The Effects of FG7142 on Overexpectation of Pavlovian Fear Conditioning

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Six experiments studied the role of GABA_A receptor activation in expression of overexpectation of Pavlovian fear conditioning. After separate pairings of CSA and CSB with shock in Stage I, rats received pairings of the compound AB with shock in Stage II, producing overexpectation of fear. The expression of overexpectation was attenuated, in a dose-dependent manner, by the benzodiazepine partial inverse agonist FG7142. FG7142 had no effect on responding to a CS paired with a low magnitude US or a CS subjected to associative blocking. These results suggest that the negative prediction error generated during overexpectation training may impose a mask on fear rather than erasing the original fear learning. They support claims that overexpectation shares features with extinction.

Keywords: overexpectation, extinction, fear, GABA, prediction error

Pavlovian fear conditioning occurs when an otherwise neutral stimulus (the conditioned stimulus [CS]) is paired with an aversive outcome (the unconditioned stimulus [US]), leading to formation of an association between the CS and the US. This association is expressed on subsequent presentations of the CS as a constellation of coordinated fear responses (species-typical defense behaviors, autonomic arousal, and endocrine activation). The actions of predictive error are central to such association formation. Learning is determined by the “error” or difference between the expected outcome of the trial and the actual outcome. Fear is acquired due to positive predictive error, when the actual outcome of the trial exceeds the expected outcome (e.g., Rescorla & Wagner, 1972).

Fear is lost due to negative predictive error, when the expected outcome of a conditioning trial exceeds the actual outcome. The prototypical example of negative predictive error is extinction. In a fear extinction experiment a CS is first paired with shock. Then, during extinction training, the CS is presented alone. The presence of the CS but absence of the otherwise expected US generates a negative predictive error (actual outcome < expected outcome) and loss of fear. There is currently intense interest in the neural mechanisms for fear extinction, at least in part because such mechanisms are relevant to treatment of human disorders of fear. However, the negative prediction error that gives rise to extinction learning can also be produced under other circumstances. Study of these other circumstances provides important insight into the nature and consequences of fear loss.

Rescorla (1970) conditioned rats to fear a tone and a flashing light separately using a shock US in Stage I. The experimental group then received conditioning of a compound of the two CSs in

Stage II, with the same shock US. Control groups received either no Stage II conditioning or continued to receive conditioning of one of the two CSs. The experimental group showed less fear on test to the CSs than the control groups. Fear of the CSs had been reduced despite continued pairings with shock. Such overexpectation occurs because Stage I training increases the associative strength of each CS. When presented in compound in Stage II, the expected outcome (the summed predictions from the CSs) exceeds the actual outcome (the US). This negative prediction error (actual outcome < expected outcome) causes a loss of associative strength to the CSs present during Stage II. Overexpectation is a robust and widespread phenomenon, having been observed in various species and conditioning preparations (e.g., Blaisdell, Denniston, & Miller, 2001; Kamin & Gaioni, 1974; Kehoe & White, 2004; Khallad & Moore, 1996; Kremer, 1978; Lattal & Nakajima, 1998; McNally, Pigg, & Weidemann, 2004).

Despite apparent differences in the circumstances that result in their production, a fundamental prediction of the Rescorla–Wagner model (Rescorla & Wagner, 1972; Wagner & Rescorla, 1972) is that overexpectation and extinction share a common cause (negative prediction error). The model therefore predicts that manipulations that affect fear extinction should exert similar influences on overexpectation of fear. There has been little investigation of this possibility but the available evidence is consistent with it. At the neurobiological level, endogenous opioids acting at opioid receptors are critical for both fear extinction and fear overexpectation learning. McNally and Westbrook (2003) showed that administrations of the opioid receptor antagonist naloxone prior to extinction training impaired extinction learning. McNally et al. (2004) showed that administrations of naloxone prior to Stage II overexpectation training also impaired overexpectation learning (see also Cole & McNally, 2007).

Rescorla likewise documented similarities between overexpectation and extinction at the behavioral level. For example, using appetitive magazine approach conditioning, Rescorla (2006, 2007) showed that the decrement in responding produced by overexpectation training was reduced by the passage of time between Stage II training and test (i.e., responding spontaneously recovered) or if test for fear to an overexpected CS occurred in a context different

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to that used for Stage II training (i.e., responding was renewed). These findings are especially striking because they suggest that the decrement produced by overexpectation training is not a partial erasure of the original learning. Rather, like extinction, these findings suggest that a mask is imposed on responding after overexpectation training and this mask can be removed with the passage of time or a change in physical context.

The experiments reported here were concerned with the nature of any such mask imposed on conditioned fear by overexpectation training. The aim was to study the role of GABA_A receptors in modulating expression of fear after overexpectation training and therefore to extend examination of similarities between overexpectation and extinction. Activation of GABA_A receptors is important for modulating fear after extinction training. Harris and Westbrook (1998) showed that administration of FG7142, a partial inverse agonist at the benzodiazepine binding site on the GABA_A receptor, prior to testing for fear extinction prevented expression of fear extinction. Moreover, because the effects of FG7142 did not summate with the effects of context change between training and test, Harris and Westbrook suggested that a GABAergic mask may mediate the temporal and context-specificity of fear extinction.

A role for changes in GABA_A receptor activity in extinction is further supported by the finding that fear extinction training up-regulates benzodiazepine binding and gephyrin (GABA_A receptor clustering protein) mRNA and protein expression in basolateral amygdala (Chhatawal, Myers, Ressler, & Davis, 2005). Taken together, these findings suggest that increases in neurotransmission through amygdala GABA_A receptors modulates the expression of fear after extinction training. If the mask on fear produced by overexpectation depends on identical mechanisms to those involved in extinction, expression of overexpectation, like extinction, should be vulnerable to a negative modulator of GABA efficacy at the GABA_A receptor. We examined this question by testing the effect of FG7142 on freezing to a tone CS after overexpectation.

Experiment 1

As only one published study has previously shown overexpectation using freezing as a measure of conditioned fear (McNally et al., 2004), the aim of Experiment 1 was to demonstrate overexpectation of a freezing CR in rats as well as to determine the amount of Stage II training optimal for its detection. The experiment used a single factor, four group design. All groups were trained to fear a visual and auditory CS in Stage I. In Stage II groups received compound presentations of the visual + auditory CS coterminating in shock. All groups were then tested for fear reactions (freezing) to the auditory CS. The groups differed in the amount of Stage II training: 0, 2, 4, or 8 compound conditioning trials.

Method

Subjects

Subjects were experimentally naive male Wistar rats obtained from a commercial supplier (Gore Hill Research Laboratories, Sydney, Australia). After arrival, rats were housed in groups of 7 to 8 in plastic cages maintained on a 12:12-hr light–dark cycle.

They were allowed access to food and water ad libitum. Rats were handled for 1-min per day for 5 to 7 days prior to any experimental procedures. The procedures were approved by the Animal Ethics Committee at the University of New South Wales and were conducted in accordance with the National Institute of Health (NIH) guidelines for the care and use of laboratory animals.

Apparatus

Behavioral procedures were conducted in a set of four identical chambers (195 mm height × 234 mm width × 204 mm length). The front and rear walls as well as the hinged lid were constructed of clear Perspex and the side walls were made of stainless steel. The floor consisted of stainless steel rods, 2 mm in diameter, spaced 13 mm apart (center to center). Each chamber stood 35 mm above a tray of paper pellet bedding (Fibercycle, Mudgeeraba, Australia). The chambers were cleaned with a damp paper towel and bedding changed between rats. These chambers were located individually within sound attenuating boxes, the inner walls of which were painted black. The boxes were illuminated with a red LED light. An extractor fan in the rear wall of each box was operating during all sessions.

The auditory CS was an 82 dB, 750 Hz tone (0.1 s rise–fall time). The visual CS was a 4 Hz presentation of a white fluorescent light mounted on the ceiling of the sound-attenuating box, producing an illumination level of 75 candela/m² within the chambers. Both CSs were 30 s in duration and during Stage I and II coterminated with a 1 s, 0.5 mA unscrambled AC 50 Hz shock from a constant-current generator that was delivered to the floor of each chamber. Digital video cameras were mounted on the rear wall of each box and connected to a digital multiplexer in an adjacent room that, in turn, was connected to a DVD recorder. The stimuli were controlled by computer (LabView, National Instruments, Austin, TX).

Procedure

Stage I. During the mornings of Days 1 to 6 all rats were placed in the conditioning chambers for 20-min sessions during which they were exposed to one CS-shock pairing (auditory CS on Days 1, 3, & 5; visual CS on Days 2, 4, & 6) commencing 9 min 30 s after placement in the chambers. On the afternoons of Days 4 to 6 rats were placed in the chambers again for 10 min each, and did not receive exposures to any CSs or shocks. These exposures were intended to reduce fear of the context.

Stage II. Rats in the control group received 30 s of handling on the morning of Day 7. Rats in the remaining groups were placed in the conditioning chambers and received either 2, 4, or 8 presentations of the compound visual + auditory CS coterminating in shock. Onset of the first trial was 4 min 30 s after placement in the chambers, and intertrial intervals were 4 min 30 s. Rats were removed from the chambers 5 min after the last shock.

Test. On Day 8 all rats were placed in the chambers and exposed to four 30 s presentations of the auditory CS. The first CS presentation began 4 min after placement in the chambers, and further CS presentations followed at an interstimulus interval (ISI) of 4 min 1 s.

Data Analysis

Rats were scored every 8 s for freezing during context exposures and every 2 s during CS presentations on each day. Freezing was defined as the absence of all movement other than that required for respiration. The data were converted into percentages of observations scored as freezing during the observation period. In this and remaining experiments, any rat that showed greater than 50% context freezing during the initial 3 min of context exposure during the final test session was excluded from further procedures. In the present experiment there were 13 rats per group at the start of the experiment. Four rats were affected by the exclusion criterion and thus final group sizes were: group control ($n = 13$); group over - 2 ($n = 12$); group over - 4 ($n = 12$); group over - 8 ($n = 11$). Data were analyzed by means of planned contrast testing. The Type I error rate (α) was controlled at .05 for each contrast tested (Hays, 1972).

Results and Discussion

Figure 1 shows the mean and standard error of the mean (*SEM*) levels of freezing during the three stages of the experiment. At the end of Stage I training there was significantly more freezing during CS presentations than during the pre-CS period, $F(1, 47) = 91.2$, $p < .05$ (M pre-CS = 11.2%, $SEM = 2.1$; M CS = 48.9%, $SEM = 3.1$).

Performance during Stage II is shown in the middle panel of Figure 1. There was significantly more freezing during CS presentations ($M = 46.4\%$, $SEM = 3.1$) than during the pre-CS period ($M = 5.3\%$, $SEM = 1.8$), $F(1, 34) = 130.4$, $p < .05$. There were no differences between groups in levels of pre-CS freezing (group over - 2, $M = 5.1$, $SEM = 2.0$; group over - 4, $M = 3.4$, $SEM = 1.3$; group over - 8, $M = 9.1$, $SEM = 4.8$), $F_s(1, 34) < 1.6$, $p > .05$. There was evidence for summation in freezing during the first Stage II trial: freezing was significantly higher to the first presentation of the compound CS as compared to the average responding observed to the individual CSs at the end of Stage I, $F(1, 34) = 16.4$, $p < .05$. This is a liberal criterion for assessing summation (e.g., Aydin & Pearce, 1997; Lattal & Nakajima, 1998; Rescorla, 1997), but a similar significant difference in freezing was observed

when the first compound trial was compared to the final Stage I presentation of the visual, $F(1, 34) = 12.2$, $p < .05$, or auditory CS, $F(1, 34) = 12.9$, $p < .05$.

Inspection of the middle panel indicates that freezing decreased as a function of the number of Stage II trials. There were no significant differences between groups in levels of freezing across the first two presentations of the compound, $F(1, 32) < 1$, $ps > .05$. There was a significant decrement in freezing across these first two trials, $F(1, 32) = 18.5$, $p < .05$, which did not interact with differences between groups, $F(1, 32) < 3.7$, $p > .05$. The linear decrease in freezing across the first four Stage II trials for groups over - 4 and over - 8 was also significant, $F(1, 21) = 17.7$, $p < .05$. These two groups did not differ over these four trials, and the between group contrast did not interact significantly with the linear decline in freezing, $F_s(1, 21) < 1$, $ps > .05$. Furthermore, the decrease in freezing across the eight Stage II trials for group over - 8 was also significant, $F(1, 10) = 26.0$, $p < .05$. The data of primary interest are those from test and are shown in the right-hand panel of Figure 1. Inspection of the panel confirms the presence of overexpectation that, surprisingly, was inversely related to the amount of Stage II training. Levels of pre-CS freezing on test were low (group control: $M = 13.3$, $SEM = 4.5$; group over - 2: $M = 10.2$, $SEM = 2.1$; group over - 4: $M = 15.6$, $SEM = 4.5$; group over - 8: $M = 18.2$, $SEM = 4.8$) and did not differ between groups, $F_s(1, 44) < 1.9$, $ps > .05$. There was significantly more freezing during CS presentations compared to the pre-CS period, averaged across groups, $F(1, 44) = 103.6$, $p < .05$. The analysis showed that among the experimental groups receiving Stage II training, freezing was significantly and inversely related to the amount of Stage II training, $F(1, 44) = 5.7$, $p < .05$, implying that overexpectation was most pronounced with fewer Stage II trials. This was confirmed by the findings that group over - 2, $F(1, 44) = 4.3$, $p < .05$, but not groups over - 4, $F(1, 44) = 1.6$, $p > .05$ and over - 8, $F(1, 44) < 1$, $p > .05$, differed significantly from group control.

These results confirm the presence of overexpectation of fear learning (Blaisdell et al., 2001; Kamin & Gaioni, 1974; Kremer, 1978; McNally et al., 2004; Rescorla, 1970). They show, that under present conditions, overexpectation is maximal after two

Figure 1. Mean and *SEM* levels of freezing during Experiment 1. There was evidence for overexpectation of fear on test that was maximal with 2 Stage II conditioning trials.

Stage II trials and is reduced with further Stage II training. The loss of overexpectation with overtraining is unexpected from the perspective of error-correcting learning rules, which predict either no effect of number of trials (if the Stage II negative error was fully corrected by learning from the first two compound trials) or an effect in the opposite direction (if further error-correction was possible beyond the first two compound trials). This result also contrasts with the results of Kremer (1978) who, using a conditioned suppression design, reported that 16, as opposed to 1, 4, or 8, Stage II trials produced optimal overexpectation. The reason for this pattern of results is unclear but it is worth noting that we have previously detected overexpectation with relatively few Stage II trials (McNally et al., 2004) and that fear learning proceeds rapidly when assessed via freezing.

Experiment 2

The aim of Experiment 2 was to study the effect of FG7142 on expression of overexpectation. FG7142 is a partial inverse agonist at the benzodiazepine binding site on the GABA_A receptor. Benzodiazepine binding sites are expressed on GABA_A receptors containing $\alpha 1$, $\alpha 2$, $\alpha 3$, or $\alpha 5$ subunits (Sieghart, 1995). FG7142 binds to receptors containing each subunit, but as a negative modulator, it reduces channel operation and therefore reduces GABAergic activation of the GABA_A receptor. As noted previously, systemic administrations of FG7142 prevent the expression of fear extinction (Harris & Westbrook, 1998).

The experiment employed a single factor, three group design. group control – vehicle received Stage I but not Stage II training and were tested for fear of the target CS. Groups over – vehicle and over – FG7142 received Stage I, Stage II training, and test. All injections occurred prior to test. Rats were injected with vehicle or 10 mg/kg FG7142. A 10 mg/kg FG7142 dose was chosen based on previous findings that this dose was maximally effective in preventing the expression of fear extinction (Harris & Westbrook, 1998).

Method

Subjects and Apparatus

Subjects were experimentally naive male Wistar rats obtained, housed, and handled as in Experiment 1. The apparatus was the same as described for Experiment 1.

Drugs

FG7142 (*N*-methyl- β -carboline-3-carboximide; Sigma, Sydney, Australia) was suspended at a concentration of 10 mg/ml in saline (0.9% w/vol) using 1 drop of Tween 80 per 5 ml saline. This suspension, or the vehicle (saline plus Tween 80) was administered in a volume of 1 ml/kg by subcutaneous injection into the dorsal region of the neck.

Procedure

Pre-exposure. Rats were exposed to the conditioning chambers during two 20-min sessions (one morning session and one afternoon session). No CS or US events were scheduled during this

time. This context pre-exposure procedure was repeated 3 days later.

Stage I. Procedures were identical to those described for Experiment 1 except that afternoon context exposures were carried out on Days 3 (10 min), 5 (20 min), and 6 (10 min). These exposures were intended to reduce levels of fear of the experimental context.

Stage II. Group control – vehicle ($n = 9$) were transported to the laboratory and handled for approximately 30 s. Groups over – vehicle ($n = 11$) and over – FG7142 ($n = 11$) received the same training as that described for group over – 2 in Experiment 1.

Test. Rats were injected with FG7142 or vehicle according to group allocations 15 min prior to test. Testing procedures were identical to those described for Experiment 1. One rat was affected by the exclusion criterion ($\geq 50\%$ pre-CS freezing) and therefore final group sizes were: group control – vehicle ($n = 9$), group over – vehicle ($n = 11$), and group over – FG7142 ($n = 10$).

Results and Discussion

The mean and *SEM* levels of freezing across the three stages of the experiment are shown in Figure 2. During the final CS presentations at the end of Stage I training there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 11.2%; $SEM = 3.1$), $F(1, 29) = 81.4$, $p < .05$.

Due to a recording failure during Stage II training, pre-CS freezing from three rats was unavailable. In the remaining rats there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 5.5, $SEM = 2.4$), $F(1, 17) = 75.8$, $p < .05$. In contrast to Experiment 1, there was no evidence for summation in freezing during the first Stage II trial: freezing during the first presentation of the compound CS was not significantly different to the average responding observed to the individual CSs at the end of Stage I, $F(1, 20) = 2.1$, $p > .05$. There was again a significant difference in freezing during Stage II, so that freezing decreased significantly between the first and second CS presentation, $F(1, 20) = 10.2$, $p < .05$.

The data of primary interest are those from test. Inspection of the panel suggests the presence of overexpectation among group over – vehicle and an attenuation of this overexpectation among group over – FG7142. The analysis confirmed this. On test there

Figure 2. Mean and *SEM* levels of freezing during Experiment 2. There was evidence for overexpectation of fear on test that was attenuated by pretreatment with FG7142 (10 mg/kg).

was significantly more freezing during CS presentations as compared to the pre-CS period, $F(1, 27) = 200.5, p < .05$ (M pre-CS freezing = 8.3%; $SEM = 4.5$). There was no significant differences between groups in levels of pre-CS freezing (group control: $M = 12.8, SEM = 5.0$; group over – vehicle: $M = 5.6, SEM = 1.8$; group over – FG7142: $M = 7.2, SEM = 4.3$), $F_s(1, 27) < 1.6, p > .05$. There was a significant overexpectation effect because there was a significant difference in freezing between group control and group over – vehicle, $F(1, 27) = 11.6, p < .05$. More importantly, there was also a significant difference between group over – vehicle and group over – FG7142, $F(1, 27) = 4.8, p < .05$, indicating that pretreatment with FG7142 prior to test attenuated expression of overexpectation.

It is possible that the difference between group over – vehicle and group over – FG7142 was due not to FG7142 attenuating expression of overexpectation, but rather to it attenuating expression of any extinction learning that might have occurred across the four nonreinforced CS presentations on test (Harris & Westbrook, 1998). To further examine this possibility, test performances across CS presentations were analyzed (see Figure 3). There was no significant linear decrease in freezing across test presentations of the CS, $F(1, 27) < 1, p > .05$. There was also no significant interaction between the contrast assessing differences between group control – vehicle versus group over – vehicle, $F(1, 27) < 1, p > .05$ and the change in freezing across CS presentations, nor was there a significant interaction between the contrast assessing differences between group over – vehicle versus group over – FG7142, $F(1, 27) < 1, p > .05$ and the change in freezing across CS presentations. The absence of any significant extinction across CS presentations, as well as of any interactions of the effects of FG7142 with CS presentations, argue strongly against the possibility that the effects of FG7142 on test were due to effects on extinction. Rather, the results from this experiment show that fear that has been reduced by overexpectation training can be increased by injection of FG7142.

Figure 3. Mean and SEM levels of freezing across test presentations of the CS. There was no evidence for extinction across CS presentations nor any interaction of the effects of FG7142 with CS presentations.

Experiment 3

The aim of Experiment 3 was to further study the effects of FG7142 on the expression of overexpectation by characterizing its dose-response properties. The experiment used a single factor, four group design. Group control received Stage I but not Stage II training and were injected with vehicle prior to test. Groups over – 0 mg/kg, over – 1 mg/kg and over – 10 mg/kg received Stage I and Stage II training and were injected with FG7142 prior to test.

Method

Subjects, Apparatus, Drugs

Subjects were experimentally naive male Wistar rats obtained from the same source and maintained under the same conditions as described previously. All apparatus was as described previously. FG7142 was prepared as described previously to obtain concentrations of 0, 1, or 10 mg/ml and was injected subcutaneously at a volume of 1 ml/kg 15 min prior to test.

Procedure

Stage I and Stage II. The procedure was similar to that described previously with the single exception that Stage I training was reduced from 6 to 4 days. Therefore, each CS received two pairings with the shock before Stage II training. This was done because freezing had reached asymptotic levels in previous experiments after 2 CS-US pairings. Groups over – 0 mg/kg ($n = 13$), over – 1 mg/kg ($n = 11$), and over – 10 mg/kg ($n = 11$) received Stage II training in the manner described for Experiment 2. Group control ($n = 12$) were transported to the laboratory and briefly handled.

Test. The following day rats were injected according to group allocations and tested 15 min later. The procedure for test was that described previously. Seven rats were affected by the exclusion criterion ($\geq 50\%$ freezing during pre-CS) and therefore final group sizes were group control ($n = 12$); group over – 0 mg/kg ($n = 11$); group over – 1 mg/kg ($n = 8$); group over – 10 mg/kg ($n = 9$).

Results and Discussion

The mean and SEM levels of freezing across the three stages of the experiment are shown in Figure 4. Stage I training proceeded uneventfully. During the final CS presentations at the end of Stage I training there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 11.8; $SEM = 2.7$), $F(1, 39) = 106.7, p < .05$. During Stage II training there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 5.8, $SEM = 1.9$), $F(1, 27) = 97.5, p < .05$. There was no evidence for summation in freezing during the first Stage II trial: Freezing during the first presentation of the compound CS was not significantly different to the average responding observed to the individual CSs at the end of Stage I, $F(1, 27) = 2.3, p > .05$. There was a significant difference in freezing across the two Stage II CS presentations, so that again freezing decreased significantly between the first and second CS presentation, $F(1, 27) = 44.8, p < .05$.

Figure 4. Mean and SEM levels of freezing during Experiment 3. There was evidence for overexpectation on test as well for a dose-dependent attenuation of overexpectation by pretest injections of FG7142.

Inspection of the test data suggests the presence of overexpectation among group over – 0 mg/kg and an attenuation of overexpectation by FG7142 in a dose-dependent manner. The analysis confirmed this observation. On test there was significantly more freezing during CS presentations as compared to the pre-CS period, $F(1, 36) = 138.7, p < .05$ (M pre-CS freezing = 11.8; $SEM = 1.8$). The only difference in pre-CS levels of freezing was between group control ($M = 5.3, SEM = 2.5$), and the overexpectation groups (over – 0 mg/kg: $M = 13.5, SEM = 2.9$; over – 1 mg/kg: $M = 18.2, SEM = 5.8$; over – 10 mg/kg: $M = 12.8, SEM = 3.0$), $F(1, 36) = 6.4, p < .05$. The overexpectation groups did not differ from each other in levels of pre-CS freezing, $F_s(1, 36) < 1.1, p > .05$.

There was evidence for overexpectation because overall, averaged across doses of FG7142, groups receiving Stage II overexpectation training showed significantly less freezing to the CS on test than did group control, $F(1, 36) = 4.3, p < .05$. There was also evidence that FG7142 attenuated expression of this overexpectation in a dose-dependent manner: Group over – 10 mg/kg showed significantly greater freezing than groups over – 1 mg/kg and over – 0 mg/kg, $F(1, 36) = 6.9, p < .05$, which did not differ from each other, $F(1, 36) < 1, p > .05$. A simple comparison of group over – 10 mg/kg and group over – 0 mg/kg likewise revealed a significant attenuation of overexpectation, $t(18) = -2.3, p < .05$, two-tailed. To further confirm that any effects of FG7142 on expression of FG7142 were not secondary to effects on any extinction that might have occurred during test, performances across the four test CS presentations were analyzed (data not shown). There was no overall significant linear decrease in freezing across CS presentations, $F(1, 36) < 1, p > .05$ and there were no interactions between the contrasts assessing differences between-groups with the contrast assessing linear decreases in freezing on test, $F_s(1, 36) < 2.1, p_s > .05$. The results of this experiment replicate the finding, reported in Experiment 2, that FG7142 attenuates expression of overexpectation and also show that this attenuation is dose dependent.

Experiment 4

Experiments 2 and 3 have shown that expression of overexpectation is blocked by injection of FG7142 prior to test. These effects

of FG7142 are similar to its effects on expression of fear that has been reduced by extinction training. One possibility is that overexpectation, like extinction, causes a masking of the fear response that is modulated by GABA_A receptor manipulations. One candidate for such modulation is state-dependent learning and memory. According to this line of reasoning, injection of FG7142 prior to test produced a shift in internal state/context that prevented overexpectation learning from Stage II generalizing to test. For fear extinction there is some evidence for such generalization decrement based on injections of benzodiazepine agonists (diazepam, chlordiazepoxide) prior to extinction training (Bouton, Kenney, & Rosengard, 1990), but there is no evidence in the literature to support this possibility for injections of the benzodiazepine partial inverse agonist FG7142. Indeed, Harris and Westbrook (1998) showed that the effects of FG7142 on extinction were not due to state-dependent learning. Moreover, in a latent inhibition paradigm, Kim, McNally, and Richardson (2006) showed that injections of FG7142 actually enhanced generalization of learning from a drug-free state and so significantly reduced expression of fear. These findings argue strongly against the possibility that injections of FG7142 on test reduced expression of overexpectation due to state-dependent memory processes. The aim of this experiment was to directly examine whether the effects of FG7142 on expression of fear overexpectation could be due to state-dependent learning/memory processes. There were four groups: group control – vehicle, group control – FG7142, group over – vehicle, and group over – FG7142. The first group designation refers to type of Stage II training whereas the second refers to the type of injection prior to both Stage II and test.

Method

Subjects, Apparatus, Drugs

Subjects were 40 experimentally naive male Wistar rats obtained, housed, and handled as in previous experiments. The apparatus was identical to that used previously. Drugs were as described for Experiment 2.

Procedure

Stage I. The Stage I conditioning procedure was identical to that described for Experiment 3.

Stage II. Compound conditioning procedures were identical to those used in Experiments 2 and 3. Fifteen minutes before compound conditioning, rats received injections of either 10 mg/kg FG7142 (group over – FG: $N = 10$) or vehicle (group over – vehicle: $N = 10$). Rats in the control group were also injected with either 10 mg/kg FG7142 (group control – FG: $N = 10$) or vehicle (group control – vehicle: $N = 10$) and briefly handled 15 min later.

Test. Fifteen minutes before test, all rats received the same injection that they had received prior to Stage II conditioning (or handling in the control groups). Test procedures were identical to those described for previous experiments.

Results and Discussion

The mean and *SEM* levels of freezing across the three stages of the experiment are shown in Figure 5. During the final CS presentations at the end of Stage I training there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 12.5; $SEM = 1.6$), $F(1, 39) = 371.0$, $p < .05$. During Stage II training there was significantly more freezing during CS presentations as compared to the pre-CS period (M pre-CS freezing = 9.8, $SEM = 3.1$), $F(1, 18) = 93.2$, $p < .05$ and there were no differences between groups in pre-CS levels of freezing, $F(1, 18) < 1$, $p > .05$. There was a significant difference in freezing across Stage II, so that again freezing decreased significantly between the first and second CS presentation, $F(1, 18) = 12.4$, $p < .05$ but there was no difference between rats injected with FG7142 or vehicle.

Inspection of the test data suggests the presence of overexpectation in group over – vehicle but not group over – FG7142. Group over – vehicle differed significantly from groups control – vehicle and control – FG7142, $F(1, 36) = 13.5$, $p < .05$. There was however no such significant difference between group over – FG7142 and groups control – vehicle and control – FG7142, $F(1, 36) = 1.9$, $p > .05$. There was also no significant difference

between groups control – vehicle and control – FG7142, $F(1, 36) < 1$, $p > .05$. A simple comparison of group over – FG7142 and group over – vehicle likewise revealed a significant attenuation of overexpectation, $t(18) = -1.9$, $p < .05$, one-tailed. In contrast to previous experiments there was an overall significant linear decrease in freezing across test CS presentations, $F(1, 36) = 10.2$, $p < .05$, indicating extinction across the test trials, but there were no interactions between the contrasts assessing differences between-groups with the contrast assessing decreases in freezing on test, $F(1, 36) < 1$, $ps > .05$. The results of this experiment confirm the presence of overexpectation of fear and extend the findings from Experiments 2 and 3 that FG7142 attenuates expression of overexpectation. These results provide no evidence to support the possibility that the effects of FG7142 on overexpectation are due to state-dependent learning or memory.

Experiments 5a and 5b

The aim of the present experiments was to examine an alternate interpretation of the effects of FG7142 on overexpectation. There is evidence in the literature that FG7142 is anxiogenic. For example, FG7142 reduces time spent in open arms of an elevated plus maze in rats at doses of 10 to 100 mg/kg (Atack et al., 2005; Cole, Hillmann, Seidelmann, Klewer, & Jones, 1995; Pellow & File, 1986) and reduces numbers of open arm entries in mice at 10 mg/kg (Rodgers, Cole, Aboualfa, & Stephenson, 1995). It follows from this that injection of FG7142 in the previous experiments may have acted simply to increase levels of fear or enhance the expression of freezing. Past research is inconsistent with this possibility (e.g., Harris & Westbrook, 1998; Kim et al., 2006; Tang, McNally, & Richardson, 2007). Moreover, it is difficult to reconcile this possibility with the absence of any effect of FG7142 in group control – FG7142 in Experiment 4. However, that design was not optimal for detecting any such potentiation of fear or freezing because it involved extensive conditioning of the CS prior to test. Such conditioning may have generated a ceiling effect that obscured detection of any effects of FG7142 on freezing or fear per se. The aim of these experiments was to further examine the effects of FG7142 on expression of conditioned fear.

Figure 5. Mean and *SEM* levels of freezing during Experiment 4. There was evidence for overexpectation on test in rats injected with vehicle (Veh) before both Stage II conditioning and test, but not in rats injected with FG7142 (FG). There was no evidence that FG7142 altered freezing during Stage II, or on test in the control group.

Experiment 5a studied the effects of FG7142 on expression of fear to a CS that had been paired with a low magnitude footshock US. Three groups of rats were trained to fear an auditory CS via pairings with a US. For one group, the US was a 1 mA, 1 s footshock (group high-vehicle) whereas for the remaining groups, the US was a 0.3 mA, 0.3 s footshock (groups low). Rats were later tested for their fear reactions to the CS. For group low – FG7142 this test was preceded by injection of 10 mg/kg FG7142 whereas for groups high – vehicle and low-vehicle this test was preceded by injection of vehicle.

Experiment 5b studied the effects FG7142 on expression of fear to a CS that had been reduced due to manipulations of CS predictiveness. Specifically, it studied the effects of FG7142 on the expression of fear that had been reduced by associative blocking (Kamin, 1968). Overexpectation is a variant of the blocking design. The key difference is that in an overexpectation design, the target CS receives Stage I training whereas in a blocking design it does not. Therefore Stage II training in an overexpectation design involves negative predictive error whereas in a blocking design it does not. By contrast, the physical events experienced by the subjects during Stage II of a blocking and overexpectation design are otherwise the same. In both cases, a compound CS is paired with a footshock US and fear is later assessed. Studying the effects of FG7142 in a blocking design enables examination of the extent to which the effects of FG7142 on expression of fear to a CS are dependent on that CS being subject to a negative prediction error during Stage II. A four group design was used. Three groups were conditioned to fear a flashing light CS during Stage I conditioning. A fourth group (group control) was briefly handled on each day of Stage I conditioning, but not exposed to any conditioning procedure. All groups then received Stage II conditioning that involved pairings of the compound flash-tone CS with shock. Rats were then tested for freezing to the tone. Prior to this test, rats that had received Stage I conditioning were injected with either vehicle (group block – 0 mg/kg), 1 mg/kg FG7142 (group block – 1 mg/kg), or 10 mg/kg FG7142 (group block – 10 mg/kg). Group control was injected with vehicle prior to this test.

Method

Subjects, Apparatus, and Drugs

Subjects were experimentally naive male Wistar rats obtained, housed, and handled as in previous experiments. The apparatus and drugs were identical to those used in Experiment 2.

Procedure

Experiment 5a: Conditioning. On the morning of Day 1 rats were placed in the conditioning chambers and exposed to two tone-shock pairings, one beginning 3 min and 47 s after placement in the chamber, and the next beginning 8 min and 18 s after placement. Group high – vehicle ($n = 11$) were conditioned using a 1 mA, 1 s shock, while Groups low – vehicle ($n = 11$) and low – FG7142 ($n = 10$) were conditioned using a 0.3 mA, 0.3 s shock. Rats were removed after a total of 14 min in the chambers. In the afternoon of the same day rats were placed in the chambers for a 10 min context exposure during which no CS or US events were scheduled.

Test. The following day rats were injected with FG7142 (10 mg/kg) or vehicle according to group allocations. Fifteen minutes later rats were placed in the chambers and exposed to six presentations of the tone beginning 4 min after introduction into the chamber, with a 2 min and 31 s ISI. Two rats were affected by the exclusion criterion ($\geq 50\%$ freezing during pre-CS) and therefore final group sizes were group high – vehicle ($n = 10$); group low – vehicle ($n = 10$); group low – FG7142 ($n = 10$).

Experiment 5b: Pre-exposure. On Days 1 and 2 rats were placed in the conditioning apparatus and exposed to four 30 s presentations each of a flashing light (4Hz) and 82 dB, 750 Hz tone at an ISI for 30 s. The order of stimulus presentation was counterbalanced. No shocks were delivered during these sessions.

Stage I. On the mornings of Days 3 to 5 rats in the blocking groups were placed in the conditioning chambers for 21 min and 30 s. During this time rats received four 30 s presentations of the flashing light coterminating with 1 s, 0.5 mA footshock at an average intertrial interval of 230 s. In the afternoons of these days, all rats in the blocking groups were exposed to the conditioning chambers for 10 min with no stimulus presentations. Rats in the control group were briefly handled once in the morning and once in the afternoon of each day of Stage I.

Stage II. On the morning of Day 6 all rats were placed in the conditioning chambers for 14 min. During this time, they received two 30 s compound presentations of the tone and flashing light coterminating with shock. The first presentation occurred 300 s after placement in the chamber. The second presentation occurred 180 s later. In the afternoon, all rats received a 10 min exposure to the chambers.

Test. On Day 7 rats in the blocking groups were injected subcutaneously with 1 ml/kg of either 0 ($N = 8$), 1 ($N = 8$), or 10 ($N = 8$) mg/kg FG7142, suspended in saline with Tween 80 as described previously. Control rats were injected with vehicle and were tested 15 min later. The test session involved four 30 s presentations of the auditory CS. The first CS presentation began 5 min after placement in the chambers, and further CS presentations followed at an ISI of 91 s. Two rats, one each in group control and block – 0 were affected by the exclusion criterion. Therefore final group sizes were: group control ($n = 7$), group block – 0 mg/kg ($n = 7$), group block – 1 mg/kg ($n = 8$), group block – 10 mg/kg ($n = 8$).

Results and Discussion

Experiment 5a

The mean and *SEM* levels of freezing on test are shown in the top panel of Figure 6. Inspection of the figure suggests that manipulation of shock magnitude resulted in different levels of freezing: group high – vehicle, conditioned with a 1 mA, 1 s footshock displayed more freezing than the remaining groups conditioned with a 0.3 mA, 0.3 s footshock. There also appeared to be no difference in levels of fear between the latter two groups as a function of injection of FG7142. These observations were confirmed by the analysis. On test there was significantly more freezing during CS presentations as compared to the pre-CS period, $F(1, 27) = 127.3$, $p < .05$ (M pre-CS freezing = 10.0; $SEM = 2.4$). There was a significant difference between groups in pre-CS freezing, so that group high – vehicle ($M = 24.1$, $SEM = 4.2$)

Figure 6. Top panel: Mean and *SEM* levels of freezing during Experiment 5a. Rats conditioned with the low magnitude footshock US (0.3 mA, 0.3 s) showed significantly less freezing than those conditioned with the high magnitude US (1 mA, 1 s). Pretest injections of FG7142 (10 mg/kg) had no effect on expression of fear. Bottom Panel: Mean and *SEM* levels of freezing on test in Experiment 5b. There was evidence of blocking of CS fear learning. Pretest injections of FG7142 had no effect on the expression of blocking.

displayed significantly more pre-CS than groups low – vehicle ($M = 4.1$, $SEM = 2.3$) and low-FG7142 ($M = 1.8$, $SEM = 1.0$), $F(1, 27) = 36.9$, $p < .05$. There was no such difference between groups low – vehicle and low – FG7142, $F(1, 27) < 1$, $p > .05$. More important, group high – vehicle showed significantly more CS freezing than groups low – vehicle and low – FG7142, $F(1, 27) = 9.7$, $p < .05$ whereas groups low – vehicle and low – FG7142 did not differ significantly from each other, $F(1, 27) < 1$, $p > .05$.

These results show that injection of FG7142 does not act simply to increase the expression of fear or augment freezing. This failure to find any effect of FG7142 cannot be due to a ceiling effect on measurement. Rats conditioned with a higher magnitude footshock displayed significantly more freezing than rats conditioned with a lower magnitude footshock. Injection of FG7142 prior to test did not increase freezing in rats conditioned with the lower magnitude footshock.

Experiment 5b

The mean and *SEM* levels of freezing on test are shown in the bottom panel of Figure 6. Inspection of the figure confirms the presence of blocking among groups Block. More interestingly, pretest injections of FG7142 had no effect on the expression of blocking. The statistical analysis confirmed these observations. On test there was significantly more freezing during CS presentations as compared to the pre-CS period, $F(1, 26) = 95.7$, $p < .05$ (M pre-CS freezing = 12.9; $SEM = 2.1$). There were no differences between groups in pre-CS freezing, $F_s(1, 26) < 1$, $ps > .05$. There was blocking of fear learning, because groups block displayed less freezing to the CS than group control, $F(1, 26) = 11.7$, $p < .05$. There was no significant effect of FG7142 on the expression of blocking because group block – 10 mg/kg did not differ from groups block – 0 mg/kg and block – 1 mg/kg, $F(1, 26) < 1$, $p > .05$, which did not differ from each other, $F(1, 26) = 1.1$, $p > .05$.

This experiment has confirmed that prior training of a CS blocks conditioning to a second CS when the two stimuli are conditioned in compound (Kamin, 1968). In contrast to the findings from an overexpectation design, however, there was no evidence here that a pretest injection of FG7142 at the same doses as used in the overexpectation design, alleviated expression of blocked fear. This result provides further support for the conclusion that FG7142 does not act to attenuate expression of overexpectation by simply increasing fear or the expression of freezing.

General Discussion

These experiments studied the role of GABA_A receptors in regulating the expression of fear after overexpectation training. Rats were trained to fear an auditory and visual CS via separate pairings with a footshock in Stage I. In Stage II, these stimuli were presented in compound and followed by shock. Across Experiments 1 through 4, fear to the compound stimuli was reduced during Stage II training and to the target auditory CS on test. Thus, overexpectation was observed (e.g., Blaisdell et al., 2001; Kremer, 1978; Lattal & Nakajima, 1998; McNally et al., 2004; Rescorla, 1970). The expression of overexpectation was prevented, in a dose-dependent manner, by injections of the benzodiazepine partial inverse agonist FG7142. The effects of FG7142 were specific to modulating expression of fear that had been reduced due to overexpectation training. FG7142 had no effect on the expression of fear to a CS that had been paired with a low magnitude US. FG7142 also had no effect on the expression of fear to a CS that had been subject to associative blocking training. Taken together, these results suggest that the effects of FG7142 were specific to modulating responding to CS that had been subject to a negative prediction error during Stage II. They support the possibility that negative prediction error produces a mask on conditioned fear, the expression of which is vulnerable to modulation of the benzodiazepine binding site of the GABA_A receptor at the time of test.

These findings add to a growing body of evidence that the reductions in conditioned responding produced by extinction training and overexpectation reflect the operation of a common decremental process. These experiments have shown that fear that has been reduced via overexpectation training can be recovered by pretest injections of a benzodiazepine partial inverse agonist. Harris and Westbrook (1998) reported the same finding for fear

extinction. Rescorla recently showed that responding to an appetitive CS that has been reduced by overexpectation training is subject to spontaneous recovery (Rescorla, 2006) and renewal (Rescorla, 2007), phenomena typically linked to extinguished stimuli. Finally, McNally reported that the actions of endogenous opioids are critical to both overexpectation (McNally et al., 2004) and extinction (McNally, & Westbrook, 2003) learning, and to error-correction learning more generally (e.g., McNally & Cole, 2006). This evidence is consistent with error-correcting learning rules, such as the Rescorla–Wagner model, which identify a common cause, negative prediction error, for both extinction and overexpectation. These commonalities underscore the point that predictive error, the discrepancy between actual and expected outcomes of the trial, is a critical determinant of fear loss.

The recovery reported here and elsewhere strongly suggests that, like extinction, fear is not erased by overexpectation training. Instead, the recovery of fear after overexpectation training suggests that a mask is imposed on responding. The generation of a negative prediction error during Stage II appears critical to imposition of this mask. The failure of FG7142 to modulate responding to a blocked CS is especially informative in this regard. The procedures for Stage II training in the blocking and overexpectation designs were similar so that for both cases a compound CS was paired with a footshock US and fear was later assessed. The key difference was the nature of the Stage II prediction error. In the overexpectation design, Stage II predictive error was negative whereas in a blocking design this error was positive but negligible. The ability of FG7142 to modulate responding to a CS previously subjected to extinction or overexpectation, but not blocking, latent inhibition (Kim et al., 2006), or simple acquisition training strongly suggests that negative predictive error is a central requirement for masking fear.

The neuroanatomical locus and mechanisms through which GABA_A receptors modulate expression of overexpectation remain to be determined. One possibility is that this occurs in the amygdala. As was noted previously, fear extinction training up-regulates benzodiazepine binding and gephyrin (GABA_A receptor clustering protein) mRNA and protein expression in basolateral amygdala (Chhatawal et al., 2005). Overexpectation may result in a similar up-regulation of amygdala GABA_A receptor function and FG7142 could reduce the consequences of this increased amygdala GABAergic function. A second, related possibility is suggested by recent electrophysiological examination of the effects of systemically administered FG7142. Stevenson, Halliday, Marsden, and Mason (2007) studied the effects of systemic administrations of FG7142 on corticolimbic interactions in rats. Systemic administrations of FG7142 modulated burst firing in units recorded from the medial prefrontal cortex as well as the basolateral amygdala, and more important, reduced the synchronized firing observed between these regions. These findings show clearly that FG7142 can disrupt medial prefrontal–amygdala interactions. They are important because it is these structures, and their interactions, which have been implicated in regulating fear after extinction training. In rats, burst firing in prefrontal units (Burgos-Robles, Vidal-Gonzales, Santini, & Quirk, 2007) and prefrontal–amygdala interactions are important for inhibiting fear memory retrieval after extinction training (Quirk, Garcia, & González-Lima, 2006; Quirk & Maren, 2004) and there is some evidence for a similar medial prefrontal contribution to human fear extinction

learning (Rauch, Shin, & Phelps, 2006). It is possible that overexpectation likewise depends on prefrontal–amygdala interactions. Further research is needed to evaluate these possibilities.

Finally, we have focused on an explanation of the overexpectation effect in terms of learning processes that operate during Stage II. A different class of models, performance-based models, supposes that overexpectation reflects operation of a comparator process on test. According to the comparator hypothesis and its derivatives (e.g., Miller & Matzel, 1988; Stout & Miller, 2007), at least three important associations are formed as a consequence of overexpectation training here: (a) an association between the tone and the US, (b) an association between the light and the US, and (c) an association between the light and the tone. Overexpectation is observed to the tone because the strengths of the latter associations (tone – light and light – US) are greater than the tone–US association. This attenuates performance to the tone. We consider this model for two reasons. First, it may predict the loss of overexpectation with extended training observed in Experiment 1. Second, more generally, it predicts recovery of fear after overexpectation. However, the model also predicts that blocking is likewise a performance deficit which shares the same mechanism as overexpectation. The difference being that, unlike overexpectation, interactions between the light and tone are asymmetrical during a test for blocking. This identification of a common mechanism for blocking and overexpectation is inconsistent with the results found here. Fear after overexpectation training, but not blocking training, was modulated by FG7142. The commonality between overexpectation and extinction, as well as their pharmacological dissociation from blocking, is generally consistent with an acquisition account and generally inconsistent with such a performance-based account.

In conclusion, these experiments have shown that fear that has been reduced via overexpectation training can be recovered via injections of the benzodiazepine partial inverse agonist FG7142. A similar sensitivity to the effects of FG7142 is seen after extinction training. These results suggest that, like fear extinction, a mask is imposed on fear after overexpectation training, the expression of which is vulnerable to modulation of the benzodiazepine binding site of the GABA_A receptor. These results add to the growing body of evidence that there are important commonalities between overexpectation and extinction (McNally et al., 2004; McNally & Westbrook, 2003; Rescorla, 2006, 2007). Such commonalities show that understanding the actions of predictive error, the discrepancy between actual and expected outcomes of the trial, is central to understanding fear loss.

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