction training.
Final Test: Fear-Potentiated Startle Reinstatement Results

Figure 126. Mean (S.E.M. ± ) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of extinction training and in context versus out of context electrical stimulation of the basolateral amygdala ( * P<0.05 relative to noise-alone trials). The mean (S.E.M. ± ) difference scores ([10 CS + noise]-[10 noise-alone]) for the Extinction + In Context BLA Stimulation and the Extinction + Out of Context BLA Stimulation groups (N=12; each) demonstrate the impact of electrical stimulation of the basolateral amygdala on fear-potentiated startle reinstatement. The results also highlight the important role played by contextual factors in influencing the magnitude of fear reinstatement exhibited by the two experimental groups ( + P<0.05 relative to the Extinction + In Context BLA Stimulation group).
Final Test: Fear-Potentiated Startle Reinstatement Score Results

Figure 127. Mean (S.E.M. ±) difference scores ([10 CS + noise]-[10 noise-alone]) for the Extinction + In Context BLA Stimulation (N=12) and the Extinction + Out of Context BLA Stimulation (N=12) groups on the final test to assess the reinstatement of fear-potentiated startle 24 hours after rats received 100 electrical stimulations of the basolateral amygdaloid complex either in the fear conditioning/startle testing apparatus (In Context) or in a completely different apparatus (Out of Context). The significant differences between the two groups highlights the importance of contextual cues and fear memories on the reinstatement of fear responding (★ P<0.05 relative to the Extinction + In Context BLA amygdala Stimulation group).
18.23: AD Threshold Results of Experiment 7A and 7B

Results obtained from the AD threshold experimental session found that prior electrical stimulation of the amygdala reduces AD current thresholds and may therefore facilitate neural sensitisation. ANOVA of the AD threshold data obtained from the EEG recordings yielded an effect of Stimulation $F(3,43)=6.04$, $p<0.002$. Newman-Keuls post hoc comparisons revealed that the Extinction + 100 Basolateral Stimulation group’s mean AD threshold was significantly lower than the Extinction + No Stimulation and the No Extinction + No Stimulation groups. The Newman-Keuls analysis also found that the No Extinction + Stimulation group’s mean AD threshold was significantly lower than the Extinction + No Stimulation group’s mean AD threshold and approached significance when compared to the No Extinction + No Stimulation group’s mean AD threshold ($P=0.06$). As shown in Figure 128, the rats in the experimental groups that received amygdaloid stimulation had lower mean AD current thresholds than did the rats in the experimental groups that received no amygdaloid stimulation.
Amygdaloid After-Discharge Thresholds
Results

Figure 128. Mean (S.E.M. ±) after discharge current thresholds recorded from the amygdala of rats in the Extinction + BLA Stimulation (N=12), Extinction + No Stimulation (N=12), No Extinction + BLA Stimulation (N=11) and No Extinction + No Stimulation (N=12) experimental groups ( * P<0.05 relative to the Extinction + BLA Stimulation group only).
The lower AD thresholds observed in the amygdala stimulated rats is an important finding because it may indicate that the stimulation of the amygdala that caused the reinstatement of FPS in fear extinguished rats may have also caused neural sensitisation in the amygdala. The amygdaloid stimulation and the sensitisation which followed could have facilitated fear-reinstatement by initially making the stimulated animals responsive to contextual fear cues associated with the training/testing apparatus. These contextual cues may not have been completely extinguished during extinction training, and as a result, eventually caused rats to become responsive to the specific fear-eliciting CS (i.e. light) once again. In other words, the repeated stimulation of the amygdala (i.e. 100 stimulations) could have produced either an internal state of fear or fear to the contextual cues of the testing apparatus. This fear, in turn, could have led to a rapid displacement of the inhibitory learning laid down by extinction training and to a reinstatement of FPS.

Alternatively, the stimulation may have facilitated the retrieval of fear memories stored locally in the amygdala and thus caused a reinstatement of FPS responding. In other words, electrical stimulation could have caused the reinstatement of FPS by reexciting or re-sensitising amygdaloid neurons and pathways that contained the fear-memory trace originally established during Pavlovian fear conditioning. Another possibility is that electrical stimulation of the amygdala may have activated other cortical brain areas that are intimately connected to the amygdala and that are involved in the storage and consolidation of fear-memories. Activation of such higher cortical regions (i.e. components of the medial temporal lobe memory system) via amygdaloid stimulation has the potential to set off a cascade of neurochemical events that may activate more complex fear memory traces that can act as mnemonic cues that allow the amygdala to retrieve fear information that is stored locally in the amygdala. Thus, amygdaloid stimulation may cause fear reinstatement by simultaneously activating fear-memories that are stored locally and remotely.

These ideas, combined with the research findings of the present experiment are important since they may explain how stressful events or perceived stressors can cause spontaneous recovery of phobias and paranoia in humans. More importantly, the findings provide additional scientific evidence that fear memories may be stored in the amygdala and that amygdaloid activation via electrical stimulation after extinction training may cause
the retrieval of fear memories that may in turn lead to autonomic and behavioural manifestations of fear. This may explain why fear manifestations and ideations are commonly associated with epilepsy and amygdaloidal after-discharge activity (for a review see Gloor, 1992). Hence, amygdaloid neural activation whether it results from electrical stimulation or epileptic seizure may reactivate fear memories by reenergising neurosynaptic pathways that had been strengthened or established after learning and experience. It is important to point out that the amygdala is reciprocally connected to various brain areas such as the temporal lobes, perirhinal and insular cortices, the hippocampus and the medial prefrontal cortex that together make up the medial temporal lobe memory system (McDonald, 1998; Alheid, et al., 1995). Such a close association with these regions would make it possible for the amygdala to tap into a vast memory system that would give it the necessary resources to reconstruct CS-UCS fear associations that have been displaced by extinction training.

18.24: Seizure Classification Results of Experiment 7A and 7B

Figure 129 depicts the typical pattern of after-discharge activity recorded from amygdala neurons in a number of rats that belonged to the Extinction + Stimulation and Extinction + No Stimulation groups of Experiment 7A. As can be seen from this figure, electrically induced after-discharge activity in the amygdala produces a distinct neuronal firing pattern that is marked by high amplitudes and a frequent firing of numerous cells all at one time. Often the interval between peaks is short and is commonly marked by small inter-spike intervals (see Figure 129). Typically, electrically induced after-discharge activity in the amygdala produces seizures that can be classified based on the degree of behavioural change they produce in the animal being studied.
Figure 129 depicts a typical after-discharge pattern recorded from neurons in the basolateral complex after electrical stimulation was used to induce after-discharge activity in the amygdala. The dark arrow represents the point at which the electrical stimulation was administered to the amygdala. To the left of the dark arrow is the cellular activity of amygdala neurons prior to the after-discharge inducing electrical stimulation and just to the right of the dark arrow is the characteristic after-discharge pattern recorded from the amygdala. As can be seen the after-discharge activity in the amygdala generally produces numerous spikes that are high in amplitude and are characterized by short inter-spike intervals. Further to the right of the after-discharge pattern the neurons have returned to normal activity level which indicates that they are either in a refractory period or they have returned back to their membrane resting potentials. The first two recordings (1 and 2) were obtained from two rats that belonged to the Extinction + Basolateral Amygdala Stimulation and the No Extinction + Basolateral Amygdala Stimulation groups of Experiment 7A whilst, the third and fourth recordings were obtained from rats belonging to the Extinction + No Stimulation group of Experiment 7A.
18.25: Seizure Classification Tables

Tables 1 to 4 list the name of each in context amygdaloid electrode experimental group and its corresponding seizure typology. Table 5 lists the Extinction + Out of Context Amygdala Stimulation group’s seizure typology and corresponding behavioural profile. These tables also provide a categorization of seizure type (i.e. Type 1 to Type 5) and contain the various behavioural changes that were associated with each seizure type (Racine, 1972a,b).
Table 1: Extinction + Basolateral Amygdala Stimulation Group (N=12)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (µA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>(X) 950</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking (squinting), head bobbing and oral movements.</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>(X) 1350</td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking, behavioural arrest and some head bobbing.</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>(X) 1100</td>
<td></td>
<td></td>
<td>Eye blinking, behavioural arrest, oral movements and head bobbing.</td>
</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td>(X) 450</td>
<td></td>
<td>Eye blinking, behavioural arrest, head bobbing, rearing (both limbs raised).</td>
</tr>
<tr>
<td>220</td>
<td></td>
<td></td>
<td>(X) 500</td>
<td></td>
<td></td>
<td>Eye blinking, behavioural arrest, head movement, one front paw shaking.</td>
</tr>
<tr>
<td>29</td>
<td></td>
<td></td>
<td></td>
<td>(X) 400</td>
<td></td>
<td>Eye squinting (blinking), behavioural arrest, head-bobbing and shaking one paw.</td>
</tr>
<tr>
<td>39</td>
<td></td>
<td></td>
<td></td>
<td>(X) 450</td>
<td></td>
<td>Facial twitching, eyes squinting and some head bobbing.</td>
</tr>
<tr>
<td>54</td>
<td></td>
<td></td>
<td></td>
<td>(X) 450</td>
<td></td>
<td>Facial twitching, eye squinting, move around a lot, and one front limb lifted a bit.</td>
</tr>
<tr>
<td>47</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 750</td>
<td>Facial twitch, eyes blinked, head bobbed, shook forelimbs, reared and fell down.</td>
</tr>
<tr>
<td>125</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 650</td>
<td>Facial twitch, eye squinting, head bobbing, and raised one front paw.</td>
</tr>
<tr>
<td>119</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 750</td>
<td>Eye squinting, head bobbing and both forelimbs were raised.</td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 850</td>
<td>Eyes squinted, head bobbing, forelimbs raised, rearing and running around a lot.</td>
</tr>
</tbody>
</table>

Table 1: As can be seen in the above table, of the twelve rats in the Extinction + Basolateral Amygdala Stimulation group N=5 exhibited Type 2 seizures, N=3 exhibited Type 3 seizures, N=3 displayed Type 4 seizures and N=1 animal showed signs of a Type 5 full blown fall down seizure.
Table 2: Extinction + No Basolateral Amygdala Stimulation Group (N=12)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure type for each animal. Right next to each (X) symbol is the electrical current level in micro-Amps (μA) that induced after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>66</td>
<td></td>
<td></td>
<td>(X) 1650</td>
<td></td>
<td></td>
<td>Eye blinking, head bobbing, forelimbs raised and rearing.</td>
</tr>
<tr>
<td>79</td>
<td></td>
<td></td>
<td></td>
<td>(X) 950</td>
<td></td>
<td>Eye squinting, head bobbing, forelimbs raised, rearing and fell to the right.</td>
</tr>
<tr>
<td>69</td>
<td></td>
<td></td>
<td></td>
<td>(X) 750</td>
<td></td>
<td>Eye blinking (squinting), head bobbing, right paw raised.</td>
</tr>
<tr>
<td>84</td>
<td></td>
<td>(X) 900</td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking, head bobbing, oral movements, teeth clicking.</td>
</tr>
<tr>
<td>72</td>
<td></td>
<td></td>
<td>(X) 1350</td>
<td></td>
<td></td>
<td>Eye blinking (squinting), head bobbing, paws raised and shaking.</td>
</tr>
<tr>
<td>82</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1150</td>
<td></td>
<td>Eye blinking and behavioural arrest, oral mastication.</td>
</tr>
<tr>
<td>88</td>
<td>(X) 1350</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Behavioural arrest and eye blinking.</td>
</tr>
<tr>
<td>89</td>
<td>(X) 1350</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking (squinting), small head twitching and behavioural arrest.</td>
</tr>
<tr>
<td>109</td>
<td>(X) 1000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking and head bobbing.</td>
</tr>
<tr>
<td>101</td>
<td>(X) 1900</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye squinting, behavioural arrest and some head bobbing.</td>
</tr>
<tr>
<td>115</td>
<td></td>
<td></td>
<td>(X) 950</td>
<td></td>
<td></td>
<td>Eye squinting, head bobbing, both paws in the air and running around shaking</td>
</tr>
<tr>
<td>140</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1350</td>
<td></td>
<td>Eye blinking, head bobbing, paws raised, rearing, running around, teeth clicking.</td>
</tr>
</tbody>
</table>

Table 2: As can be seen in the above table, of the twelve rats in the Extinction + No Amygdala Stimulation group N=1 exhibited a Type 1 seizure, N=5 exhibited Type 2 seizures, N=3 displayed Type 3 seizures and N=2 animals showed signs of a Type 4 seizure and N=1 had a Type 5 seizure.
Table 3: No Extinction + Basolateral Amygdala Stimulation Group (N=11)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure Type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (µA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>4</td>
<td>(X) 550</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking, head bobbing oral chewing action.</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>(X) 700</td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking, head bobbing, single paw shaking and teeth clicking.</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td></td>
<td>(X) 850</td>
<td></td>
<td></td>
<td>Eye blinking, head movement, teeth clicking, running and some rearing.</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td></td>
<td></td>
<td>(X) 600</td>
<td></td>
<td>Eye blinking, head bobbing, one paw elevated and twitching.</td>
</tr>
<tr>
<td>15</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1050</td>
<td>Eye blinking (squinting), head bobbing and front paw in the air.</td>
</tr>
<tr>
<td>73</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 550</td>
<td>Eye blinking, head bobbing and teeth click (mastication).</td>
</tr>
<tr>
<td>53</td>
<td></td>
<td></td>
<td></td>
<td>(X) 500</td>
<td></td>
<td>Eye blinking, behavioural arrest, head bobbing and head shaking.</td>
</tr>
<tr>
<td>37</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1300</td>
<td>Eye blinking, head bobbing, lifting one paw in air and shaking it.</td>
</tr>
<tr>
<td>60</td>
<td></td>
<td></td>
<td></td>
<td>(X) 850</td>
<td></td>
<td>Eye blinking, head bobbing, both front paws raised maybe rearing.</td>
</tr>
<tr>
<td>59</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 600</td>
<td>Behavioural arrest, eye twitch, head bob, rearing, and paws lifted up.</td>
</tr>
<tr>
<td>105</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 700</td>
<td>Eyes squinting, head bob, teeth clicking, rearing, and fell down.</td>
</tr>
</tbody>
</table>

Table 3: As can be seen in the above table, of the eleven rats in the No Extinction + Basolateral Amygdala Stimulation group N=2 exhibited Type 2 seizures, N=6 displayed Type 3 seizures and N=2 animals showed signs of a Type 4 seizure and N=1 had a Type 5 seizure.
Table 4: No Extinction + No Amygdala Stimulation Group (N=12)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure Type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (µA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>55</td>
<td></td>
<td></td>
<td>(X) 1750</td>
<td></td>
<td></td>
<td>Eye blinking, head movement, forelimb shaking and running.</td>
</tr>
<tr>
<td>77</td>
<td></td>
<td></td>
<td>(X) 1100</td>
<td></td>
<td></td>
<td>Eye blinking, teeth clicking, head bobbing, and front leg was raised.</td>
</tr>
<tr>
<td>78</td>
<td></td>
<td></td>
<td>(X) 800</td>
<td></td>
<td>(X) 1300</td>
<td>Eye blinking, behavioural arrest, head bobbing and teeth chattering.</td>
</tr>
<tr>
<td>80</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1450</td>
<td>Eyes blinking, front paws raised, rearing and tilted to one side (fall).</td>
</tr>
<tr>
<td>81</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1050</td>
<td>Behavioural arrest, eye blinking and head bobbing.</td>
</tr>
<tr>
<td>71</td>
<td></td>
<td>(X) 650</td>
<td></td>
<td></td>
<td></td>
<td>Eye blink, head bobbing and behavioural arrest.</td>
</tr>
<tr>
<td>28</td>
<td></td>
<td></td>
<td></td>
<td>(X) 950</td>
<td></td>
<td>Eye blinking, head twitching, teeth clicking and one paw was raised.</td>
</tr>
<tr>
<td>86</td>
<td></td>
<td></td>
<td>(X) 1150</td>
<td></td>
<td></td>
<td>Eye squinting, raised two front paws, head bob and teeth clicking.</td>
</tr>
<tr>
<td>122</td>
<td>(X) 850</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking and behavioural arrest.</td>
</tr>
<tr>
<td>129</td>
<td></td>
<td></td>
<td></td>
<td>(X) 700</td>
<td></td>
<td>Eye squinting, head bobbing and shaking one paw in the air.</td>
</tr>
<tr>
<td>153</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1200</td>
<td></td>
<td>Eye squinting, head bob, jaw movements and paw in the air.</td>
</tr>
</tbody>
</table>

Table 4: As can be seen in the above table, of the twelve rats in the No Extinction + No Amygdala Stimulation group N=1 exhibited a Type 1 seizure, N=4 exhibited Type 2 seizures, N=5 displayed Type 3 seizures, N=1 animal showed signs of a Type 4 seizure and N=1 had a Type 5 seizure.
**Table 5**: Extinction + Out of Context Basolateral Amygdala Stimulation Group (N=12)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure Type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (μA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>118</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1050</td>
<td>Eyes squinting, head bobbing, rearing, paws raised and then fell over.</td>
</tr>
<tr>
<td>124</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1800</td>
<td></td>
<td>Eye squinting, raised paws, jaw chewing and some head bobbing.</td>
</tr>
<tr>
<td>120</td>
<td></td>
<td>(X) 850</td>
<td></td>
<td></td>
<td></td>
<td>Eye twitching, head bobbing, oral movements and some paw lifting.</td>
</tr>
<tr>
<td>121</td>
<td></td>
<td>(X) 550</td>
<td></td>
<td></td>
<td></td>
<td>Eye squinting, jaw and teeth clicking, and head bobbing.</td>
</tr>
<tr>
<td>113</td>
<td>(X) 1050</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eyes squinting, oral movements and head bobbing.</td>
</tr>
<tr>
<td>108</td>
<td></td>
<td>(X) 1350</td>
<td></td>
<td></td>
<td></td>
<td>Eye blinking, oral movements, head bobbing and paw lifting and shaking.</td>
</tr>
<tr>
<td>107</td>
<td>(X) 1300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye twitching, head bobbing, and jaw twitching or chewing action.</td>
</tr>
<tr>
<td>111</td>
<td>(X) 700</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Eye twitching, head bobbing, and front paw was raised in the air.</td>
</tr>
<tr>
<td>104</td>
<td></td>
<td>(X) 850</td>
<td></td>
<td></td>
<td></td>
<td>Eyes twitching, head bobbing, jaw movements and a paw was raised.</td>
</tr>
<tr>
<td>126</td>
<td></td>
<td></td>
<td></td>
<td>(X) 600</td>
<td></td>
<td>Eyes squinting, head bobbing and quiet mastication movements (oral).</td>
</tr>
<tr>
<td>134</td>
<td></td>
<td></td>
<td></td>
<td>(X) 900</td>
<td></td>
<td>Eyes squinting, head bobbing, jaw movements and paw lifted into the air.</td>
</tr>
<tr>
<td>139</td>
<td></td>
<td></td>
<td></td>
<td>(X) 800</td>
<td></td>
<td>Eye blinking, facial twitching and rearing with both front paws raised.</td>
</tr>
</tbody>
</table>

**Table 5**: As can be seen in the above table, of the twelve rats in the Extinction + Out of Context Basolateral Amygdala Stimulation group N=5 exhibited Type 2 seizures, N=5 displayed Type 3 seizures, N=1 animal showed signs of a Type 4 seizure and N=1 had a Type 5 fall down seizure.
19. Experiment 8:
Electrical Stimulation of the Granular and Dysgranular Insular Cortices, the Posterior Agranular Insular Region and the Ectorhinal and Perirhinal Cortical Areas and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats that have received Extinction Training

19.1: Rationale

Experiment 7A determined that FPS could be reinstated in fear-extinguished rats by electrical stimulation of the amygdala. The perirhinal and insular regions of the temporal lobe are intimately connected with the lateral and basal amygdaloid nuclei (see McDonald 1998; also see Chapter 3 of this thesis). Furthermore, the perirhinal and insular cortical regions provide auditory, visual, and nociceptive input to the lateral and basolateral amygdalar nuclear groups (McDonald, 1998; Shi and Davis, 1998a,b; Shi and Cassell, 1999; Shi and Davis, 1999; LeDoux, et al., 1990; LeDoux, et al., 1991; Ono, et al., 1995; Uwano, et al., 1995; Romanski and LeDoux, 1993; Turner and Zimmer, 1984; McDonald and Jackson, 1987; Dostrovski and Craig, 1996; McDonald and Mascagni, 1996). Research has shown that lesions of the rostral perirhinal cortex block the expression of fear-induced defensive freezing behaviour elicited either by auditory or visual conditioned cues (Corodimas and LeDoux, 1995). In a similar fashion, Falls and colleagues (1997) demonstrated that lesions of the perirhinal cortex disrupt the expression of FPS. This finding is consistent with earlier research demonstrating that perirhinal cortex lesions interfere with the expression of FPS in rats (Falls, et al., 1992; Rosen, Hitchcock, Miserendino, Falls, Campeau, and Davis, 1992; also see Campeau and Davis, 1995b).

With reference to the insular cortex, research by Shi and Davis, (1999) has shown that combined lesions of the caudal insular cortex (IC) and the posterior intralaminar nucleus of the thalamus (PIN) block the acquisition of FPS in rats trained using both auditory and visual CS. Furthermore, this combined lesioning technique employed by Shi and Davis (1999) was also successful in blocking the shock sensitisation of startle. Interestingly, combined posttraining lesions of the PIN and IC did not block the expression of FPS. More recently, combined posttraining lesions of the PIN and IC blocked defensive freezing behaviour to an auditory CS that was previously paired with aversive footshock but did not
interfere with conditioned fear responses evoked by contextual cues (Brunzell and Kim, 2001).

Since the perirhinal and insular cortical regions appear to be involved in conditioned fear learning and expression (Falls, et al., 1992; Rosen, et al., 1992; Campeau and Davis, 1995b; Shi and Davis, 1999; Corodimas and LeDoux, 1995; Brunzell and Kim, 2001), and provide sensory input to the lateral and basolateral amygdaloid nuclei (see McDonald, 1998; also see Chapter 3), it is logical to investigate whether or not the perirhinal, ectorhinal and insular cortical regions contribute to FPS reinstatement. Thus, the purpose of the present experiment is to see if electrical stimulation of the rhinal/insular cortical region will restore FPS reinstatement in fear-extinguished rats. The methods and results of this particular experiment are listed below.

19.2: Method

19.21: Subjects

Twenty-two naive, male Wistar rats bred and housed in the psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 340 grams at the beginning of the experiment (range 265-370 grams). A constant temperature of 20° Celsius (±1° Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulation took place during the light portion of this cycle.

19.22: Stereotaxic Surgery

Rats were anesthetised with sodium pentobarbitone (95.0 to 110 mg/kg), mounted in a Stoelting stereotaxic instrument (Wood Dale, IL), and then surgically implanted with a single bipolar stainless steel twisted electrode (MS-303/1; Plastics-One, Roanoke, VA) aimed at the medial portion of the temporal lobe region that contains the ectorhinal and perirhinal cortices, and granular and dysgranular insular cortices. Prior to surgery all electrodes were prepared as described in Experiment 7A. Electrodes were cut to length, insulation was scraped from their tips (0.4 mm), and the wires were gapped (0.5 mm). All
electrodes were implanted using the flat skull technique described in Experiment 7A except that the coordinates used were A.P. - 2.90 mm from bregma, M.L. ± 6.40 mm from the sagittal suture, and D.V. -6.80 mm from the skull surface. The coordinates used for stereotaxic surgery were obtained from the rat brain atlas of Paxinos and Watson (1986 and 1998 versions) and took into consideration the results obtained from three lesion studies that evaluated the contribution made by the perirhinal/insula region to FPS (Rosen, et al., 1992; Campeau and Davis, 1995b; Shi and Davis, 1999). This electrode placement was designed to target the granular and dysgranular insular cortices along with the perirhinal and ectorhinal cortices. Essentially, the goal was to place the bipolar electrodes in an area between the ventral insular cortices (i.e. the granular, dysgranular, and agranular insula) and the rhinal cortical regions (i.e. the ectorhinal and perirhinal cortices). It was hoped that such a placement would result in the activation of both the insular and rhinal cortical regions when the 400 µA current was passed through the electrode during FPS reinstatement training. These areas were chosen for investigation since various anatomical tracing experiments have demonstrated that the amygdaloid complex receives rich innervation from the granular and dysgranular cortices and that these cortices may be involved in the processing of nociceptive information during fear conditioning (McDonald, 1998; Shi, and Davis, 1997; 1999). The reason the ectorhinal and perirhinal cortical regions were also selected for experimental manipulation is that they convey visual information to the lateral and basolateral amygdala during fear conditioning (Ono, et al., 1995; McDonald, 1998; Shi and Davis, 2001) and seem to be involved in the expression of FPS (Falls, et al., 1992; Rosen, et al., 1992; Campeau and Davis, 1995b). The eventual names assigned to the two experimental groups (i.e. Extinction + In Context Perirhinal/Insular Cortex Stimulation group vs. Extinction + No Perirhinal/Insula Cortex Stimulation group) were chosen as it was felt they provided a reasonable description of the cortical regions that were to be stimulated in this particular study.

Due to the extreme laterality of the coordinates used, the muscle connected to the temporal bone of the skull was cut with a scalpel and retracted to allow for accurate implantation of the bipolar electrode. Electrodes were unilaterally implanted with half of the animals receiving an electrode in the right hemisphere and the remaining animals implanted in the left hemisphere. The electrode was secured to the skull surface via a head
cap constructed from dental acrylic. This head cap was attached to five stainless steel self-tapping jeweller screws (3.20 mm in length, Lomat Precision Tools, Montreal Quebec, Canada) embedded in the skull surface. Specially designed dummy dust caps (303DC and 303DCA; Plastics-One, Roanoke, VA) were screwed onto the secured electrode to prevent rats from chewing the plastic portion of the electrode and making it inoperative. Following surgery, rats were placed into a recovery cage until the anaesthetic wore off and then were returned to normal group housing conditions. Rats were allowed a 14 day recovery period to ensure their wounds were adequately healed before any behavioural testing or experimental manipulation commenced.

19.23: Procedure

19.24: Baselining, Fear Conditioning, and Pretesting

All details and procedures concerning testing apparatus, baselining, fear conditioning, pretesting for FPS and histology are the same as those described in the methods section of Experiment 7A. The baselining procedure was used to establish stable acoustic startle responding in the experimental groups and to reduce startle variability both within-subjects and between-groups. The pretest administered 48 hours after fear conditioning was used to help assign rats to experimental groups in a manner that ensured that the two experimental groups were equated on FPS responding.

19.25: Group Assignment, Extinction Training, and Unsignalled Electrical Stimulation of the temporal lobe Rhinal and Insular Cortices

Rats were assigned to one of two experimental groups (N=11 per group) with similar averaged startle scores, noise intensity levels, and levels of FPS. Twenty-four hours after the pretest, rats were placed in their designated test chambers, and after a 5 min habituation period, exposed to 140 light-alone extinction trials. The interstimulus interval for the light presentation during extinction training was 15 s. The two experimental groups were the Extinction + In Context Perirhinal/Insular Cortex Stimulation group (N=11) and the Extinction + No Stimulation Perirhinal/Insular Cortex group (N=11). This extinction training technique was used to extinguish conditioned fear
responding to the CS (i.e. light) and this method has been shown to reliably extinguish FPS in rats (see Experiment 2 and 7A; also see Walker and Davis, 2002; Davis, et al., 2003; Borowski and Kokkinidis, 1998; on extinction methods used inhibit FPS).

Forty-eight hours after extinction, rats were connected to a programmable constant current stimulator via an insulated bipolar electrode cable (305-S304-CCT 80CM TT2 CS, Plastics One, Roanoke VA) which connected to the MS- 303/1 bipolar electrode. The wire cable was protected by a stainless steel coil spring to prevent rats from damaging the wire. After rats were connected to the stimulator, they were then placed into their designated chambers and allowed 5 min to acclimatise to the test environment. Rats assigned to the Extinction + In Context Perirhinal/Insular Stimulation group were administered 100 unsignalled electrical brain stimulations.

The current intensity used to stimulate the perirhinal and insular regions of the temporal lobe was 400 µA (base to peak current) with a pulse-duration of 0.1 ms, and a frequency of 100 Hz. The interstimulus interval used in this experimental manipulation was 3 s and the duration of the stimulation was 500 ms. The programmable constant current stimulator was connected to the 386 computer that ran the startle testing and fear conditioning programs, and a specially designed computer program was used to trigger the electrical brain stimulation. After the electrical stimulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment. Rats assigned to the In Context No Stimulation Perirhinal/Insular group did not receive any electrical brain stimulation.

19.26: Final Test

Twenty-four hours after stimulation or no stimulation administration, rats were tested for the reinstatement of FPS. The final test employed a quasi-counterbalanced design. This procedure entailed presenting half of the rats in each experimental group with 10 noise-alone trials first followed by 10 light + noise trials, whilst the other half of the rats in each experimental group were exposed to 10 light + noise trials first followed by 10 noise-alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the white-noise burst was presented during the last 100 ms of the light interval. The purpose of
the final test was to ascertain whether electrical stimulation of the rhinal/insular cortical region would cause a reinstatement of FPS in fear-extinguished rats.

19.27: After Discharge Thresholds and Seizure Classification

Ninety-six hours after the final test all rats were assessed for after-discharge (AD) threshold levels and classification of seizure type produced when the first AD was recorded. This was done in order to see if 100 electrical pulses administered earlier caused neural sensitisation within the rhinal/insular region. Rats were placed into the Plexiglas after-discharge testing chamber and had their electrode connected to the insulated bipolar electrode cable. The bipolar electrode cable was connected to a programmable constant current stimulator that was in turn connected to a model 50 Grass EEG machine. After a 2 minute habituation period elapsed, an EEG recording period began so as to establish baseline EEG activity in the rhinal/insular cortex. The baseline recording lasted for a period of 2 minutes. Rats then received the first electrical rhinal/insular cortex stimulation to try and initiate AD activity in the perirhinal/insula cortex.

The first current intensity used to initiate AD activity in the perirhinal/insula cortex was 400 μA (base to peak current; 0.1 ms pulse duration; 100 Hz frequency; 500 ms duration) which was the same current used to try reinstate FPS. EEG activity in the perirhinal/insular cortex was then recorded for a period of 1 min after stimulation. If no AD activity was recorded, the current was raised by 50 μA, and the rat’s brain was again stimulated. Following the electrical stimulation, EEG activity was again recorded in the perirhinal/insula cortex for a 1 min period. The current was raised in increments of 50 μA followed by 1 min EEG recordings until an AD was recorded in the perirhinal/insula cortex. Once an AD was recorded and confirmed, recording continued for 2 minutes after the AD occurred. The level of seizure associated with the AD was classified on a scale of Type 1 to Type 5 for each rat (Racine, 1972a,b).

19.28: Statistical Analysis and Dependent Measures

A 2 Stimulation Group X 2 Stimulus Condition (light + noise vs. noise-alone) ANOVA was used to analyse the pretest and final test data. Newman-Keuls multiple comparisons (α = 0.05) were used to assess differences between experimental group means. The Newman-
Keuls post hoc test followed the same statistical protocol as described in Experiment 7A in dealing with any between group comparisons. Shock reactivity scores were evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 CS-UCS trials. Independent T-tests were used to statistically analyse several dependent measures, including, noise intensity level, baseline startle amplitude scores, and FPS difference scores (Difference Score = [CS + noise trials] - [noise-alone trials]) that were calculated for both the pretest and final test.

Two rats from the Perirhinal/Insular Extinction + Stimulation group lost their head caps before AD thresholds testing was initiated, leaving this experimental group with N= 9 before perfusion and histology. One animal in the Perirhinal/Insular Extinction + No Stimulation control group died and another rat in this group had its head cap broken when trying to remove the aluminium dust cap leaving this group with an N= 9.
19.3: Results and Discussion

19.3.1: Histological Results

The histological techniques used in this study are the same as those described earlier and were used to determine the composition of the two experimental groups in this study. Microscopic examination of brain sections determined that one animal out of the Perirhinal/Insular Extinction + Stimulation group had to be removed from the study because the electrode was misplaced and was located in the caudate putamen. This left the Perirhinal/Insular Extinction + Stimulation group with N= 8 and the Perirhinal/Insular Extinction + No Stimulation control group with an N= 9. Figure 130 depicts the location of electrodes stereotaxically placed in the Perirhinal/Insular Cortex region of the rats for the two experimental groups.

One of the major concerns regarding the surgical placement of the electrodes was that they did not descend far enough ventrally to actually come into contact with the perirhinal cortex. Rather, most of the bipolar electrodes were located in the granular and dysgranular insular region. Also, some electrode tips did intrude into the posterior agranular insula and the ectorhinal cortex slightly dorsal to the perirhinal cortex proper. While these electrode placements would be considered perirhinal according to the 1986 version of the rat brain atlas of Paxinos and Watson, they would not be defined as perirhinal by the 1998 version of Paxinos and Watson (see Paxinos and Watson 1986 vs. Paxinos and Watson, 1998). Basically, the 1998 version of this rat brain atlas has divided what was once considered the perirhinal cortex (see Paxinos and Watson, 1986) into several distinct cortical regions. These include, the granular and dysgranular insula cortical areas, the agranular posterior insula cortex, the ectorhinal cortex, the entorhinal cortex and finally the perirhinal cortex which is located around -2.80 mm to -3.30 mm posterior to bregma and -7.40 mm ventral to the skull surface at ± 6.40 mm lateral to the sagittal suture (see Paxinos and Watson, 1998).

Of course, it would have been favourable to have implanted the electrodes a bit more ventral, perhaps using a dorsoventral coordinate of -7.30 mm in relation to the skull surface instead of the -6.80 mm used in this experiment. This would have increased the probability of activating the perirhinal cortex as well as the granular and dysgranular insular regions.
Also, it would have been better had more of the electrodes been implanted in a region between -2.56 mm and -3.14 mm posterior to bregma. This would have ensured a greater activation of not only the ectorhinal and perirhinal cortical regions but it would have still meant that the granular, dysgranular and agranular insular regions would have activated as well. In any event, the placement of the electrodes as they stand, and given that a 400 μA current can spread between 0.3 mm and 0.5 mm from a non-insulated electrode tip (Watson, Troiano, Poulakos, Weiner, Block and Siegel, 1983) probably meant that the granular and dysgranular insula, the posterior agranular insula and the ectorhinal region were significantly activated by the 100 electrical pulses (i.e. 400 μA, 100 Hz, 0.5 s duration) administered to rats belonging to the Extinction + Stimulation group of this study. In contrast, the perirhinal cortex may have only been minimally activated by such a current spread.

Indeed, if one examines the electrode placements of the Extinction + Perirhinal/Insula Stimulation group depicted in Figure 130, only perhaps 3 or 4 rats would have had a realistic chance of receiving some activation of the perirhinal cortex from the 400 μA current delivered during FPS reinstatement training. These animals would have probably been the rats with electrodes situated at around -2.80 mm posterior to bregma (see Figure 130). For the most part, the electrodes of these rats would have activated the granular/dysgranular insular region and the ectorhinal cortex however, some activation of the dorsal perirhinal cortex may have occurred as well. It is therefore possible that these few rats (i.e. 3 or 4) could be classified as a subgroup within the Extinction + Perirhinal/Insula Stimulation group. Thus, it may be possible to divide the Extinction + Perirhinal/Insula Stimulation group into two groups in order to statistically compare FPS reinstatement final test scores from rats with electrodes located in more anterior insular regions that are farther away from the perirhinal cortex (i.e.-1.80 mm to -2.30 mm posterior to bregma; N=4) to rats with more caudally placed electrodes that are closer to the perirhinal regions (i.e. -2.56 mm to -2.80 mm posterior to bregma; N=4). The results of these statistical analyses will be included to supplement the normal statistical comparisons made between the Extinction + No Perirhinal/Insula Stimulation and the Extinction + Perirhinal/Insula Stimulation groups. The benefit of these additional comparisons is that they may reveal if there are any differences in FPS reinstatement exhibited by rats with
bipolar stimulating electrodes located close (proximal and posterior; N=4) to the perirhinal cortex versus those situated farther away (distal and anterior; N=4). Also, such comparisons may provide some valuable insight into whether the anterior insula or the posterior insula/ECTORHINAL area is more critically involved in stimulation-induced FPS reinstatement.

In any event, it should again be noted that the main objective of this experiment was to activate both the insular (i.e. granular and dysgranular insular nuclei) and the rhinal (i.e. ectorhinal and perirhinal nuclei) cortical regions in order to evaluate their contribution to FPS reinstatement and the placement of electrodes may reflect this fact as attempts were obviously made to place electrodes between the insular and rhinal cortical regions. This may have led to the less than ideal placement of bipolar stimulating electrodes (i.e. no electrodes in the perirhinal cortex). In hind-sight, it may have been better to target each insular or rhinal brain region separately in order to determine their individual contribution to FPS reinstatement. Perhaps, future research may take up this challenge.

19.32: Experimental Results

An independent T-test of the baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the two perirhinal/insular cortex experimental groups (t(2,15)=0.745; p=0.467 [n/s]). The T-test results indicate that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 131). The independent T-test of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result t(2,15)=0.737; p=0.472 [n/s], demonstrating that the groups were matched on this measure (see Figure 132). The independent T-test of the shock reactivity data obtained during fear conditioning yielded a non-significant result t(2,15)=0.604, p=0.554 [n/s], suggesting that both perirhinal/insular cortex experimental groups showed similar levels of movement amplitude during light + footshock presentations. The reactivity results as a function of shock administration during the 30 light + shock fear conditioning trials are shown in Figure 133.
Figure 130 schematically depicts the location of bipolar electrodes in the granular, dysgranular, and posterior agranular insular cortices bordering the ectorhinal and perirhinal cortices. These electrode locations are represented by the solid filled circles (●). On the left are the approximate location of implanted electrodes for the Extinction + Perirhinal/Insular Cortex Stimulation group (N=8) and on the right are the electrode placements for the Extinction + Perirhinal/Insular No Stimulation control group (N=9) of Experiment 8. Bipolar electrodes were unilaterally implanted into either the left or right Perirhinal/Insular cortex region of the temporal lobe (coordinates A.P. - 2.90 mm from bregma, M.L. ± 6.40 mm from the midline sagittal suture, D.V. - 6.80 mm from the skull surface). Representative sections (top to bottom; - 1.80 mm, - 2.12 mm, - 2.30 mm, - 2.56 mm and - 2.80 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1998.
Figure 131. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the Perirhinal/Insular Cortex Extinction + Stimulation (N=8) and the Perirhinal/Insular Cortex Extinction + No Stimulation (N= 9) groups.
White-noise Intensity Used to Induce Startle

Figure 132. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that were used to induce stable acoustic startle responses in animals belonging to the Perirhinal/Insular Cortex Extinction + Stimulation (N= 8) and Perirhinal/Insular Cortex Extinction + No Stimulation (N=9) groups.
Shock Reactivity During Fear Conditioning

Figure 133. Mean (S.E.M. ± ) movement amplitudes recorded in response to footshocks administered during the presentation of 30 light + footshock fear conditioning trials to rats in the Perirhinal/Insular Cortex Extinction + Stimulation (N= 8) group and the Perirhinal/Insular Cortex Extinction + No Stimulation (N= 9) control group.
19.33: Pretest Results

The pretest results indicate that both experimental groups displayed FPS 48 hours after 30 light + footshock pairings. ANOVA (2 Stimulation Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1, 15)= 41.20$, $p<0.00002$, with no significant effect of Group, or Group X Stimulus Condition interaction. Newman-Keuls post hoc tests for the main effect of Stimulus Condition (3 CS + noise trials vs. 3 noise-alone trials) confirmed that rats in both groups showed significantly higher mean startle responses on the 3 CS + noise trials than on the last 3 noise-alone trials of the pretest. These results are depicted in Figure 134, and show that rats in both experimental groups displayed robust FPS after fear conditioning. Also, the two experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. An independent T-test of the pretest difference score data (Difference Score= [3 light + noise trials]-[last 3 noise alone trials]) confirms that the two perirhinal experimental groups had similar levels of fear $t(2,15)=1.12$, $p=0.279$ [n/s]. Figure 135 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the experimental groups were matched on fear prior to any experimental manipulation involving either extinction training or administration of 100 electrical stimulations of the perirhinal and insular cortices.
Figure 134. Mean (S.E.M. ±) acoustic startle amplitudes for the Perirhinal/Insular Cortex Extinction + Stimulation (N= 8) and Perirhinal/Insular Cortex Extinction + No Stimulation (N= 9) groups on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (☆ P<0.05 relative to noise-alone trials). The mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the nearly equivalent levels of fear-potentiated startle exhibited by both experimental groups 48 hours after exposure to 30 light + footshock fear conditioning trials.
Figure 135. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the near equal and robust levels of fear exhibited by the Perirhinal/Insular Cortex Extinction + Stimulation (N= 8) and the Perirhinal/Insular Cortex Extinction + No Stimulation (N= 9) groups 48 hours after fear conditioning.
19.34: Final Test Results and Discussion

The main finding of this experiment is that electrical stimulation of the perirhinal/insular cortices nearly reinstated FPS in a group of fear-extinguished rats. A 2 Stimulation Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA failed to reveal a significant main effect of Stimulus Condition $F(1,15)=1.43$, $p=0.249 \ [n/s]$. The Stimulation Group X Stimulus Condition interaction approached but did not reach statistical significance $F(1,15)=4.00$, $p=.063$. Despite this non-significant result, it is important to point out that the perirhinal/insular cortex stimulation rats did show a trend towards higher acoustic startle amplitudes on the 10 light + noise trials than on the 10 noise-alone trials (see Figure 136). On the other hand, the Extinction + No Stimulation control group showed no such upward trend between the 10 light + noise trials and 10 noise-alone trials on the final test. These results indicate that the non-stimulated rats exhibited no FPS, whereas perirhinal/insular cortex stimulated rats showed a positive trend towards fear reinstatement.

Another important observation was that the mean acoustic startle amplitude on the noise-alone trials appeared depressed in the Perirhinal/Insular Cortex Extinction + Stimulation group relative to the Extinction + No Stimulation control group (see Figure 136). Newman-Keuls post hoc comparisons revealed that these differences on the noise-alone trials were not statistically significant. Nevertheless, it is still possible that perirhinal/insular cortex stimulation 24 hours prior to final testing dampened noise-alone startle amplitudes in the Extinction + Perirhinal/Insular Cortex Stimulation group. It is tempting to speculate that electrical stimulation inhibited noise-alone startle but at the same time freed CS + noise startle responding from the inhibitory controls imposed by extinction training. If these two events occurred in a simultaneous fashion during electrical stimulation of the perirhinal/insular region then it is at least possible to envisage how and perhaps why the Extinction + Perirhinal/Insular Cortex Stimulation group exhibited a positive trend towards fear-reinstatement but still showed low noise-alone acoustic startle. Thus, increases in startle amplitudes on the CS + noise trials over the noise-alone trials resulting from stimulation-induced fear reinstatement could have combined with the dampening effects on noise-alone startle to produce the positive trend towards fear reinstatement observed in the Extinction + Perirhinal/Insular Cortex Stimulation group.
One could speculate that electrical stimulation of these temporal cortical regions could have served to inhibit a sub-population of neurons amygdala and BNST that were devoted to elevating startle responding. However, whether these events had any impact on the final test results is difficult to clarify without further experimentation.
Figure 136. Mean (S.E.M. ±) acoustic startle amplitudes on the 10 noise-alone and 10 CS + noise trials of the final test for fear potentiated startle reinstatement as a function of Perirhinal/Insular cortex stimulation or no stimulation 24 hours earlier (★ P<0.05 relative to noise-alone trials). The mean (S.E.M. ±) difference scores of the final test ([10 CS + noise]−[10 noise-alone]) for the Perirhinal/Insular Cortex Extinction + Stimulation group (N=8) and the Perirhinal/Insular Cortex Extinction + No Stimulation group (N=9) demonstrate the impact on fear reinstatement that occurred as a result of electrical stimulation of the perirhinal and insular cortical areas 24 hours earlier († P<0.05 relative to the Perirhinal/Insular Cortex Extinction + Stimulation group mean difference score).
An Independent T-test of the difference scores failed to yield an effect of Stimulation \( t(2,15)=2.00, p=0.063 \) [ns]. Figure 137 displays the difference in the magnitude of fear between the two experimental groups (Difference Score = 10 light + noise trials - 10 noise-alone trials) as a function of electrical stimulation. As depicted in Figure 136, the mean difference score obtained for the Extinction + Perirhinal/Insular Cortex Stimulation group (N= 8) was 141.9 SEM ± 33.7 compared to the mean difference score for the Extinction + No Stimulation control group (N= 9) -35.6 SEM ± 77.8. In our lab FPS is operationally defined as a 50% increase in startle amplitude on the CS + noise trials compared to the noise-alone trials. However, this 50% increase from noise-alone startle to CS + noise startle is used as a criterion for the inclusion of rats into a study following fear conditioning and pretesting and is therefore not generally employed to evaluate whether animals express conditioned fear on final tests. Nevertheless, the given operational definition of fear was applied to this particular experiment and the increase in startle amplitudes from the noise-alone trials to the CS + noise trials on the final test was analysed as a percent increase. This was done to see how close the Extinction + Perirhinal/Insular Cortex Stimulation group came to actually meeting an operational definition of FPS.

The formula used to convert the final test scores to percent increases for each animal was; Percent Increase or Change = (Larger startle score) / (Smaller startle score) – 1 x 100. When differences between 10 CS + noise trials and 10 noise-alone trials were converted to a percentage increase in startle responding, an independent T-test of these data revealed a significant effect of Stimulation \( t(2,15)=2.98, p < 0.01 \). Figure 138 shows that as a percent increase from noise-alone to CS + noise, the level of FPS exhibited by the Extinction + Perirhinal/Insular Cortex Stimulation group was significantly greater than the Extinction + No Stimulation control group. The mean percent increase from noise-alone to CS + noise for the Extinction + Perirhinal/Insular Cortex Stimulation group (N=8) was 107.9% (SEM ± 38.9%) compared to the Extinction + No Stimulation control group’s (N=9) mean of -25.1% (SEM ± 24.0%). These results seem to indicate that 100 electrical stimulations of the perirhinal and insular cortical regions in the same context where fear conditioning took place had a positive effect on the reinstatement of FPS in rats that received extinction training.
Although it is still too early to make any definitive claims regarding the role of the perirhinal and insular regions in stimulation-induced FPS reinstatement given the fact that the sample size was lower than desired, it is still important to point out that there was a trend towards fear reinstatement in this experiment. As such, the results of this study are consistent with other research that has implicated perirhinal and insular cortices involvement in FPS responding and nociceptive information processing (Shi and Davis, 1997; 1999; Rosen, et al., 1992). Moreover, these results indicate that some fear-memories or perhaps some key elements that are closely related to specific fear memories are stored in the perirhinal and insular cortical region. Excitation of the perirhinal and insular cortical regions of the temporal lobe via electrical stimulation may have activated populations of neurons that contained these fear-memory traces or their related elements. This activation could have set off a stimulation-induced sensory cascade to the amygdala that was able to generate the non-significant positive trend toward fear reinstated startle that was observed in the Extinction + Perirhinal/Insular Stimulation group on the final test. This view is quite attractive when one considers the extensive projection from the perirhinal and insular cortical regions that target the basolateral amygdaloid nuclear complex (see McDonald, 1998; Shi and Davis, 1997; also see Chapter 3 of this thesis).

In order to further evaluate the role of the insular and rhinal cortical regions in stimulation-induced FPS reinstatement, the Extinction + Perirhinal/Insula Cortex Stimulation group was divided into two groups based on the position of electrodes in relation to the perirhinal cortex. This resulted in the formation of two Extinction + Stimulation groups with N=4 each. One group was made up of rats with electrodes situated in the anterior insular regions (i.e. granular and dysgranular insula cortex) that is farther away from the perirhinal cortex around the -1.80 mm to -2.12 mm mark relative to bregma (see Figure 130). The second group was made up of rats with electrodes located in the posterior insular regions and ectorhinal cortex which is much closer to the perirhinal cortex (i.e. around -2.40 mm to -2.90 mm posterior to bregma).

A 2-way ANOVA (Group X Stimulus Condition) carried out on the final test data compared startle on the 10 CS + noise versus the 10 noise-alone trials for these two groups. The ANOVA obtained a significant main effect of Stimulus Condition (i.e. 10 CS + noise vs. 10 noise-alone) F(1,6)=17.45, p<0.006 only, with no significant main effect of Group or
Group X Stimulus Condition interaction. This latter result indicates that anterior insular electrode rats did not differ from posterior insula/ectohinal electrode rats in FPS reinstatement. However, Newman-Keuls post hoc tests of the main effect of Stimulus Condition did reveal that the anterior insula electrode rats (N=4) did exhibit significantly higher mean startle amplitudes on the 10 CS + noise trials than on the 10 noise-alone trials (CS + noise mean= 390.5; SD=24.9 versus noise-alone mean= 216.7; SD=131.1). In contrast, the posterior insula/ectohinal electrode group (N=4) also exhibited higher startle on the 10 CS + noise trials than the 10 noise-alone trials but Newman-Keuls post hoc tests determined that this difference was not statistically significant (CS + noise mean= 278.9; SD=93.4 versus noise-alone mean= 168.9; SD=58.0). These results seem to indicate that stimulation of the anterior insular region does have a slightly more positive impact on CS-induced startle reinstatement than stimulation of the posterior insular/ectohinal cortex (see Figure 138a). In fact, an independent T-test carried out on the mean startle scores of the CS + noise trials between these two experimental groups did approach statistical significance \(t(2,6)= 2.31, p=0.060 \{n/s\}\). This suggested that the anterior insula electrode rats generally exhibited higher startle on the CS + noise trials during the final test than did the posterior insula/ectohinal electrode group.

To scrutinize the final test data further, an independent T-test of the posttest difference scores (i.e. Difference score=[10 CS + noise trials]-[10 noise-alone trials]) was carried out to determine if there were any differences between the anterior and posterior electrode groups in the magnitude of FPS reinstatement expressed during final testing. The T-test of the final test difference scores failed to yield any significant effect \(t(2,6)=0.938, p=0.384, \{n/s\}\), indicating that the anterior insula and posterior insula/ectohinal electrode groups (N=4 each) exhibited levels of conditioned fear during FPS reinstatement testing that were not different from each other (see Figure 138a). The mean difference score for the anterior insula electrode group was 173.7 (SD= 126.7), whilst the mean difference score for the posterior insula/ectohinal electrode group was 110.0 (SD= 48.8). To further evaluate the data from these two groups, the percentage increase from noise-alone to CS + noise trials were calculated for each rat in each group and then analysed using an independent T-test. The results obtained from this T-test revealed that there were no statistically significant between-group differences in the percent increase of startle from the noise-alone to CS +
noise trial type \( t(2,6)=1.06, p=0.332 \text{[n/s]} \). Although, it should be noted that the anterior insula electrode group did have a somewhat higher percent increase in startle from the noise-alone to the CS + noise trial type than did the posterior insula/ectohinal electrode group. The mean percent increase for the two groups are, 148.5% increase (SD= 151.3) for the anterior insula electrode group and 67.0% increase (SD= 30.7) for the posterior insula/ectohinal electrode group (see Figure 138a).

Even though the above data analysis did not find any statistically significant effects, it is still interesting in that the anterior insula electrode group exhibited a slightly larger magnitude of conditioned fear than the posterior insula/ectohinal electrode group (i.e. mean difference= 173.7 versus mean difference= 110.0). In a similar fashion, the anterior insula electrode group’s percent increase in startle from noise-alone to CS + noise trials is reasonably higher than that of the posterior insula/ectohinal electrode group (mean= 148.5% versus mean= 67.0%). Furthermore, the Newman-Keuls tests of the main effect of Stimulus Condition did reveal that the anterior insula electrode rats exhibited significantly higher startle amplitudes on the 10 CS + noise trials than on the 10 noise-alone trials, whereas the posterior insula/ectohinal rats did not. Moreover, the independent T-test between the two groups on the means of the 10 CS + noise trials during FPS reinstatement testing did approach statistical significance \( t(2,6)=2.31, p=0.060 \text{[n/s]} \), indicating that the anterior insula electrode group did exhibit marginally higher startle levels during the presentation of CS + noise trials than did their counterparts with more posterior fixed electrodes. Thus, it seems as though electrical stimulation of the anterior granular, dysgranular and agranular insular cortical regions may have a slightly more positive impact on FPS reinstatement than stimulation of the posterior insula/ectohinal regions that are closer to the perirhinal cortex. Whether these findings are scientifically meaningful is debatable since the sample sizes used in making these comparisons were quite small (i.e. N=4 each). Nevertheless, it is still interesting that electrical stimulation of the insular and rhinal cortical regions did have a positive impact on FPS reinstatement. It is important to remember that the rhinal and insular cortical regions do provide auditory, visual and nociceptive input to the lateral and basolateral amygdala (McDonald, 1998; Shi and Davis, 1998a,b; Shi and Cassell, 1999; Shi and Davis, 1999; LeDoux, et al., 1990; LeDoux, et al., 1991; Ono, et al., 1995; Uwano, et al., 1995; Romanski and LeDoux, 1993; McDonald and
Mascagni, 1996), thus making it possible for electrical stimulation of these cortical regions to reunite the CS (light) to the UCS (footshock). In addition, the insular and rhinal cortical regions have been shown to contribute to FPS responding and fear-induced defensive freezing behaviour in rats as lesions confined to these areas tend to disrupt FPS and conditioned freezing behaviour (Falls, et al., 1992; Rosen, et al., 1992; Campeau and Davis, 1995b; Falls, et al., 1997; Shi and Davis, 1999; Corodimas and LeDoux, 1995; Brunzell and Kim, 2001). In fact, the location of the bipolar stimulating electrodes in Experiment 8 (i.e. -1.80 mm to -2.90 mm posterior to bregma) were approximately where electrolytic and NMDA lesions of the rhinal/insular regions (i.e. perirhinal according to Paxinos and Watson, 1986) were shown to be very effective in blocking FPS expression in rats (see Rosen, et al., 1992; Campeau and Davis, 1995b). Thus, the FPS reinstatement results presented in Experiment 8 do seem consistent with much of the research carried out on the rhinal and insular cortical regions by other scientists, however more experimental work is definitely required to fully understand and appreciate the contribution made to FPS reinstatement behaviour by the various individual nuclei of the insular and rhinal cortical group. Perhaps future experiments may be specifically tailored to stimulate discrete nuclei in the insular and rhinal cortical regions so as to determine how each nucleus located in these cortical areas contributes to the FPS reinstatement effect.
Figure 137. Mean (S.E.M. ±) difference scores ([10 CS + noise]-[10 noise-alone]) of the final test for the Perirhinal/Insular Cortex Extinction + Stimulation group (N= 8) and the Perirhinal/Insular Cortex Extinction + No Stimulation group (N= 9) twenty-four hours after either stimulation of the perirhinal and insular cortical regions or no stimulation.
Final Test FPS Reinstatement Results as Percent Increases of CS + Noise over Noise-Alone

Figure 138. Mean (S.E.M. ±) percentage increase of CS + noise over noise-alone trials for the Perirhinal/Insular Cortex Extinction + Stimulation group (N=8) and the Perirhinal/Insular Cortex Extinction + No Stimulation group (N=9) based on the formula: Percent Increase or Decrease = ([Larger startle score + Smaller startle score] - 1) x 100 (★ P<0.05 relative to the Perirhinal/Insular Cortex Extinction + Stimulation group's percent increase score).
Final Test for FPS Reinstatement

![Graph showing mean startle amplitudes and percent increase units for two groups: Anterior Insula Electrode Stimulation Group and Posterior Insula/Ectorhinal Electrode Stimulation Group.]

Figure 138(a). Mean (S.E.M. ±) acoustic startle amplitudes on 10 noise-alone and 10 CS + noise trials of the final test for fear-potentiated startle reinstatement as a function of electrical stimulation of either the anterior Insula region (N=4) or the posterior Insula/Ectorhinal region (N=4) (★ P<0.05 relative to noise-alone trials). As can be seen, only the anterior Insula electrode Extinction + Stimulation group exhibited significant CS-induced startle on the FPS reinstatement test. The mean (S.E.M. ±) difference scores of the final test ([10 CS + noise]–[10 noise-alone]) for the anterior Insula electrode group and the posterior Insula/Ectorhinal electrode group were found not to be significantly different from each other. Also, the two experimental groups depicted above did not differ from each other on the percent increase in startle from noise-alone to CS + Noise trials. However, by just visually inspecting the graphs above, it does seem as though stimulation of the anterior insula region did generally have a more positive impact on FPS reinstatement than insula/ectorhinal stimulation.
19.35: AD Threshold Results

As was mentioned previously one animal in each experimental group had its electrode chewed or broken before the AD thresholds were able to be measured, leaving \( N= 8 \) in the Extinction + Stimulation group and \( N= 9 \) animals in the Extinction + No Stimulation control group for the data analysis. One animal in the Perirhinal/Insular Cortex Extinction + Stimulation group during the AD threshold data collection appeared to have a poor connection develop where the cable connected from the stimulator to the electrode. As a result this animal was excluded from the AD threshold data analysis, thus leaving the Perirhinal/Insular Cortex Extinction + Stimulation group with an \( (N= 7) \) for this particular analysis.

An independent T-test of the AD threshold data obtained from EEG recordings obtained from the perirhinal/insular cortex region yielded a non significant result \( t(2,14)=1.18, p=0.254 \) [ns]. Figure 139 depicts the mean AD thresholds for the Extinction + Perirhinal/Insular Cortex Stimulation group \{mean AD= 978.6, SEM ± 76.3; SD= 201.8 \( (N= 7) \}\} and the Extinction + No Stimulation control group \{mean AD= 1133.3, SEM ± 97.9; \( (N= 9) \}\}. These results indicate the perirhinal cortex stimulation produced a slight decrease in AD thresholds, however it was not significantly different than the mean AD threshold measured in the Extinction + No Stimulation rats. Despite the non-significant difference in AD thresholds, it is still possible that stimulation of the perirhinal/insular cortex after extinction training did cause neural excitation in the perirhinal/insular cortex and other proximal brain nuclei such as the amygdala which receives numerous inputs from both the perirhinal and insular cortices. Thus, electrical stimulation of the insular cortex and nearby perirhinal cortex could have facilitated the positive trend towards fear-reinstatement in the Extinction + Perirhinal/Insular Cortex Stimulation group by enhancing neuronal firing rates in the amygdala and other brain areas known to be involved in mediating fear behaviours.
After-Discharge Current Threshold Scores

Figure 139. Mean (S.E.M. ±) after discharge current thresholds for the Perirhinal/Insular Cortex Extinction + Stimulation group (N= 7) and the Perirhinal/Insular Cortex Extinction + No Stimulation control group (N= 9).
19.36: After Discharge Activity and Seizure Classification Results

Figure 140 depicts the pattern of after-discharge activity recorded from temporal lobe neurons (i.e. insular cortex and perirhinal cortex neurons) as a function of electrical stimulation of these temporal lobe regions. As can be seen, electrically induced after-discharge activity of the temporal lobe neurons produces after-discharge activity that is somewhat different to that observed in the amygdala. Generally speaking, the frequency of after-discharge cell firing recorded from the temporal cortex was faster than that seen in the amygdala. Also in some instances this period of activity was followed by a very quiet period during which almost no cell firing occurred at all. This period of non-activity can be seen in Figure 140 just after the after-discharge activity has stopped. In some cases, this quiet period was followed by a few random single spikes and then a return to more normal neuronal firing patterns. For an accurate comparison between the amygdala and perirhinal/insular cortex after-discharge neuronal firing patterns see Figure 129 and 140 respectively.
Figure 140 depicts a typical after-discharge pattern recorded from neurons in the perirhinal/insular cortical region of the temporal lobe after electrical stimulation was used to induce after-discharge activity in this region. The dark arrow represents the point at which the electrical stimulation was administered to the perirhinal/insular cortical region. To the left of the dark arrow is the cellular activity of perirhinal/insular cortex neurons prior to the after-discharge inducing electrical stimulation and just to the right of the dark arrow is the characteristic after-discharge pattern recorded from the insular and perirhinal cortical area. As can be seen the after-discharge activity in the perirhinal/insular cortices of the temporal lobe generally produces more frequent spikes that are as high or slightly higher in amplitude than those recorded from the amygdala during after-discharge activity. The perirhinal/insular cortex after discharge pattern is also characterized by short inter-spike intervals however the frequency of spikes seems to occur more rapidly than those in the amygdala. Further to the right of the after-discharge pattern the neurons exhibit an uncharacteristic quiet period lasting several seconds (perhaps indicative of a refractory period) followed by intermittent large spikes separated by smaller spiking activity. It is important to point out that the length of this quiet period varied from animal to animal and sometimes animals exhibited significant neuronal activity after the quiet period. The first two after-discharge recording patterns presented above were obtained from rats that belonged to the Extinction + Perirhinal/Insular cortex Stimulation group of Experiment 8. The third recording comes from an animal belonging to the Extinction + No Stimulation Perirhinal/Insular cortex control group of Experiment 8.
19.37: Seizure Classification Tables (Tables 6 and 7)

In most instances, electrically induced after-discharge activity in the cortical constituents of the temporal lobe region produces different behavioural manifestation that can be used to classify the severity and type of epileptic seizure that has occurred. Tables 6 and 7 list the name of the two Perirhinal/Insular cortex electrode experimental groups and their corresponding seizure typology. These tables list the seizure Type for each experimental group in the perirhinal/insular cortex FPS reinstatement study along with the typical behaviour associated with the seizure in question.
Table 6: Extinction + In Context Insular/Perirhinal Cortex Stimulation Group (N=7)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure Type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (μA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>13</td>
<td>(X) 1300</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Facial twitching, oral movements, teeth clicking, and head bobbing.</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>(X) 700</td>
<td></td>
<td></td>
<td></td>
<td>Eyes squinting, head bobbing and one paw raised in air and shaking.</td>
</tr>
<tr>
<td>17</td>
<td></td>
<td></td>
<td>(X) 800</td>
<td></td>
<td></td>
<td>Eyes squinting, head bobbing and one front paw lifted slightly in the air.</td>
</tr>
<tr>
<td>18</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1150</td>
<td></td>
<td>Eyes squinting, whiskers twitching, head bobbed, reared up and fell down.</td>
</tr>
<tr>
<td>19</td>
<td></td>
<td></td>
<td></td>
<td>(X) 900</td>
<td></td>
<td>Eyes squinted, oral movements, head bobbing and both paws were raised.</td>
</tr>
<tr>
<td>201</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1000</td>
<td></td>
<td>Eyes squinting, head bobbing, shaking, paws in air, rearing and fell.</td>
</tr>
<tr>
<td>22</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 950</td>
<td>Eyes blinking, head bobbing, reared up and tried to bite electrode cord.</td>
</tr>
</tbody>
</table>

Table 6: As can be seen in the above table, of the eight rats in the Extinction + Insular/Perirhinal Cortex Stimulation group seven could be assessed for after discharge activity. Of these seven rats N=1 exhibited a Type 2 seizure, N=2 displayed Type 3 seizures, N=2 showed signs of a Type 4 seizure and N=2 had behavioural profiles that were characteristic of Type 5 fall down seizures.
Table 7: Extinction + No Insular/Perirhinal Cortex Stimulation Group (N=9)

Seizure Type and Behavioural Classification Scheme

**Type 1 Seizure** = Behavioural Arrest; **Type 2 Seizure** = Behavioural Arrest, Oral Movements and Head Bobbing; **Type 3 Seizure** = Head Bobbing and Rapid Shaking of Unilateral Forelimbs; **Type 4 Seizure** = Clonus of Both Forelimbs and Rearing Behaviour. **Type 5 Seizure** = Rearing, Head Bobbing and Falling Down. The Symbol (X) denotes the seizure Type for each animal. Right next to the (X) symbol is the level of electrical current in micro-Amps (μA) that induce the after-discharge activity and the subsequent seizure for each rat.

<table>
<thead>
<tr>
<th>Rat #</th>
<th>Type 1 Seizure</th>
<th>Type 2 Seizure</th>
<th>Type 3 Seizure</th>
<th>Type 4 Seizure</th>
<th>Type 5 Seizure</th>
<th>Observed Behaviour</th>
</tr>
</thead>
<tbody>
<tr>
<td>161</td>
<td></td>
<td>(X) 950</td>
<td></td>
<td></td>
<td></td>
<td>Oral chewing, small head bobbing, but no behavioural arrest.</td>
</tr>
<tr>
<td>162</td>
<td></td>
<td>(X) 800</td>
<td></td>
<td></td>
<td></td>
<td>Oral movements, running around and eye twitching and squinting.</td>
</tr>
<tr>
<td>163</td>
<td></td>
<td>(X) 800</td>
<td></td>
<td></td>
<td></td>
<td>Oral jaw movements, circling to the right and shaking of the head.</td>
</tr>
<tr>
<td>165</td>
<td></td>
<td>(X) 1200</td>
<td></td>
<td></td>
<td></td>
<td>Twitching/blinking eyes, turns to the left side and head bobs.</td>
</tr>
<tr>
<td>157</td>
<td></td>
<td></td>
<td>(X) 1450</td>
<td></td>
<td></td>
<td>Head bobbing, paws raised to mouth, and lots of motor activity.</td>
</tr>
<tr>
<td>173</td>
<td></td>
<td></td>
<td></td>
<td>(X) 1050</td>
<td></td>
<td>Turns in circle to the left, eyes squinting and paw raised.</td>
</tr>
<tr>
<td>175</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 950</td>
<td>Squinted both eyes, turns to the left and head bobs a fair bit.</td>
</tr>
<tr>
<td>171</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1600</td>
<td>Both eyes squinted, twisted to the left and raised one paw in the air.</td>
</tr>
<tr>
<td>155</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(X) 1450</td>
<td>Squinting and twitching of eyes, one paw raised and teeth clicking.</td>
</tr>
</tbody>
</table>

Table 7: As can be seen in the above table, of the nine rats in the Extinction + No Insular/Perirhinal Cortex Stimulation group that could be assessed for after discharge activity; N=5 exhibited a Type 2 seizure, and N=4 exhibited Type 3 seizures. Behavioural profiles characteristic of Type 4 and Type 5 seizures were not observed.
20. Experiment 9:

*Electrical Stimulation of the Ventral Tegmental Area and the Rostrodorsal Periaqueductal Gray Area and its Effects on the Reinstatement of Fear-Potentiated Startle in Rats that have Received Fear Extinction Training:*

20.1: Rationale

The justification for investigating the role of the VTA and PAG in fear reinstatement emerges out of the various scientific efforts that have verified the involvement of these two mid-brain regions in mediating various forms of conditioned fear expression (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Gifkins, et al., 2002; LeDoux, et al., 1988; De Oca and Fanselow, 1995; Fanselow, et al., 1995; Fanselow, 1991; Fendt, et al., 1994; 1996; Fendt, 1998; Walker and Davis, 1997d). As a matter of fact, a wealth of research evidence and literature reviews demonstrate that the PAG is responsible for mediating conditioned defensive freezing behaviour (LeDoux, et al., 1988; De Oca and Fanselow, 1995; Fanselow, et al., 1995; Fanselow, 1991), defensive jumping and flight behaviours (Bandler and Shipley, 1994; Behbehani, 1995; Bandler, 1982; Fernandez de Molina, et al., 1962), and FPS (Fendt, et al., 1994; 1996; Fendt, 1998; Walker and Davis, 1997c). Furthermore, electrical or chemical stimulation of the PAG often produces fear-like responses and flight behaviours, heightened acoustic startle and conditioned fear responding when it is used as a UCS (see Bandler, 1982; Borowski and Kokkinidis, 1996; Di Scala, et al., 1987). Moreover, the central amygdaloid nucleus efferents pass through the ventrolateral PAG on route to the startle circuit at the level of the reticularis pontis caudalis (RPC) (Rosen, et al., 1991; Hitchcock and Davis, 1991) and electrical stimulation of the dPAG sensitises acoustic startle responding in rats (Borowski and Kokkinidis, 1996). These factors when combined with the research reports mentioned above, makes investigations into whether electrical stimulation of the PAG will lead to FPS reinstatement a valuable scientific pursuit.

With regard to the VTA, electrical stimulation of this dopamine rich mid-brain area has been shown to amplify acoustic startle responding in rats (Borowski and Kokkinidis, 1996) and alter the fast and slow firing rates of amygdaloid neurons (Rosenkranz and Grace, 1999). The DA cell group of the VTA projects to the amygdala (Swanson, 1982; Loughlin...
and Fallon, 1983; Brinley-Reed and McDonald, 1999; Oades and Halliday, 1987) and appears to play an important role in mediating conditioned fear expression and stress related responses (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997; Nader and LeDoux, 1999b; Gifkins, et al., 2002; Coco, et al., 1992; Deutch, et al., 1985). Furthermore, kindling of VTA neurons generates AD activity in the amygdaloid complex and triggers unconditioned fear responding in laboratory animals (Gelowitz and Kokkinidis, 1999; Stevens and Livermore, 1978). Based on these findings and earlier discussion concerning the involvement of the mesoamygdaloid DA system in fear expression, it is reasonable to pursue experiments that evaluate the role of the VTA in the reinstatement of FPS. Hence the purpose of this experiment is to determine if electrical stimulation of either the VTA or dPAG will cause FPS to be reinstated in rats that have experienced inhibitory fear training (i.e. extinction training). The method and results section of this experiment are list below.

20.2: Method

20.21: Subjects

Thirty-eight naive, male Wistar rats bred and housed in the psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 340 grams at the beginning of the experiment (range 265-370 grams). A constant temperature of 20°C Celsius (±1°C Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulation took place during the light portion of this cycle. It is also important to mention that the role of the caudoventral PAG in stimulation induced reinstatement was also being investigated at the same time this experiment was being run. This meant that if some animals in the ventral PAG met the histological criteria for assignment into the rostroventral PAG, these animals would be transferred over to the rostroventral PAG group when the statistical analysis for Experiment 9 was run.
20.22: Stereotaxic Surgery

Rats were anesthetised with sodium pentobarbitone (95.0 to 110 mg/kg), mounted in a Stoelting stereotaxic instrument (Wood Dale, IL), and then surgically implanted with a single bipolar stainless steel twisted electrode (MS-303/1; Plastics-One, Roanoke, VA) aimed at the medial portion of either the ventral tegmental area (VTA) or the dorsolateral periaqueductal gray (dPAG). Prior to surgery each electrode was prepared using the same methods described in Experiment 7A. Each electrode was then implanted employing the same flat skull stereotaxic surgical technique that was described in Experiment 7A. The coordinates used in this experiment were taken from the rat brain atlas of Paxinos and Watson (1986) and were \{A.P. - 4.80 mm from bregma, M.L. ± 0.80 mm from the sagittal suture, and V -8.20 mm from the skull surface\} for the VTA and \{A.P. -6.30 mm from bregma, M.L. ± 0.3 mm from the sagittal suture, and D.V. -5.00 mm from the skull surface\} for the dorsolateral PAG.

Electrodes were unilaterally implanted with half of the animals in each group receiving an electrode in the right hemisphere and the remaining animals receiving implantation in the left hemisphere. The electrode was secured to the skull surface via a head cap constructed from dental acrylic. This head cap was attached to five stainless steel self-tapping jeweller screws (3.20 mm in length, Lomat Precision Tools, Montreal Quebec, Canada) embedded in the skull surface. Specially designed dummy dust caps (303DC and 303DCA; Plastics-One, Roanoke, VA) were screwed onto the secured electrode to deter rats from chewing the plastic portion of the electrode and making it inoperative. Following surgery, rats were placed into a recovery cage until the anaesthetic wore off and then were returned to normal group housing conditions. Rats were allowed a 14 day recovery period to ensure their wounds were adequately healed before any behavioural testing or experimental manipulation commenced.

20.23: Procedure
20.24: Baselining, Fear Conditioning, and Pretesting

The methods used to baseline, fear condition, and pretest rats in this particular experiment are the same as those described in Experiment 7A and 8. Baselining was used to establish stable acoustic startle responding and acclimatise rats to the startle testing apparatus. The baselining techniques were also designed to reduce within subjects and between group startle variability. Scores obtained on the pretest were used to assign animals to experimental groups.

20.25: Group Assignment, Extinction Training, and Unsignalled Electrical Stimulation of the VTA or Dorsal PAG

After pretesting was completed, rats were assigned to one of three experimental groups (N=11 per group) with similar averaged startle scores, noise intensity levels, and levels of FPS as reflected through the difference scores calculation and analysis. Twenty-four hours later animals were placed in their designated test chambers, and after a 5 min habituation period, exposed to 140 light-alone extinction training trials. The interstimulus interval for the light presentation during extinction training was 15 seconds.

The three experimental groups were the Extinction + VTA Stimulation group (N=14), the Extinction + dPAG Stimulation group (N=12) and the Extinction + No Stimulation control group (N=11) that was made up of 6 VTA electrode implanted rats and 5 dPAG electrode implanted rats since one dPAG animal expired prematurely. Forty-eight hours after extinction training, rats were connected to a programmable constant current stimulator via an insulated bipolar electrode cable (305-S304-CCT 80CM TT2 CS, Plastics One, Roanoke VA) which connected to the MS-303/1 bipolar electrode. The wire cable was protected by a stainless steel coil spring to prevent rats from damaging the wire. After rats were connected to the stimulator, they were then placed into their designated chambers and allowed 5 min to acclimatise to the test environment.

Rats assigned to either the Extinction + VTA Stimulation group or the Extinction + dPAG Stimulation group were then administered 100 unsignalled electrical brain stimulations. The current intensity used to stimulate the VTA and dPAG was 400 μA (base to peak current) with a pulse-duration of 0.1 ms, and a frequency of 100 Hz. The interstimulus interval used in this experimental manipulation was 3 s and the duration of
the stimulation was 500 ms. The programmable constant current stimulator was connected to the 386 computer that ran the startle testing and fear conditioning programs, and a specially designed computer program was used to trigger the electrical brain stimulation. After the electrical stimulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment. Rats assigned to the Extinction No Stimulation control group did not receive any electrical brain stimulations.

20.26: Final Test

Twenty-four hours after stimulation or no stimulation administration, rats were tested for the reinstatement of FPS. The final test employed a quasi-counterbalanced design. This procedure entailed presenting half of the rats in each experimental group with 10 noise-alone trials first followed by 10 light + noise trials, whilst the other half of the rats in each experimental group were exposed to 10 light + noise trials first followed by 10 noise-alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the white-noise burst was presented during the last 100 ms of the light interval. The purpose of this final test was to determine if electrical stimulation of either the VTA or the dPAG would cause a reinstatement of FPS in fear-extinguished rats. It is also important to point out that no after-discharge current threshold levels were recorded from the VTA or the dPAG simply because raising the current significantly above 400 μA mark would have caused severe stimulation-induced locomotor activity that would likely have caused injury to the animals.

20.27: Statistical Analysis and Dependent Measures

Analysis of variance (ANOVA) was used to statistically examine several dependent variables, these included; acoustic startle amplitude data obtained during baselining, shock reactivity data gathered during fear conditioning, and the level of FPS measured during the pretest and fear reinstatement tests. A 3 Stimulation Group X 2 Stimulus Condition (light + noise vs. noise-alone) ANOVA was used to analyse the pretest and final test data. Newman-Keuls multiple comparisons (α = 0.05) were used to assess differences between experimental group means. Any significant interactions arising from the ANOVA carried
out on the data were subjected to Newman Keuls post hoc tests that incorporated a pooled error term to calculate the critical ranges. Shock reactivity was evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 CS-UCS trials. ANOVA was also used to demonstrate that the three experimental groups were equated on decibel level, baseline startle amplitude, and FPS, via the difference score calculated from the pretest results.

20.3: Results

20.3.1: Histological Results

Two animals from the rostro-dorsal PAG Extinction + Stimulation group had electrodes located more dorsal than desirable. This made it difficult to dissociate the electrode tract from the invagination caused by the longitudinal fissure. As a result these two animals were discarded from the dPAG group. Fortunately, two rats from the vPAG group in Experiment 10 met the histological criteria for assignment to the dPAG group and as a consequence these two rats replaced the two that were lost, leaving the dPAG Extinction + Stimulation group with (N=11) rats. The electrode placements of the dPAG Extinction + Stimulation group have been reconstructed on figures adopted from the rat brain atlas of Paxinos and Watson (1986) and can be seen in Figure 141.

Most of the animals in the dPAG Extinction + Stimulation group had their electrodes located just dorsal to the very anterior portions of the PAG between -5.10 mm to -5.60 mm posterior to bregma. Only two animals (i.e. those transferred from the ventral PAG Extinction + Stimulation group in Experiment 10) had electrodes placed right in the very heart of the dorsal PAG and these were found at -6.04 mm and -6.80 mm posterior to bregma respectively. It would have been more desirable had the electrodes for the dPAG Extinction + Stimulation group been placed more caudal to bregma and more ventral to the skull surface.

Three rats in the VTA Extinction + Stimulation Group had electrodes that were surgically misplaced. In two of the rats, the electrodes were located anterior to the VTA in the area of the lateral hypothalamus at the level of the medial forebrain bundle from about -4.00 mm posterior to bregma. The third rat’s electrode was located at approximately -4.16
mm posterior to bregma. As a result these three rats were excluded from the study and subsequent data analysis. A fourth rat of the VTA Extinction Stimulation Group also had an electrode placement that was located just anterior to the anterior portions of the VTA (between -4.30 mm and -4.52 mm posterior to bregma). This rat was kept in the VTA Extinction + Stimulation group and data analysis for two obvious reasons. First, this particular rat’s electrode was so close to the anterior segment of the VTA, and second, a 400 μA electrical current most likely spread to the near by anterior VTA and activated neurons residing in the region.

Based on the histological analysis the VTA Extinction + Stimulation group was left with (N=11) animals. The electrode placements for this group have been reconstructed on figures adopted from the rat brain atlas of Paxinos and Watson (1986) and can be seen in Figure 142. As can be seen from Figure 142, some of the electrodes are located either in or near the anterior VTA (-4.52 mm posterior to bregma), whilst others are found in the anterodorsal portions of the VTA located between -4.52 mm to -4.80 mm posterior to bregma. Generally speaking, the surgical procedures and coordinates used resulted in most electrodes in the VTA Extinction + Stimulation Group being located in the more anterior and dorsal parts of the VTA.

Based on these placements it is likely that only the anterior and dorsal one-third to one-half of the VTA was activated by the series of 400 μA electrical current pulses administered 24 hours before testing for FPS reinstatement. Other neighbouring brain regions and fibre pathways that may have been activated by this level of electrical current include; the rostral interstitial nucleus, the preruberal field, the supramammillary nucleus, the lateral hypothalamic area, the mammillotegmental tract, and the medial aspects of the medial forebrain bundle (MFB) which is a massive longitudinal fibre tract that courses through the VTA and lateral hypothalamus on route to the amygdala, septum, nucleus accumbens, BNST, insula and prefrontal cortex. Since a portion of the MFB contains fibres arising from out of the DA-ergic cell group of the VTA (Swanson, 1982; Oades and Halliday, 1987; Björkland and Lindvall, 1986), it is possible that electrical stimulation of either the VTA or the MFB may serve to elevate DA levels in the amygdala and other corticolimbic regions (i.e. nucleus accumbens, mPFC, BNST, insula and septum) and induce FPS reinstatement.
Of course, it must be acknowledged that it would have been desirable to have surgically implanted the VTA electrodes a bit more posterior relative to bregma (i.e. between -4.60 mm and -5.30 mm) and slightly more ventral in relation to the skull surface (i.e. -8.40 mm instead of -8.20 mm). However, caution needed to be exercised so as not to implant the bipolar electrodes too deep into the VTA as the placement of such foreign objects may have resulted in an accidental unilateral lesioning of the VTA. This was an obvious concern since NMDA lesions of the VTA have been shown to block the expression of FPS in rats (Borowski and Kokkinidis, 1996). Thus, any possible unilateral lesioning of the VTA produced by ventrally placed electrodes could have potentially made it more difficult for rats to express FPS. Obviously, such a situation would have created even more difficulties in terms of FPS behavioural testing outcomes and data interpretation. To avoid this potential problem and minimise any damage to the VTA, it was important to surgically implant the non-insulated portions of the bipolar stimulating electrodes into the dorsal one-half of the VTA and allow current spread from the 400 μA electrical pulses (0.5 s in duration) to activate the remainder of this midbrain nucleus.

As it stands, the placement of the electrodes in relation to the VTA as depicted in Figure 142 would have meant that no meaningful physical damage to this area would have occurred and that at the very least, the anterior and dorsal aspects of the VTA and parts of the MFB would have been activated by the current spread produced by the 400 μA electrical pulses administered during stimulation-induced FPS reinstatement training. This seems plausible as a 400 μA electrical current has the capacity to spread approximately 0.3 mm up to 0.5 mm in all directions from the non-insulated electrode wire (Watson, et al., 1983). While it is true that other near adjacent brain nuclei and fibre pathways may have been activated by the 400 μA current emanating from the bipolar electrodes (i.e. see the various brain areas and fibre tracts mentioned above), it does not negate the fact that the VTA was also probably activated by current spread. Having made this point, it is still recommended that any future scientists who assess the contribution of VTA stimulation to FPS reinstatement implant stimulating electrodes slightly more posterior relative to bregma and a fraction more ventral relative to the skull surface. This type of accurate electrode placement would help keep much of the electrical current confined to the VTA. In addition, it would be wise to use smaller gauged wires on bipolar electrodes if they are...
available, as this would help minimise any damage to the VTA when electrodes are implanted into its neural tissue.

The electrode placements for the mid-brain Extinction + No Stimulation control group which was made up of N= 6 VTA electrode rats and N=5 dPAG electrode rats (total N=11) is depicted in Figure 143. These rats were used in the statistical analysis as the Extinction + No Stimulation control group in this experiment.
Figure 141. A schematic depiction of the bipolar electrodes located in or near the anterior portions of the dorsal periaqueductal gray (dPAG) for the Extinction + dPAG Stimulation group (N=11). The electrode locations are represented by the solid filled circles ( ). Bipolar electrodes were unilaterally implanted into either the left or right dPAG nuclei (coordinates A.P. - 6.30 mm from bregma, M.L. ± 0.30 mm from the midline sagittal suture, D.V. - 5.00 mm from the skull surface). Representative sections (from top to bottom; - 5.20 mm, - 5.30 mm, - 5.60 mm, - 5.80 mm, - 6.04 mm and - 6.80 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 142. A schematic depiction of the bipolar electrodes located in or near the ventral tegmental area (VTA) for the Extinction + VTA Stimulation group (N=11). The electrode locations are represented by the solid filled circles (●). Bipolar electrodes were unilaterally implanted into either the left or right VTA nuclear group (coordinates A.P. - 4.80 mm from bregma, M.L. ± 0.80 mm from the midline sagittal suture, D.V. - 8.20 mm from the skull surface). Representative sections (top to bottom; - 4.16 mm, - 4.30 mm, - 4.52 mm, - 4.80 mm and - 5.20 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
Figure 143. A schematic depiction of the bipolar electrodes located in or near the ventral tegmental area (VTA; N=6) on the left and anterior portions of the dorsal periaqueductal gray (dPAG; N=5) on the right for the Mid-Brain Extinction + No Stimulation Control group (N=11). This Mid-Brain Extinction + No Stimulation group (N=11) served as the no stimulation control group for the Extinction + VTA Stimulation and Extinction + dPAG Stimulation groups (N=11; each). The electrode locations are represented by the solid filled circles (●) and all bipolar electrodes were unilaterally implanted into either the left or right VTA and dPAG (VTA coordinates A.P. - 4.80 mm from bregma, M.L. ± 0.80 mm from the midline sagittal suture, D.V. - 8.20 mm from the skull surface; dPAG coordinates A.P. - 6.30 mm from bregma, M.L. ± 0.30 mm from the midline sagittal suture, D.V. - 5.00 mm from the skull surface). Representative sections (Left side: from top to bottom VTA; - 4.30 mm, - 4.52 mm and - 4.80 mm from bregma; Right side: from top to bottom dPAG; - 5.20 mm, - 5.30 mm and - 5.60 mm from bregma) were taken from *The Rat Brain in Stereotaxic Coordinates*, by G. Paxinos and C. Watson, 1986.
20.32: Experimental Results

An ANOVA of the baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the three experimental groups $F(2,30)=0.02$, $p=0.9777$ [n/s]. The ANOVA results indicate that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 144). ANOVA of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result $F(2,30)=1.14$; $p=0.331$ [n/s], demonstrating that the groups were matched on this measure (see Figure 145). The ANOVA of the shock reactivity data obtained during fear conditioning yielded a non-significant result $F(2,30)=0.01$, $p=0.989$ [n/s], suggesting that all three experimental groups showed similar levels of movement amplitude during CS + footshock administration. The shock reactivity results obtained during 30 light + footshock fear conditioning trials for all three experimental groups are displayed in Figure 146.
Figure 144. Mean (S.E.M. ±) baseline startle amplitudes recorded on the third baselining block for the mid-brain + No Stimulation, rostro-dorsal periaqueductal gray + Stimulation and the ventral tegmental area + Stimulation experimental groups (N= 11 per group).
White-noise Intensity Used to Induce Startle

Figure 145. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that were used to induce stable acoustic startle responses in the Extinction + Midbrain No Stimulation group (N=11), the rostrodorsal Periaqueductal Gray + Stimulation group (N=11) and the ventral tegmental area Stimulation group N=11) respectively.
Shock Reactivity During Fear Conditioning

Figure 146. Mean (S.E.M. ± ) movement amplitudes recorded in response to footshock presentation during the administration of 30 light + footshock fear conditioning trials to rats of the mid-brain Extinction + No Stimulation group (N= 11), the rostroventral periaqueductal gray Extinction + Stimulation group (N= 11) and the ventral tegmental area Extinction + Stimulation group (N= 11).
20.33: Pretest Results

The pretest results demonstrate that all three experimental groups in this study displayed FPS 48 hours after the presentation of 30 light + footshock training trials. ANOVA (3 Stimulation Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition $F(1,30)= 50.65, p<0.000001$, with no significant effect of Group, or Group X Stimulus Condition interaction. Newman-Keuls post hoc tests of the ANOVA results revealed that all groups showed higher mean startle amplitudes on the 3 CS + noise trials than on the last 3 noise-alone trials. These pretest results are depicted in Figure 147, and show that rats in all three experimental groups exhibit robust fear-potentiated after fear conditioning. Also, the three experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. An ANOVA of the pretest difference score data (Difference Score= [3 light + noise trials]-[last 3 noise alone trials]) confirms that the three experimental groups had similar levels of fear $F(2,30)=0.01, p=0.985 [n/s]$. Figure 148 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the experimental groups were closely matched on fear prior to any experimental manipulation involving either extinction training or administration of 100 electrical stimulations of either the VTA or the dPAG.
Figure 147. Mean (S.E.M. ±) acoustic startle amplitudes for the mid-brain Extinction + No Stimulation, rostroventral periaqueductal gray Extinction + Stimulation and ventral tegmental area Extinction + Stimulation groups (N= 11 per group) on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only ( * P<0.05 relative to noise-alone trials). The mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the nearly equivalent magnitude of fear-potentiated startle exhibited by all three experimental groups 48 hours after 30 fear conditioning trials were administered to each group.
Figure 148. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) depict the equally robust levels of fear exhibited by all three experimental groups ([mid-brain Extinction + No Stimulation group; N=11][rost-dorso periaqueductal gray Extinction + Stimulation group; N=11][ventral tegmental area Extinction + Stimulation group; N=11]) forty-eight hours after fear conditioning.
20.34: Final Test Results and Discussion

The main finding of this experiment was that only electrical stimulation of the VTA seemed to produce FPS reinstatement in rats that had been exposed to fear-extinction training. A 3 Stimulation Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. This ANOVA approached but did not reach a main effect of Stimulus Condition F(1,30)=3.37, p=0.07 [n/s]. Similarly, the Stimulation Group X Stimulus Condition interaction approached but did not reach significance F(2,30)=3.05, p=0.06. Thus although Newman-Keuls post hoc tests revealed that VTA-stimulated rats showed higher acoustic startle amplitudes on the 10 light + noise trials than on the 10 noise-alone trials, this result just failed to reach statistical significance (see Figure 149). On the other hand, the Extinction + No Stimulation group (midbrain control group) and the dPAG-stimulation group showed no significant differences between the 10 light + noise trials and 10 noise-alone trials on the final test. These results indicate that whereas VTA stimulated rats exhibited a strong trend towards fear reinstatement, both the non-stimulated control group rats, and the dPAG stimulated rats exhibited no FPS reinstatement.

ANOVA of the difference scores (Difference Score= 10 light + noise trials - 10 noise-alone trials) calculated from the final test data just failed to yield an effect of Stimulation F(2,30)=3.05, p=0.06. As depicted in Figure 150, the mean difference score obtained for the Extinction + VTA Stimulation group (N=11) was 142.3 (SEM ± 50.8 compared to the mean difference score for the Extinction + dPAG Stimulation group (N=11) 54.4 (SEM ± 50.7), and the Extinction + No Stimulation group (N=11) -35.7 SEM ± 47.6. Newman-Keuls post hoc comparisons carried out on the difference scores revealed that only the VTA stimulation group’s magnitude of fear was significantly greater than that exhibited by the Extinction + No Stimulation control group. Although the dPAG stimulated group did exhibit a small increase in CS-induced startle, Newman Keuls post hoc tests found that it was not significantly different from the Extinction + No Stimulation control group.

These results indicate that only electrical stimulation of the VTA and not the dPAG was able to cause rats to exhibit a trend towards the reinstatement of FPS. Since the VTA Extinction + Stimulation group appeared to be showing a strong trend toward the
reinstatement of FPS it was decided that it would be necessary to only compare this group to the mid-brain Extinction + No Stimulation control group. Running this analysis would reduce the between-subjects variability and provide a more accurate representation of whether or not the VTA Extinction + Stimulation group was exhibiting a stimulation-induced reinstatement of FPS responding.

A 2 Stimulation Group X 2 Stimulus Condition (10 CS + noise vs. 10 noise-alone) ANOVA was run on the data obtained from the VTA Extinction + Stimulation and mid-brain Extinction + No Stimulation control group's final test results. The ANOVA of this data yielded a significant Stimulation Group X Stimulus Condition interaction $F(1,20)=6.32, p<.03$. Newman-Keuls post hoc within group comparisons revealed that the VTA Extinction + Stimulation group displayed significantly higher acoustic startle responding on the 10 CS + noise trials than on the 10 noise-alone trials during the final test (see Figure 151). Although not statistically significant, the VTA Extinction + Stimulation group did exhibit higher mean startle levels during the 10 CS + noise presentations than did the mid-brain Extinction + No Stimulation controls. This indicates that the VTA group has a significantly higher magnitude of fear when compared to the control group and a T-test of the difference scores ($\text{Difference score} = 10 \text{ CS + noise trials} - 10 \text{ noise-alone trials}$) confirmed this notion as it was shown to yield a main effect of Stimulation $t(1,20)=6.32, p<.03$.

These difference score results are shown in Figure 152 and they indicate that the VTA Extinction + Stimulation Group did indeed show a significantly higher magnitude of fear-reinstatement than the non-stimulated mid-brain control group. Thus, when compared only to the mid-brain Extinction + No Stimulation control group, the VTA Extinction + Stimulation group seems to exhibit a marked reinstatement of FPS responding. Although the results presented here are preliminary in nature, they are still important, since they demonstrate that excitation of VTA neurons and the mesolimbic DA pathway can, under the right circumstances, induce fear reinstatement.
Figure 149. Mean (S.E.M. ±) acoustic startle amplitudes for the mid-brain Extinction + No Stimulation group (N=11), the rostro-dorsal periaqueductal gray Extinction + Stimulation group (N=11) and the ventral tegmental area Extinction + Stimulation group (N=11) on the 10 noise-alone and 10 CS + noise trials of the fear-potentiated startle reinstatement test as a function of stimulation or no stimulation that occurred 24 hours earlier ( ★ P<0.05 relative to noise-alone trials). The mean difference scores (S.E.M. ±) of the reinstatement test ([10 CS + noise]-[10 noise-alone]) depict the effect of electrical stimulation of the periaqueductal gray and ventral tegmental area on the magnitude of fear-potentiated startle reinstatement exhibited ( + P<0.05 relative to the VTA Extinction + Stimulation group only).
Figure 150. Mean (S.E.M. ±) difference scores ([10 CS + noise] - [10 noise-alone]) for the mid-brain Extinction + No Stimulation, rostromedial periaqueductal gray Extinction + Stimulation, and ventral tegmental area Extinction + Stimulation groups (N=11 per group) on the fear-potentiated startle test carried out 24 hours after electrical stimulation or no stimulation of the rostromedial PAG and ventral tegmental area ( * P<0.05 relative to the Extinction + VTA Stimulation group only).
Figure 151. Mean (S.E.M. ± ) acoustic startle amplitudes for the mid-brain Extinction + No Stimulation Control group (N=11) and the Extinction + VTA Stimulation group (N=11) on the 10 noise-alone and 10 CS + noise trials of the fear-potentiated startle reinstatement test as a function of either stimulation or no stimulation that occurred 24 hours earlier ( * P<0.05 relative to noise-alone trials). The mean (S.E.M. ± ) difference scores of the reinstatement test ([10 CS + noise]-[10 noise-alone]) depict the effect of electrical stimulation of the ventral tegmental area (VTA) on the magnitude of fear-potentiated startle reinstatement expressed by this group when it is compared to the mid-brain Extinction + No Stimulation controls ( + P< 0.05 relative to the Extinction + VTA Stimulation group).
Fear-Potentiated Startle Reinstatement Difference Score Results

Figure 152. Mean (S.E.M. ±) difference scores ([10 CS + noise]-[10 noise-alone]) for the mid-brain Extinction + No Stimulation and Extinction + ventral tegmental area (VTA) Stimulation groups on the fear-potentiated startle reinstatement test carried out 24 hours after either electrical stimulation of the VTA or no stimulation of the mid-brain control group (N=11 per experimental group; * P< 0.05 relative to the Extinction + VTA Stimulation group).
This reinstatement of FPS may be accomplished through several neural substrates, but it most likely involves direct VTA neuronal projections that target the lateral, basolateral, and central amygdaloid nuclei (Swanson 1982). One possibility is that the excitation of VTA neurons through repeated stimulation served to elevate the firing rates and metabolic activity levels of amygdaloid neurons that possess the memory-trace of the CS-UCS association that was formed during Pavlovian fear conditioning. The findings reported in this experiment are generally consistent with the electrophysiological and pharmacological research literature that demonstrates the importance of both the mesoamygdaloid DA and non-DA neural systems in influencing amygdaloid kindling and the expression of FPS and defensive freezing responses (Gelowitz and Kokkinidis, 1999; Borowski and Kokkinidis, 1996; Greba, Munro and Kokkinidis, 2000; Munro and Kokkinidis, 1997; Gifkins, Greba and Kokkinidis, 2002; Nader and LeDoux, 1999). While it is still too early to make any definitive claims regarding the role of the VTA in fear reinstatement, several lines of research evidence seem to suggest that this midbrain region does have the capacity to influence conditioned fear expression and fear acquisition. For example, electrical stimulation of the VTA has been shown to alter the firing pattern of amygdala neurons (Rosenkranz and Grace, 1999) and increase acoustic startle responses in rats (Borowski and Kokkinidis, 1996). Furthermore, electrically induced kindling of the VTA generates after-discharge activity in the amygdala and triggers unconditioned fear responding in laboratory animals (Gelowitz and Kokkinidis, 1999; Stevens and Livermore, 1978). Moreover, chemical stimulation of the VTA increases levels of corticosterone and c-fos in the amygdala and prefrontal cortex (Cornish and vanden Buuse, 1996) and chemical lesions of the VTA block the expression of FPS as do pharmacological manipulations which inhibit the activity of DA neurons residing in this midbrain region (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997). This suggests that the VTA may, through its intimate connections with the amygdala, gate the level of emotional arousal within the amygdala-based fear circuit and influence fear-reinstatement and other conditioned fear responses (Borowski and Kokkinidis, 1996; Munro and Kokkinidis, 1997).

Another possibility is that electrical stimulation of the medial forebrain bundle (MFB) may have contributed to the FPS reinstatement effect observed in the Extinction + VTA Stimulation group of Experiment 9 since some bipolar electrodes may have been close
enough to activate this fibre pathway. As was mentioned in the histology results section, the MFB travels through the VTA and lateral hypothalamus and eventually targets several limbic and cortical regions such as the amygdala, septum, nucleus accumbens, BNST, entorhinal cortex, insula and prefrontal cortex. In addition, the MFB contains numerous fibres that emerge from out of the DA cell group of the VTA (Swanson, 1982; Oades and Halliday, 1987; Björkland and Lindvall, 1986). It is therefore quite possible that electrical stimulation of either the VTA or the MFB carrying VTA efferents may have contributed to the positive increases in FPS reinstatement observed in the Extinction + VTA Stimulation group. It is tempting to speculate that MFB stimulation, like VTA stimulation, may have produced this effect on FPS reinstatement either by elevating DA metabolites and increasing neuronal excitability in the amygdala and other limbic and corticolimbic structures (i.e. nucleus accumbens, BNST, septum, insula and prefrontal cortex) or by reducing cellular activity in discrete neuronal populations and brain regions which are thought to impose inhibitory control over the amygdaloidal complex. Thus, electrical stimulation of the VTA and/or MFB may have caused excitation of the amygdala neurons that are devoted to excitatory forms of fear learning and expression, whilst suppressing the activities of intrinsic amygdaloidal and extrinsic mPFC inhibitory neural networks that work to reduce amygdaloidal excitability levels and preserve the behaviours that develop from fear-extinction training. In other words, repeated electrical stimulation of the VTA and MFB may have somehow caused neural mechanisms that coordinate inhibitory learning and behaviour to breakdown. Such an event would have made it possible for the neurons in the amygdala to displace inhibitory learning and resurrect the CS-UCS fear associations and memory traces that had been formed during Pavlovian fear conditioning. This might explain why the Extinction + VTA Stimulation rats of Experiment 9 exhibited FPS reinstatement when they were compared to the Extinction + No Stimulation midbrain control group. A detailed discussion of these and other relevant issues is given in the general discussion section of this thesis.

A final possibility is that some electrical current may have spread to activate caudal portions of the lateral hypothalamic area located just anterior and ventrolateral to the rostral VTA (i.e. between -4.16 mm and -4.52 mm relative to bregma). The lateral hypothalamic area is thought to be involved in regulating autonomic conditioned fear responses as both
chemical and electrolytic lesions of this hypothalamic region disrupt CS-evoked elevations of the mean arterial blood pressure response in rats that have experienced CS + footshock fear training (LeDoux, et al., 1988). Furthermore, the lateral hypothalamic area is connected to the periventricular nucleus of the hypothalamus (Silverman, Hoffman and Zimmerman, 1981), an area that together with the amygdala regulates corticosteroid release during times of fear or stress (for a review see Davis, 1992a,b; Chapter 4 section 4.4). In addition, the lateral hypothalamic area cells project to the dorsal motor nucleus of the vagus and the nucleus of the solitary tract, two prominent nuclei in the medulla that control fear/stress-induced urination, defecation, bradycardia and ulcer formation (van der Kooy, et al., 1984; Schwansel-Fukuda, Morrel and Pfaff, 1984; Davis, 1992a,b) and electrical or chemical stimulation of this particular hypothalamic region does elevate systemic arterial pressure, lumbar sympathetic neuronal discharge and excitability of premotor neurons of the ventrolateral medulla where the solitary and vagus nucleus are located (Sun and Guyenet, 1987). Thus, it is conceivable that electrical activation of the lateral hypothalamic area and neighbouring MFB may have been sufficient to provoke changes in blood pressure, heart rate and various other autonomic responses during stimulation-induced FPS reinstatement training. Such an occurrence in fear-extinguished rats may have served as an internal autonomic cue that somehow worked to convoke CS-UCS memory traces and other related internal physiological states that were experienced during Pavlovian fear conditioning. However, caution needs to be taken when making these types of hypothesis since many of the electrodes in the Extinction + VTA Stimulation rats of Experiment 9 were either in the anterior and dorsal VTA or at the very least were located closer to the VTA than to the lateral hypothalamus. This means that the positive impact on FPS reinstatement measured in the Extinction + VTA Stimulation group was probably due to an electrical activation of VTA neurons and projection pathways to the amygdala.

Nevertheless, the contribution made by hypothalamic neurons to FPS reinstatement should not be simply dismissed as research has shown that neurons in the dorsomedial nucleus of the hypothalamus of rats exposed to FPS testing tend to exhibit elevated levels of DA and norepinephrine (NE) (Shekhar, Katner, Rushe, Sajdyk and Simon, 1994). Also, fear-induced escape and flight responses provoked by the administration of an aversive sensory stimulus (e.g. footshock) can be significantly enhanced in laboratory animals if the
medial hypothalamus is stimulated (Redgrave and Horrel, 1975). Therefore, future research into FPS reinstatement should be directed at evaluating the contribution made by certain hypothalamic nuclear regions such as the periventricular nucleus and the dorsomedial nucleus that have been shown to play an integral role in mediating fear and stress-related responses (see Chapter 4 section 4.4; Shekhar, et al., 1994). This type of research would be quite interesting in light of the fact that the amygdala sends extensive projections to the hypothalamus (McBride and Sutin, 1977; Krettek and Price, 1978a; Gray, et al., 1989; Rosen, et al., 1991; Price, et al., 1991), receives input from the hypothalamus (Ottersen, 1980), and interacts with several hypothalamic nuclei in order to regulate autonomic responses, corticosteroid release and ulcer formation during times of intense fear and stress (Brown and Gray, 1988; Iwata, et al., 1987; Gray, et al., 1989; Feldman and Conforti, 1981; Feldman, et al., 1994; Henke, 1980; Ray, et al., 1988a; 1988b; Gelsema, et al., 1987; also see Chapter 4 section 4.4).

The lack of FPS reinstatement in the dPAG Extinction + Stimulation group could be due to the fact that electrodes were generally more anterior and rostrally situated than desired. As a result neurons in the dPAG may not have been excited to sufficient levels to facilitate fear reinstatement. In addition, axonal projections from amygdala neurons to the PAG are robust however there are fewer reciprocal projections from the PAG directly back to the amygdala. Moreover, the PAG sends most of its projections to midbrain, hindbrain, and spinal cord nuclei. This may mean that excitation of the PAG by stimulation may have produced dramatic increases in heart rate, respiration and defensive jumping behaviour without activating the necessary amygdaloid neurons that are involved in fear memory storage and retrieval. As a result, electrical stimulation of the PAG may have produced all the autonomic and behavioural correlates typically associated with a central fear state without directly activating the amygdala or higher brain regions that contain a memory-trace of the CS-UCS association that was established during fear conditioning.

In essence, one may speculate that dPAG stimulation may have produced all the autonomic and behavioural correlates associated with fear, but whether fear reinstatement occurred or not depends to a large degree on whether or not the amygdala was activated enough to link up the autonomic, visceral and behavioural reactions with prior learning that occurred during fear conditioning. It is possible that electrical stimulation of the brain
regions downstream of the amygdala that typically produce increases in heart rate, respiration and jumping behaviour (i.e. aversive events other than footshock) may not tap into or necessarily be sufficient to cause extinguished fear responses to be reinstated. Perhaps, direct excitation of the amygdala neurons and pathways that are involved in the storage and retrieval of fear memories may be required. Hence not just any aversive event or internal bodily state produced by electrically stimulating the PAG would be sufficient to cause a reinstatement of FPS.

Nevertheless, Borowski and Kokkinidis (1996) did report that electrical stimulation of the dPAG did elevate acoustic startle responding in rats. Also, electrical or chemical stimulation of the PAG produces a number of physiological and behavioural responses that are indicative of a central fear state (Bandler, 1982; Di Scala, 1987; Borowski and Kokkinidis, 1996) and an extensive research literature has shown that the PAG is involved in mediating conditioned defensive freezing behaviour (LeDoux, et al., 1988; De Oca and Fanselow, 1995 Fanselow, et al., 1995; Fanselow, 1991), defensive attack and flight (Bandler and Shipley, 1994; Behbehani, 1995; Bandler, 1982; Fernandez de Molina, et al., 1962) and FPS (Fendt, et al., 1994; 1996; Fendt, 1998). In this regard, it is noteworthy that the dPAG Extinction + Stimulation group did show higher albeit non-significant acoustic startle responding on the CS + noise trials than on the noise-alone trials during final testing. Given this observation, it may be necessary to re-examine the dPAG’s role in the reinstatement of FPS at some future date. Although it is beyond the scope of this thesis, future research may use lower current levels to stimulate this midbrain region and perhaps adjust several other stimulation parameters as well. Also, implanting electrodes slightly more caudal and ventral in relation bregma would be advisable.
21. Experiment 10:

Electrical Stimulation of the Caudoventral Periaqueductal Gray and its Impact on Reinstating Fear-Potentiated Startle in rats that have been Exposed to Extinction Training

21.1: Rationale

The ventral PAG has been shown to be involved in mediating FPS and defensive freezing behaviour (Fendt and Koch, 1993, 1994; Fendt, et al., 1996; Fendt, 1998; Fanselow, 1995; LeDoux, et al., 1988). Since the vPAG role in mediating fear behaviours is well documented it was necessary to determine if stimulation of the vPAG after extinction training would cause a reinstatement of FPS in rats. To test this hypothesis, I carried out an additional experiment designed to assess the role of the vPAG in the reinstatement of FPS. The methods and results of this experiment are list below.

21.2: Method

21.21: Subjects

Fourteen, male Wistar rats bred and housed in the psychology animal facility at the University of Canterbury served as subjects in this experiment. On average, rats weighed 340 grams at the beginning of the experiment (range 265-375 grams). A constant temperature of 20°C Celsius (±1°C Celsius) was maintained and food and water were continuously available. Animals were group housed and experienced a 12:12 hour light-dark cycle with lights on at 8:00 am and off at 8:00 pm. All behavioural testing and experimental manipulation took place during the light portion of this cycle.

21.22: Stereotaxic Surgery

Rats were anesthetised with sodium pentobarbitone (95.0 to 110 mg/kg), mounted in a Stoelting stereotaxic instrument (Wood Dale, IL), and then surgically implanted with a single bipolar stainless steel twisted electrode (MS-303/1; Plastics-One, Roanoke, VA) aimed at the medial portion of the caudal ventral periaqueductal gray (vPAG). Prior to surgery, all electrodes were cut to length, gapped and scraped in a manner identical to that
described in Experiment 7A. Electrode implantation followed the same flat skull stereotaxic surgical technique and incisor bar adjustment that was described in Experiment 7A. The vPAG coordinates were A.P. - 7.80 mm from bregma, M.L. ± 0.70 mm from the sagittal suture, and D.V. -5.80 mm from the skull surface and were taken from the rat brain atlas of Paxinos and Watson (1986).

Electrodes were unilaterally implanted with half of the animals in each group receiving an electrode in the right hemisphere and the remaining animals receiving implantation in the left hemisphere. The electrode was secured to the skull surface via a head cap constructed from dental acrylic. This head cap was attached to five stainless steel self-tapping jeweller screws (3.20 mm in length, Lomat Precision Tools, Montreal Quebec, Canada) embedded in the skull surface. Specially designed dummy dust caps (303DC and 303DCA; Plastics-One, Roanoke, VA) were screwed onto the secured electrode to prevent rats from chewing the plastic portion of the electrode and making it inoperative. Following surgery, rats were placed into a recovery cage until the anaesthetic wore off and then were returned to normal group housing conditions. Rats were allowed a 14 day recovery period to ensure their wounds were adequately healed before any behavioural testing or experimental manipulation commenced.

21.23: Procedure

21.24: Baselining, Fear Conditioning, and Pretesting

The method used to baseline, fear condition, and pretest rats for FPS in this particular experiment are identical as those described in Experiments 7A, 8 and 9. For a review see the method section of Experiment 7A. As with the previous experiments, baselining was used to produce stable baseline startle and acclimatise the rats to the startle testing apparatus. In addition, FPS scores obtained from the pretest were used to assign rats to experimental groups in a fashion that ensured that groups were equated on level of conditioned fear.
21.25: Group Assignment, Extinction Training, and Unsignalled Electrical Stimulation of the Ventral PAG

After pretesting for FPS, rats were assigned to one of two experimental groups (N= 7; per group) with similar averaged startle scores, noise intensity levels, and levels of FPS as reflected through the difference score calculation and analysis. Twenty-four hours later rats were placed in their designated test chambers, and after a 5 min habituation period, exposed to 140 light-alone extinction trials. The interstimulus interval for the light presentation during extinction training was 15 s.

The two experimental groups were formed and they included; the Extinction + 100 vPAG Stimulation group (N= 7) and the Extinction + No Stimulation control group (N= 7). Forty-eight hours after extinction, rats were connected to a programmable constant current stimulator via an insulated bipolar electrode cable (305-S304-CCT 80CM TT2 CS, Plastics One, Roanoke VA) which connected to the MS- 303/1 bipolar electrode. The wire cable was protected by a stainless steel coil spring to prevent rats from damaging the wire. After rats were connected to the stimulator, they were then placed into their designated chambers and allowed 5 min to acclimatise to the test environment. Rats assigned to the Extinction + vPAG Stimulation group were then administered 100 unsignalled electrical brain stimulations.

The current intensity used to stimulate the vPAG was 400 μA (base to peak current) with a pulse duration of 0.1 ms, and a frequency of 100 Hz. The interstimulus interval used in this experimental manipulation was 3 s and the duration of the stimulation was 500 ms. The programmable constant current stimulator was connected to the 386 computer that ran the startle testing and fear conditioning programs, and a specially designed computer program was used to trigger the electrical brain stimulation. After the electrical stimulation was completed, rats were left in the test chambers for an additional minute and then returned to the home cage environment. Rats assigned to the No Stimulation vPAG control group did not receive any electrical brain stimulation. It was noticed that electrical stimulation of the vPAG with the 400 μA current caused excessive locomotor activity and violent jumping behaviour that could have caused injury to the animals or damage to the equipment. Given this observation, and for the obvious ethical reasons, it was decided to
terminate the study early, thus leaving the Extinction + vPAG stimulation group with N = 7
before histology was completed.

21.26: Final Test

Twenty-four hours after stimulation or no stimulation administration, rats were tested
for the reinstatement of FPS. The final test employed a quasi-counterbalanced design.
This procedure entailed presenting half of the rats in each experimental group with 10
noise-alone trials first followed by 10 light + noise trials, whilst the other half of the rats in
each experimental group were exposed to 10 light + noise trials first followed by 10 noise-
alone trials. The interstimulus interval for the CS + noise and the noise-alone trials in the
final test was 30 s. During the CS + noise trials the duration of the light was 3.5 s and the
white-noise burst was presented during the last 100 ms of the light interval. The purpose of
the final test was to determine if electrical stimulation of the vPAG caused fear-
extinguished rats to exhibit a reinstatement of FPS.

Once again, it is important to note that after-discharge threshold current levels were not
collected for the vPAG electrode rats since electrical currents of 400 µA already caused
significant locomotor and jumping behaviour. Increasing the current higher than 400 µAs
would have caused uncontrollable locomotor activity and possible injury to the animals.
As a result, no after-discharge data was collected from the vPAG as it had the potential to
cause too much trauma to the animals. After the experiment was terminated, perfusion and
histology was carried out as previously described.

21.27: Statistical Analysis and Dependent Measures

Independent T-tests were used to statistically examine several dependent variables, these
included; acoustic startle amplitude data obtained during baselining, shock reactivity data
gathered during fear conditioning, and the magnitude of FPS calculated from noise-alone
and CS + noise trials on the pretest and fear reinstatement test. A 2 Stimulation Group X 2
Stimulus Condition (CS + noise vs. noise-alone) ANOVA was also employed to analyse the
pretest and final test data. Newman-Keuls multiple comparisons (α = 0.05) were used to
assess differences between experimental group means. Also as mentioned in Experiment
7A, Newman-Keuls post hoc tests that relied on a pooled error term were used to
statistically evaluate any between group difference when and if any significant interactions occurred with the ANOVA statistical method. Shock reactivity was evaluated in this experiment by comparing the averaged movement amplitudes obtained during the first 100 ms of each electric shock presentation over the 30 CS-UCS trials. Results obtained from ANOVA and independent T-tests were used to demonstrate that the two experimental groups were equated on noise intensity level, baseline startle amplitude, and FPS prior to extinction training or electrical stimulation of the vPAG.

21.3: Results

21.31: Histology Results

As mentioned earlier in Experiment 9, two animals from the caudoventral PAG Extinction + Stimulation group were transferred to the dorsal PAG Extinction + stimulation group because the histological profile regarding the location of the electrodes more closely matched the criteria of the dorsal PAG group. As a result, the caudoventral PAG Extinction + Stimulation group was left with N= 5, whereas the caudoventral PAG Extinction + No Stimulation group was left with (N= 7). Figure 153 depicts the location of electrodes in the caudoventral PAG for both experimental groups.

21.32: Experimental Results

An independent T-test of the baseline startle amplitude data revealed that there were no significant between group differences on baseline startle amplitudes when comparing the two experimental groups t(2,10)=0.002, p=0.999 [n/s]. The independent T-test results indicate that groups had been properly matched and had similar baseline acoustic startle responding (see Figure 154). The T-test of the noise intensity levels used to initiate stable baseline startle scores also yielded a non-significant result t(2,10)= 0.669; p=0.518 [n/s], demonstrating that the groups adequately were matched on measure (see Figure 155). The T-test of the shock reactivity data obtained during fear conditioning yielded a non-significant result t(2,10)=0.198, p=0.846 [n/s], indicating that the two experimental groups showed similar levels of movement amplitude during CS + footshock administration. The
shock reactivity scores obtained during fear conditioning are graphically depicted in Figure 156.
Figure 153. A schematic depiction of the bipolar electrodes located in or near the caudal portions of the ventral periaqueductal gray (vPAG). The electrode locations are represented by the solid filled circles (●). On the left are the electrode locations for the Extinction + vPAG Stimulation group (N=5) while on the right are the electrode locations for the Extinction + vPAG No Stimulation control group (N=7). Bipolar electrodes were unilaterally implanted into either the left or right vPAG nuclei for both experimental groups (coordinates A.P. -7.80 mm from bregma, M.L. ±0.70 mm from the midline sagittal suture, D.V. -5.80 mm from the skull surface). Representative sections (top to bottom; -6.80 mm, -7.04 mm, -7.30 mm, -7.64 mm and -7.80 mm from bregma) were taken from The Rat Brain in Stereotaxic Coordinates, by G. Paxinos and C. Watson, 1986.
Figure 154. Mean (S.E.M. ± ) baseline startle amplitudes recorded on the third baselining block for the ventral periaqueductal gray Extinction + No Stimulation group (N=7) and the ventral periaqueductal gray Extinction + Stimulation group (N=5).
Figure 155. Mean white-noise intensity levels (S.E.M. ±) recorded in decibels that were used to induce stable acoustic startle responses for the ventral periaqueductal gray Extinction + No Stimulation (N=7) and the ventral periaqueductal gray Extinction + Stimulation group (N=5).
Shock Reactivity During Fear Conditioning

![Graph showing shock reactivity during fear conditioning.]

Figure 156. Mean (S.E.M. ±) movement amplitudes recorded in response to footshocks presented during the administration of 30 light + footshock fear conditioning trials to ventral periaqueductal gray Extinction + Stimulation (N=5) and Extinction + No Stimulation (N=7) experimental groups.
21.33: Pretest Results

The pretest results indicate that both experimental groups in this study displayed FPS after being exposed to a Pavlovian fear conditioning regime (i.e. 30 light + footshock trials). ANOVA (2 Stimulation Group X 2 Stimulus Condition) of the pretest data revealed only a main effect of Stimulus Condition F(2,12)= 22.75, p<0.0005, with no significant effect of Group, or Group X Stimulus Condition interaction. These results are depicted in Figure 157, and show that rats in both experimental groups displayed robust FPS after fear conditioning. Also, the two experimental groups had similar levels of fear as indicated by the non-significant Group effect and by the lack of a significant Group X Stimulus Condition interaction. An independent T-test of the pretest difference score data (Difference Score= [3 light + noise trials]-[last 3 noise alone trials]) confirms that both experimental groups had similar levels of fear t(2, 10)=0.155, p=0.879 [n/s]. Figure 158 depicts the mean difference scores obtained from the pretest and clearly demonstrates that the experimental groups were closely matched on fear prior to any experimental manipulation involving either extinction training or administration of 100 electrical stimulations of the vPAG.
Figure 157. Mean (S.E.M. ±) acoustic startle amplitudes for the ventral periaqueductal gray Extinction + No Stimulation (N=7) and Extinction + Stimulation (N=5) groups on the 3 noise-alone and 3 CS + noise trials of the pretest as a function of fear conditioning only (★ P<0.05 relative to the noise-alone trials). The mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]-[3 noise-alone]) display the equally robust magnitude of fear expressed by the two experimental groups 48 hours after Pavlovian fear conditioning.
Figure 158. Mean difference scores (S.E.M. ±) of the pretest ([3 CS + noise]- [3 noise-alone]) for the ventral periaqueductal gray Extinction + No Stimulation (N=7) and Extinction + Stimulation (N=5) experimental groups depicts the nearly equivalent and robust levels of fear exhibited by both groups 48 hours after exposure to 30 light + footshock fear conditioning trials.
21.34: Final Test Results and Discussion

The main finding of this experiment was that vPAG stimulation failed to cause FPS reinstatement in a group of fear-extinguished rats. A 2 Stimulation Group X 2 Stimulus Condition (10 light + noise trials vs. 10 noise-alone trials) ANOVA was carried out on the final test data. ANOVA failed to obtain a main effect of Stimulus Condition F(1,10)=0.104, p=0.753 [n/s]. The Stimulation Group X Stimulus Condition interaction also failed to reach statistical significance F(1,10)=0.839, p=0.381 [n/s]. These results indicate that vPAG-stimulated rats did not exhibit fear reinstatement because they did not differ significantly from the non-stimulated vPAG control group rats (see Figure 159).

An independent T-test of the difference scores (Difference Score= [10 light + noise trials] – [10 noise-alone trials]) calculated from the final test data confirmed that vPAG stimulation did not cause a reinstatement of FPS {t(2,10)= .916, p=0.381 [n/s]}. Figure 160 displays the mean difference score obtained for the Extinction + vPAG Stimulation group {((N= 7), ~61.4 SEM ± 11.6} and the mean difference score for the Extinction + No Stimulation control group {((N= 5), 29.4 SEM ± 118.6}. These results indicate that electrical stimulation of the vPAG was not able to cause a reinstatement of FPS. Thus although the electrical stimulation of the vPAG produced violent jumping behaviour, a visible increase in respiration and other autonomic and behavioural correlates typically associated with fear or exposure to an aversive sensory stimulus, it did not cause a reinstatement of FPS in animals that had received extinction training.
Final Test Scores for the Reinstatement of FPS

Figure 159. Mean (S.E.M. ±) acoustic startle amplitude for the ventral periaqueductal gray Extinction + No Stimulation (N=7) and Extinction + Stimulation (N=5) experimental groups on the 10 noise-alone and 10 CS + noise trials of the test designed to measure the reinstatement of fear-potentiated startle as a function of electrical stimulation of the ventral periaqueductal gray 24 hours earlier (★ P<0.05 relative to noise-alone trials). The mean difference scores (S.E.M. ±) of the final test ([10 CS + noise]-[10 noise-alone]) show the effect of electrical stimulation of the ventral periaqueductal gray on the reinstatement of fear-potentiated startle in rats that were exposed to fear-extinction training.
Figure 160. Mean (S.E.M. ±) difference scores ([10 CS + noise] - [10 noise-alone]) for the ventral periaqueductal gray Extinction + No Stimulation (N=7) and Extinction + Stimulation (N=5) groups on the final test conducted 24 hours after either electrical stimulation or no electrical stimulation of the caudal portions of the ventral periaqueductal gray.
A possible explanation for this apparent lack of FPS reinstatement to a specific CS is that vPAG stimulation may have produced very high levels of contextual fear and this stimulation induced contextual fear may have effectively masked CS specific fear expression and reinstatement. This view is supported by the fact that the vPAG Extinction + Stimulation group generally exhibited much higher levels of acoustic startle responding during the noise-alone trials than did the vPAG Extinction + No Stimulation control group. Figure 159 clearly shows that rats of the vPAG Extinction + Stimulation group have acoustic startle response amplitudes that are elevated on the noise-alone and light + noise stimulus conditions when compared to the vPAG Extinction + No Stimulation controls. Although an independent T-test on just the noise-alone trials of final test for the vPAG Extinction + No Stimulation and vPAG Extinction + Stimulation groups failed to yield a significant result $t(2,10)= 1.01 \ p= 0.333$ [n/s], it is still possible that acoustic startle responding and contextual fear may have been elevated in the vPAG Extinction + Stimulation group enough to disrupt fear-reinstatement. It is important to note that this increase in noise-alone acoustic startle responding exhibited by the vPAG-stimulated rats is generally symptomatic of contextual fear and is similar in some ways to fear responses that are evoked after exposure to unsignalled and aversive footshocks (see Gifkins, Greba and Kokkinidis, 2002; Richardson and Elsayed, 1998; Davis, 1989). It is therefore possible that the observed stimulation-induced increase in noise-alone responding and contextual fear may have been sufficient enough to interfere with CS-induce fear reinstatement.

However, given the small sample sizes used, and the fact that a single electrical current intensity was employed in this study, makes any definitive claims regarding the contribution of the vPAG to fear reinstatement difficult. Taking this into consideration, future research may need to focus on using lower electrical currents to stimulate the vPAG that produce less violent motor behaviours and autonomic arousal. Introducing such measures would provide a more accurate assessment of the vPAG's role in reinstatement of FPS.