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The anxiolytic action of mGlu2/3 receptor agonist, LY354740, in the fear-potentiated startle model in rats is mechanistically distinct from diazepam

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Abstract

The fear-potentiated startle paradigm has been characterized for drugs that act via ionotropic (NMDA and AMPA/kainate receptor) glutamate receptor mechanisms. Previous studies have shown that the potent systemically active mGlu2/3 receptor agonist, LY354740, effectively reduced the expression of fear-potentiated startle responses in rats. The present study examined the effects of LY354740 in a pre- versus post-fear conditioning paradigm and compared the effects to diazepam. Diazepam (0.3, 0.6, and 1.0 mg/kg ip) attenuated both pre- and post-fear conditioning startle responses in a dose-related manner. In contrast, LY354740 (0.03, 0.3, and 3.0 mg/kg ip) did not disrupt preconditioning startle responses at doses that attenuated post-fear conditioning responses. The benzodiazepine antagonist, flumazenil, at a dose (2 mg/kg sc) that did not alter fear-potentiated startle per se, selectively reversed suppression of fear responses to diazepam (0.6 mg/kg ip) while not affecting fear suppression induced by LY354740 (0.3 mg/kg ip). At a dose of 1 mg/kg ip, the mGlu2/3 receptor antagonist, LY341495, did not disrupt fear-enhanced startle per se, but completely reversed the postconditioning anxiolytic effects of LY354740 in this model. This dose of LY341495 had no effect on fear suppression by diazepam. These results demonstrate that fear suppression by diazepam and LY354740 involves different neuronal mechanisms. While diazepam acts via the facilitation of GABAergic transmission, LY354740 induces its actions via the glutamatergic system, specifically mGlu2/3 receptor activation. Furthermore, in contrast to disruption of fear conditioning as well as fear suppression by diazepam, LY354740 had selective effects on fear expression, suggesting anxiolytic actions without the associated memory impairment.

Keywords: Metabotropic glutamate receptors; Fear conditioning; Fear expression

1. Introduction

Since their introduction in the 1960s, benzodiazepines (e.g., diazepam) have remained the most widely used drugs for the treatment of anxiety disorders. While benzodiazepines are highly efficacious in some anxiety disorders (i.e., generalized anxiety), their utility is greatly hampered by side effects that include CNS depression, cognitive impairment, and dependence and withdrawal liabilities. Benzodiazepines act at the cellular level to modulate GABA, the major inhibitory neurotransmitter system in the brain. In most synapses, the actions of GABA are opposed by glutamate, the major excitatory neurotransmitter acting on metabotropic and ionotropic glutamate (iGlu) receptors. "Anxiety/panic" has been linked to enhanced excitatory transmission in specific brain regions (e.g., amygdala), which ultimately leads to the expression of fear and arousal states in animals (for reviews, see Davis, 1997; Davis et al., 1993). Thus, targeting the reduction of glutamatergic excitatory transmission in key limbic brain areas and neuronal circuits involved in anxiety responses represents a novel approach to finding novel, effective, and possibly safer anxiolytic agents.

Glutamate receptors are classified into iGlu receptors, which are ligand-gated ion channels, and metabotropic glutamate (mGlu) receptors, which are coupled via G-proteins to alterations in second messengers. As a novel class of G-protein-coupled receptors, mGlu receptors func-

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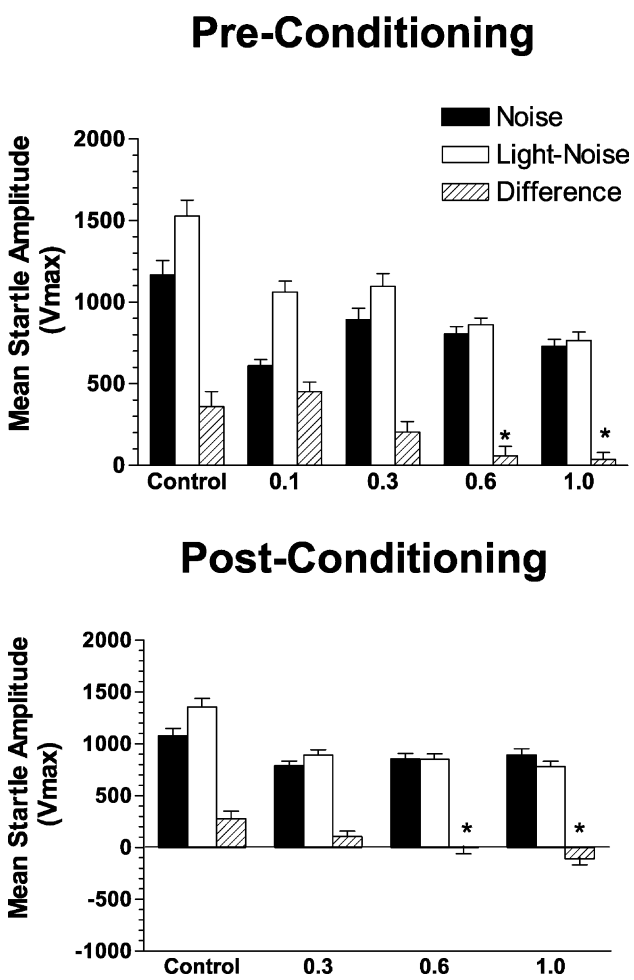

Fig. 1. The effects of diazepam on fear conditioning and fear expression in male Sprague–Dawley rats ($n=8$ rats/treatment). Intraperitoneal administration of diazepam (0.1, 0.3, 0.6, 1.0 mg/kg) occurred 30 min prior to conditioning on Day 2 (preconditioning) or (0.3, 0.6, 1.0 mg/kg) testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P<.05$.

tion to modulate glutamatergic transmission by pre- and postsynaptic and glial mechanisms (Schoepp et al., 1999). There are currently eight different subtypes of mGlu receptors that have been cloned from human and rat species, which are designated mGlu1–mGlu8. These have been subdivided into three subgroups (I, II, and III) based on higher structural homology and shared pharmacology within each mGlu receptor group. Group II mGlu receptors include mGlu2 and mGlu3 receptors, which are negatively coupled to cAMP formation when expressed and can be potently and selectively activated by the agonist compound, LY354740 (Monn et al., 1997; Schoepp et al., 1997). Numerous electrophysiological studies have demonstrated that mGlu2/3 agonists, including LY354740, suppress glutamatergic excitatory transmission in limbic synapses including hippocampus, amygdala, and prefrontal cortex (reviewed by Anwyl, 1999). The suppression of glutamate transmission by mGlu2/3 agonists involves presynaptic decreases of the evoked release of glutamate (Battaglia et al., 1997).

The potential role of mGlu2/3 receptor agonists in the treatment of anxiety disorders was initially elucidated using LY354740 in the fear-potentiated startle model of anxiety (Helton et al., 1998). Here, LY354740 has been shown to block the expression of fear-potentiated startle in conditioned rats at doses, unlike diazepam, producing no CNS impairments (Helton et al., 1998). At 100–1000 times higher doses of LY354740, which suppresses fear responses in rats, acute LY354740 did not produce the side effects (e.g., sedation, memory impairments) or withdrawal reactions (i.e., seizures, enhanced startle) that are associated with other marketed agents such as benzodiazepines (e.g., diazepam). More recently, LY354740 was also shown to be

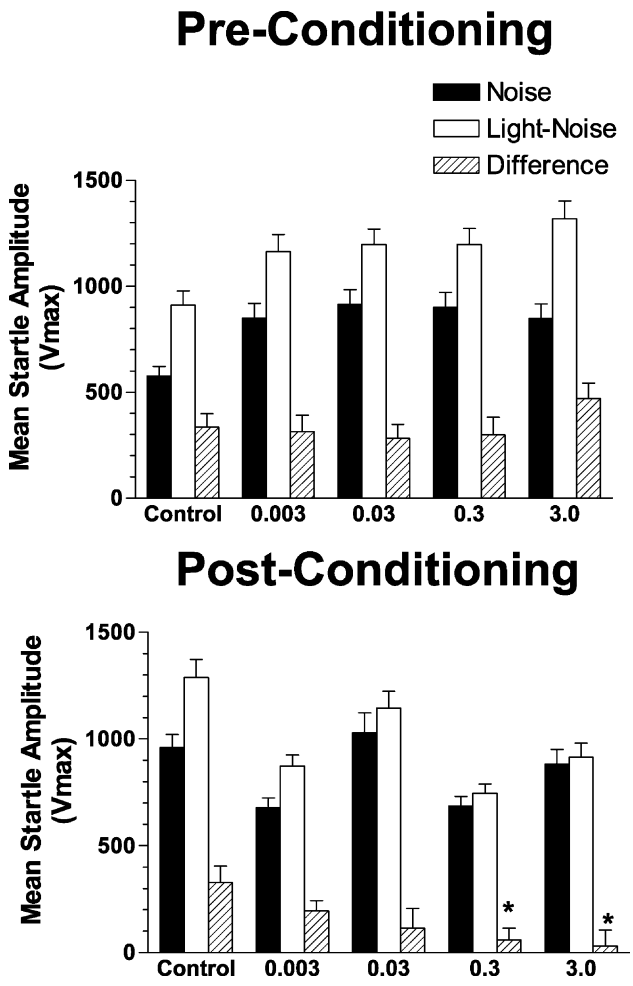

Fig. 2. The effect of compound LY354740 on fear conditioning and fear expression in male Sprague–Dawley rats ($n=8$ rats/treatment). Intraperitoneal administration of LY354740 (0.003, 0.03, 0.3, 3.0 mg/kg) occurred 30 min prior to conditioning on Day 2 (preconditioning) or testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P<.05$.

active in the animal model of panic disorder (Skekhar and Keim, 2000) and in the conflict drinking test in rats (Klodzinska et al., 1999).

In the fear-potentiated startle paradigm, animals are preconditioned by the pairing of an adversive stimulus (foot shock) with a neutral stimulus (e.g., presentation of a light). Following this preconditioning paradigm, animals respond with an enhanced acoustic startle reaction when presented with only the neutral stimulus (Davis, 1992; Davis et al., 1994). It has been shown previously that administration of either benzodiazepines or NMDA receptor antagonists will block both preconditioning as well as the expression of potentiated startle in fear-conditioned animals (Kim et al., 1993). These actions may be associated with the amnestic properties that have been associated with these agents in rats and humans.

Little is known about the role of mGlu2/3 receptor activation in pre- versus postconditioning of fear-potentiated startle. Thus, the present study examined the effects of LY354740 in pre- versus post-fear conditioning paradigms and compared the effects to diazepam. In addition, we have studied the influence of the benzodiazepine antagonist, flumazenil (Davis et al., 1988), and the mGlu2/3 receptor antagonist, LY341495 (Kingston et al., 1998), on LY354740 versus diazepam-induced suppression of fear-potentiated startle. A preliminary report of this work has been previously published in abstract form (Tizzano et al., 1999).

2. Methods

2.1. Animals

All experiments were carried out in accordance with Eli Lilly and Company (Indianapolis, IN) animal care and use policy. Male Sprague–Dawley rats (325–400 g) were obtained from Harlan Sprague Dawley (Cumberland, IN). Animals were individually housed in suspended wire cages and standard animal care procedures were followed. Animals were given Purina Rodent Chow (PMI International, St. Louis, MO) and water ad libitum and were maintained on a 12-h light–12-h dark cycle. Rats were acclimated for at least 4 days prior to testing.

2.2. Materials

Diazepam (Sigma, St. Louis, MO) and flumazenil were prepared in a suspension of 5% ethanol, 0.5% CMC, 0.5% Tween 80, and 99% water. Compounds (+) LY354740 monohydrate (1*S*, 2*S*, 5*R*, 6*S*-2-aminobicyclo [3.1.0] hexane-2, 6-dicarboxylate monohydrate) (Monn et al., 1997) and LY341495 (2*S*-2-amino-2-(1*S*, 2*S*-2-carboxycyclopropyl-1-yl)-3-(xanth-9-y 1) propanoic acid) (Ornstein et al., 1998) were dissolved in a vehicle of purified water and neutralized with 5 N NaOH to a pH of approximately 7–9. Control rats were given the respective vehicle.

2.3. Apparatus and procedures

SR-LAB (San Diego Instruments, San Diego, CA) chambers were used for conditioning sessions and for producing and recording startle responses. A respondent conditioning procedure was used to produce the potentiation of startle responses. The fear-potentiated startle paradigm was conducted over three consecutive days. All three days began with a 5-min adaptation period before the trial started. On Day 1 (baseline startle) after the adaptation period, the animals received 30 trials of 120-dB auditory noise. Startle responding was measured through transducers located under the startle platforms. Recorded values represent a maximum change in voltage (V_{max}). The mean startle amplitude (V_{max}) was used to assign animals to groups ($n=8$) with similar means before conditioning began. Day 2 consisted of conditioning the animals. Each animal received 0.5 mA of shock for 500 ms, preceded by a 5-s presentation of light (15 W) that remained on for the duration of the shock. Ten presentations of the light and shock were administered. Animals tested in the preconditioning paradigm were

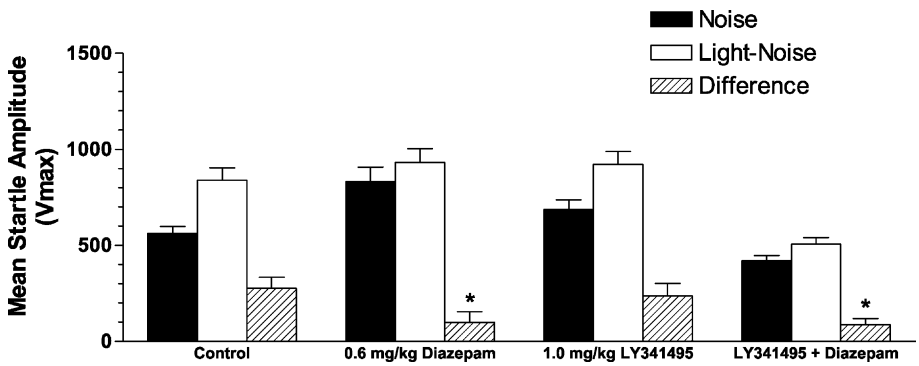

Fig. 3. The effects of diazepam, LY341495, and LY341495 + diazepam on fear expression in male Sprague–Dawley rats ($n=8$ rats/treatment). Compound LY341495 was administered subcutaneously 60 min prior to testing on Day 3 (postconditioning). Diazepam was administered intraperitoneally 30 min prior to testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P<.05$.

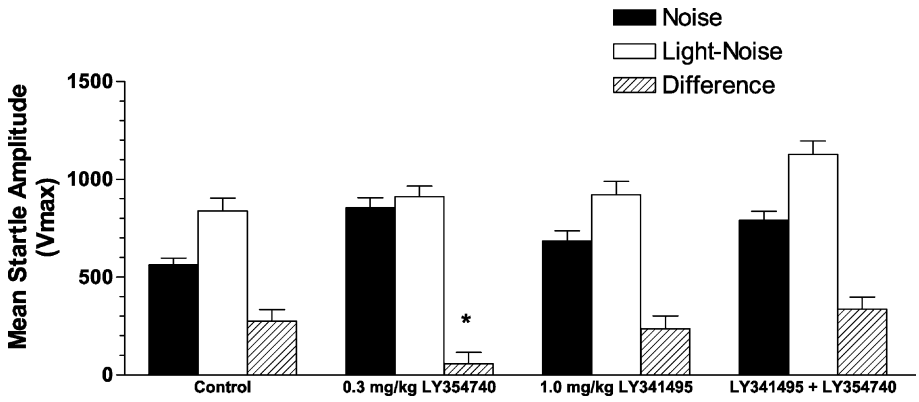

Fig. 4. The effects of compounds LY354740, LY341495, and LY341495 + LY354740 on fear expression in male Sprague–Dawley rats ($n = 8$ rats/treatment). Compound LY341495 was administered subcutaneously 60 min prior to testing on Day 3 (postconditioning). Compound LY354740 was administered intraperitoneally 30 min prior to testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P < .05$.

administered the compound prior to conditioning. Day 3—24 h after conditioning—startle testing sessions were conducted. Ten trials of acoustic startle (120 dB), nonlight-paired, were presented at the beginning of the session to minimize the influence of the initial rapid phase of habituation to the stimulus. This was followed by 20 random trials of the noise-alone and 20 random trials of noise preceded by light. Animals tested in the postconditioning paradigm were administered the compounds prior to testing on Day 3.

2.4. Statistical analysis

Excluding the first 10 trials, the startle response amplitudes for each trial type were averaged for each animal. Data were presented as noise, light + noise, and the difference between light + noise and noise-alone. Differences in startle response amplitudes were analyzed by JMP statistical software using a one-way analysis of variance (ANOVA) followed by a Dunnett's posthoc analysis to determine differences between control and treatment groups. Interaction experiments between agonists and antagonists were analyzed using a Student's t test. Group differences were considered significant at $P < .05$.

3. Results

The administration of diazepam (Fig. 1) resulted in a significant dose-dependent decrease in both fear conditioning (preconditioning paradigm [$F(4,719) = 8.08$, $P < .001$]) and fear expression (postconditioning paradigm [$F(3,639) = 7.43$, $P < .001$]). Posthoc evaluation revealed a significant attenuation at doses of 0.6 and 1.0 mg/kg in both paradigms (Dunnett's, $P < .05$). Diazepam administra-

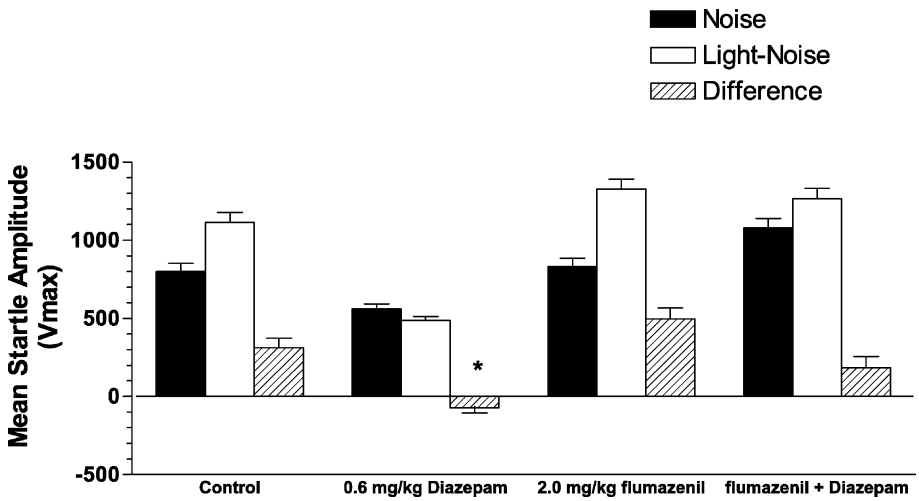

Fig. 5. The effects of diazepam, flumazenil, and flumazenil + diazepam on fear-potentiated startle in male Sprague–Dawley rats ($n = 8$ rats/treatment). Diazepam and flumazenil were administered intraperitoneally 30 and 60 min, respectively, prior to testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P < .05$.

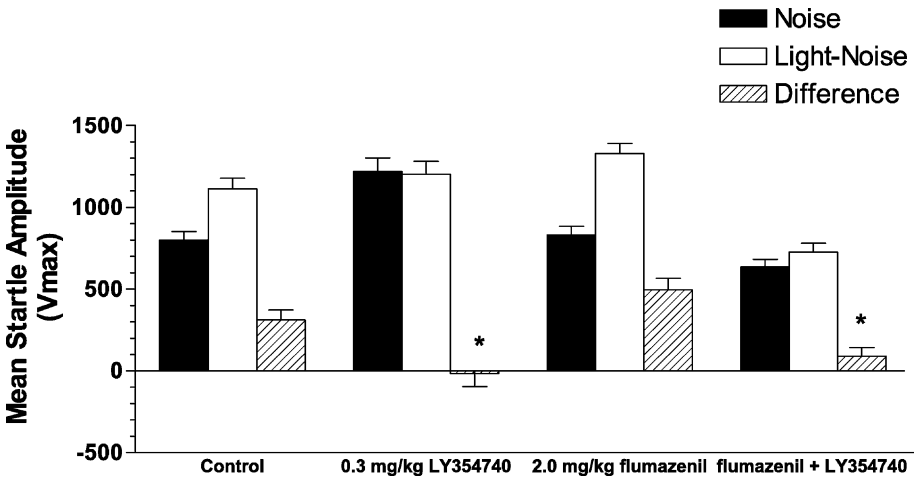

Fig. 6. The effects of compounds LY354740, flumazenil, and flumazenil + LY354740 on fear-potentiated startle in male Sprague–Dawley rats ($n = 8$ rats/treatment). Compound LY354740 and flumazenil were administered intraperitoneally 30 and 60 min, respectively, prior to testing on Day 3 (postconditioning). Animals received 0.5 mA of shock during conditioning. Values represent the means of noise-alone, light + noise, and differences between light + noise and noise-alone (startle amplitude, V_{max}). * Significantly different from control, $P < .05$.

tion did not disrupt startle responding during the noise-alone trials in either the preconditioning or postconditioning paradigm, indicating a lack of secondary pharmacological effects.

The administration of LY354740 (Fig. 2) did not block fear conditioning (preconditioning paradigm [$F(4,722) = 1.08$, $P > .05$]), but attenuated fear expression in a dose-dependent manner (postconditioning paradigm [$F(4,559) = 2.70$, $P < .029$]) in rats with significant reductions at 0.3 and 3.0 mg/kg (Dunnett's, $P < .05$). LY354740 administration did not disrupt startle responding during the noise-alone trials in either the preconditioning or postconditioning paradigm, indicating a lack of secondary pharmacological effects.

The mGlu2/3 receptor antagonist, LY341495, had no effect on fear expression in the postconditioning paradigm ($t = 0.12$, $P > .05$; Fig. 3). The anxiolytic actions of diazepam at 0.6 mg/kg ($t = 2.20$, $P < .028$; Fig. 3) were not reversed by LY341495 ($t = 0.16$, $P > .05$; Fig. 3). In contrast, the mGlu 2/3 receptor antagonist, LY341495, reversed the anxiolytic actions of LY354740 at 0.3 mg/kg ($t = 3.20$, $P < .001$; Fig. 4).

The benzodiazepine antagonist, flumazenil, alone had no effect on fear expression in the postconditioning paradigm ($t = 1.90$, $P > .05$; Fig. 5). However, flumazenil (Fig. 5) reversed the anxiolytic action of diazepam at 0.6 mg/kg ($t = 3.49$, $P < .0006$). In contrast, the anxiolytic actions of LY354740 at 0.3 mg/kg ($t = 3.19$, $P < .001$) were not reversed by flumazenil ($t = 1.05$, $P > .05$; Fig. 6).

4. Discussion

In the search to find novel anxiolytics, one major goal is to find agents with the efficacy of benzodiazepines, with no associated side effects and abuse liabilities. In particular, benzodiazepines are well documented to produce CNS depression, which manifests as sedation/amnesia in both animals and humans (Shader and Greenblatt, 1993). The neuronal substrates, which are responsible for the anxiolytic actions of benzodiazepines, have been elucidated. Benzodiazepines target $GABA_A$ receptor complexes in the CNS, which are composed of multiple subunits forming a chloride-permeable heteromeric ligand-gated ion channel. Recent studies indicate that the anxiolytic effects of benzodiazepines are mediated by $\alpha 2$ $GABA_A$ receptors (Low et al., 2000), while the CNS-depressant effects of benzodiazepines involve $\alpha 1$ $GABA_A$ receptors (McKernan et al., 2000). In animals where a point mutation of these specific subunits was introduced to prevent benzodiazepine binding, there was a loss of the anxiolytic effects in both light/dark and elevated plus maze tests in the $\alpha 2$ mutant mice while the anxiolytic activity was retained in $\alpha 1$ mutants. Importantly, the $\alpha 1$, but not $\alpha 2$, mutation produced loss of diazepam-induced CNS depression as measured by locomotor activity or rotorod tests. Consistent with these data, the compound L838417, which is an $\alpha 2$ allosteric agonist, but $\alpha 1$ antagonist, was shown to block expression of fear-potentiated startle in rats (McKernan et al., 2000). Thus, the anxiolytic activity of benzodiazepines in blocking the expression of fear-potentiated startle also appears to involve $\alpha 2$ $GABA_A$ receptors.

Our studies here show that fear conditioning was disrupted at the same doses of diazepam, which blocked expression of "anxiety" in the fear-potentiated startle test. Likewise, the blockade of fear expression by diazepam was prevented by flumazenil, a nonselective benzodiazepine antagonist. These studies established the usefulness of the fear-potentiated startle test to study how potential anxiolytic compounds such as LY354740 might be distinguished from benzodiazepines, in terms of their ability to prevent plasticity associated with fear learning versus expression of fear behavior. It should be noted that Davis (1979) previously reported that diazepam blocked the expression of potenti-

ated startle at doses having no effects on fear conditioning in this model. The reasons for this difference in results between our studies and this earlier one are not clear. From our literature review, it has been consistently shown that glutamatergic transmission, specifically NMDA receptor activation, is involved in plasticity mechanisms of fear learning (Davis, 1997; Davis et al., 1993; Wiley, 1997), but little else has been reported on the effects of benzodiazepines in paradigms of fear conditioning.

In addition to fear-potentiated startle, LY354740 has been reported to be active in the elevated plus maze test for anxiety in mice (Helton et al., 1998), and will block the effects of acute stress on elevated plus maze performance in rats (Schoepp et al., 2001). Very recently, Ferris et al. (2001) reported that the benzodiazepine antagonist, flumazenil, but not the opioid antagonist, naloxone, blocked the anxiolytic effects of LY354740 in rats on the elevated plus maze. These data indicate that unlike our results here in the fear-potentiated startle test, the anxiolytic effect of LY354740 in elevated plus maze may involve the modulation of GABAergic pathways in the brain. Group II mGlu receptor agonists are known to inhibit the release of glutamate and GABA in the CNS (Cartmell and Schoepp, 2000). Thus, the net effect of mGlu2/3 receptor activation on brain excitability depends on the relative tone of both excitatory versus inhibitory input in the system (see Schoepp, 2001). Furthermore, the suppression of GABAergic transmission via presynaptic mGlu2/3 receptors can lead to either reductions or enhancements of excitation to excitatory cells depending on which population of GABAergic interneurons is affected and the locations of inhibitory synapses in the neuronal circuit (Poncer et al., 2000; Schoepp, 2001). The studies here clearly show that the actions of LY354740 in blocking the expression of fear-potentiated startle are mechanistically distinct from the actions of the benzodiazepine diazepam. The mGlu2/3 antagonist blocked LY354740 while having no effects on diazepam. In contrast to what was reported for the elevated plus maze test by Ferris et al. (2001) using flumazenil, the effects of LY354740 in fear-potentiated startle were not affected by a dose of the benzodiazepine antagonist, flumazenil, shown to block diazepam. Thus, the data here indicate that the brain regions or neuronal circuits involved in the elevated plus maze performance and fear-potentiated startle may be different. The brain regions and neuronal system involved in fear-potentiated startle have been extensively studied. In particular, the amygdala is a key brain structure involved in fear-potentiated startle behavior (Davis, 1997), and the amygdala is thus a potential target for mediating the actions of mGlu2/3 receptor agonists.

As LY341495 selectively blocked the suppression of fear-potentiated startle by LY354740, it would appear that mGlu2/3 receptor activation is clearly responsible for the actions of LY354740. In this respect, mGlu2/3 receptor activation has been clearly shown to suppress evoked excitations in synapses of the amygdala (Rainnie et al., 1991; Holmes et al., 1996; Anwyl, 1999; Neugebauer et al., 2000). Mechanistically, the mGlu2/3 agonist induces postsynaptic hyperpolarization and will suppress evoked AMPA receptor-mediated excitatory synaptic currents by reducing glutamate release. Interestingly, reductions in AMPA neuronal transmission induced by local injections of the AMPA receptor antagonist, CNQX, also blocked the expression of fear-potentiated startle (Kim et al., 1993). As excitatory neuronal transmission within the amygdala is critical for expression of fear-potentiated startle, we hypothesize that the actions of LY354740 in blocking fear-potentiated startle may be mediated via its actions within the amygdala. Indeed, Stanek et al. (2000) have recently shown that the local injection of LY354740 into the amygdala blocks fear-potentiated startle. Thus, in this respect, LY354740 may be acting like a ''functional'' AMPA receptor antagonist, reducing synaptic AMPA receptor-mediated excitations. As NMDA receptor antagonists have been reported to block acquisition of conditioned fear (Miserendino et al., 1990; Lee and Kim, 1998), it would appear that LY354740 does not appreciably alter NMDA receptor-mediated excitations in vivo. This would be consistent with other studies which show that, in fact, LY354740 blocks certain in vivo actions of NMDA antagonists such as phencyclidine (Moghaddam and Adams, 1998; Cartmell et al., 1999). Here we hypothesize that the in vivo actions of LY354740 may also involve reductions in postsynaptic AMPA-mediated excitations of neurons, but in this case are due to their ability to counteract the disinhibitory actions of NMDA antagonists in certain limbic brain regions (Marek et al., 2000). In any case, studies here suggest that LY354740 does not share the actions of NMDA receptor antagonists in the fear-potentiated startle model. In other animal models of anxiety such as the punished responding, NMDA receptor antagonists have potent anticonflict activity, greatly increasing response rates for a reinforcer (e.g., food) under the threat of punishment (shock) (Clineschmidt et al., 1982; Wiley, 1997). However, unlike NMDA receptor antagonists, LY354740 was reported to be ineffective in increasing rates of responding suppressed by electric shock in the rat and pigeon conflict test, unless the shock was not delivered coincident with the reinforcement (Benvenga et al., 1999; Moore et al, 1999). Thus, LY354740 does not have a profile similar to an NMDA receptor antagonist in the conflict anxiety test as well.

In summary, results here demonstrate that LY354740 potently suppressed fear-potentiated startle in rats in a manner that was mechanistically distinguished from benzodiazepines. Furthermore, unlike what has been reported for other glutamatergic agents (e.g., NMDA receptor antagonists), the LY354740 anxiolytic activity in this test occurred at doses that did not alter fear conditioning. These animal studies further support the potential anxiolytic activity of LY354740, but importantly suggest that the clinical profile of LY354740 may also be distinguished from existing anxiolytics. It is hypothesized that novel agents such as LY354740 will have potent anx-

iolytic activities in humans, but without the side effects of current agents such as benzodiazepines. Clinical studies with LY354740 are currently in progress to test this hypothesis (Bourin and Hascoet, 2001).

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