Brief Communication

Like Extinction, Latent Inhibition of Conditioned Fear in Mice Is Blocked by Systemic Inhibition of L-Type Voltage-Gated Calcium Channels

Mark Barad,^{1,3} Ashley M. Blouin,² and Chris K. Cain²

¹Department of Psychiatry and Biobehavioral Sciences, Brain Research Institute and Neuropsychiatric Institute, and

²Interdepartmental Program in Neuroscience, University of California, Los Angeles, Los Angeles, California 90095-1761, USA

Having recently shown that extinction of conditioned fear depends on L-type voltage-gated calcium channels (LVGCCs), we have been seeking other protocols that require this unusual induction mechanism. We tested latent inhibition (LI) of fear, because LI resembles extinction except that cue exposures precede, rather than follow, cue-shock pairing. Systemic injections of two LVGCC inhibitors, nifedipine and diltiazem, before pre-exposure blocked LI completely with no evidence of state-dependent learning. The results indicate that extinction and LI share a common molecular requirement and may support the notion that LI, like extinction, is a form of inhibitory learning.

Latent inhibition (LI) describes a decrease in conditioned responding after a neutral cue, the conditioned stimulus (CS), is paired with an unconditional stimulus (US), if animals are first pre-exposed (PE) to the CS, compared with animals that are not pre-exposed (NPE; Lubow and Moore 1959). LI is of considerable interest both clinically and as a paradigm of learning. LI has attracted considerable clinical interest in psychiatry, because it appears to be disrupted during the acute stages of schizophrenic illness, and thus may point to an important elemental lesion of this disease (Lubow and Gewirtz 1995; Gray 1998; Weiner 2003). It is important theoretically because it is a form of learning that takes place during presentation of a neutral stimulus before any pairing with a US, whereby the animal learns that the stimulus is not followed by any event of consequence. Although some researchers believe that LI causes an interference with the expression of an intact learned association (Miller and Matzel 1988; Bouton 1993; Escobar et al. 2002; Weiner 2003), others maintain a traditional view that nonreinforced pre-exposure decreases attention to the CS or CS associability, making it harder to associate with a US during paired training (Mackintosh 1975; Pearce and Hall 1980; Wagner 1981; Ayres et al. 1987; Lubow 1989). A variation of this idea is that the context of pre-exposure becomes an “occasion setter” for the recall of the CS–no US association, accounting for the decrease in latent inhibition when fear acquisition is performed in a context different from that of pre-exposure (Lubow 1989).

We approached LI as an extension of our study of extinction. We recently demonstrated that both acute and long-lasting extinction, but not acquisition or expression, of conditioned fear depends on L-type voltage-gated calcium channels (LVGCCs; Cain et al. 2002). No acute or lasting extinction occurs with CS presentations after systemic injections of the LVGCC blockers nifedipine and nimodipine. As far as we know, extinction is the only behavioral protocol that has been shown to be completely dependent on LVGCC activity. In contrast, NMDA receptors are not necessary for the initial induction of extinction learning, but only for its consolidation (Falls et al. 1992; Santini et al. 2001), but these receptors are absolutely crucial to both the acquisition

and the consolidation of conditioned fear itself (Miserendino et al. 1990; Kim et al. 1991; Fanselow and Kim 1994). Together these findings indicate that the acquisition and the extinction of conditioned fear depend on distinct molecular mechanisms, and we wondered why this should be so. One difference between acquisition and extinction of fear is that acquisition involves the pairing of an intrinsically neutral cue, the CS (e.g., a tone), with an intrinsically aversive US (e.g., a footshock), whereas extinction involves presentation of the CS alone. We therefore hypothesized that other forms of learning about CSs without paired USs might be mediated by LVGCCs. On the other hand, during extinction the internal representation of the CS has already been changed by previous CS–US pairing, so that it is no longer “neutral.” That is, it is now linked to a representation of the US. Thus, it may be that LVGCC inhibitors block new learning about this linked CS, rather than learning about a truly neutral CS, before any conditioning has taken place.

To differentiate between these hypotheses, we chose to study the LVGCC-dependence of LI in mice. Both extinction and LI involve conflicts between associations formed at two temporally separated phases. However, because LI involves giving CS presentations before CS–US pairing, the learning is necessarily about a neutral stimulus, before it can be changed in any way by CS–US pairing, and thus provides a direct means to test whether LVGCCs play a role in learning about a truly “neutral” CS. In our LI protocol, we used conditions similar to those we previously used for extinction (Cain et al. 2002), with the exception of giving our CS presentations 1 d before fear conditioning instead of 1 d after. This protocol generated robust LI of conditional fear to the CS in the pre-exposure context as measured by behavioral freezing. In this paper, we report that, like extinction, LI is blocked by systemic injections of two LVGCC antagonists, nifedipine and diltiazem.

To test whether LI shares LVGCC-dependence with extinction, we pre-exposed the PE group of mice to 60 2-min CSs in Context B, while NPE animals spent equal time in the context without CS presentations. We fear-conditioned the animals 1 d later in Context A with two pairings of the 2-min CS coterminating with a 2-sec footshock (0.7 mA), and then tested the animals for fear of the CS 1 d after conditioning in Context B. We intentionally used different contexts for pre-exposure and conditioning, because context effects are known to disrupt LI. We

³Corresponding author.

E-MAIL mbarad@mednet.ucla.edu; FAX (310) 267-0040.

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wanted to isolate any effect of learning about the pre-exposure context from the paired training by performing that training in a separate context. Although a change of context for conditioning is known to reduce LI during acquisition, LI is intact when testing occurs in the same context as PE (Westbrook et al. 2000). The LI effect that we observed is smaller than is usually obtained. However, it remains significant, and can be attributed to decreased learning (or expression of learning) about the explicit CS, and not to some combination of learning about CS and context. In addition, this procedure is similar to that of our extinction training protocol.

Half of each group was injected with 40 mg/kg nifedipine 50 min before the pre-exposure session, and the other half with vehicle (Fig. 1). The mixed ANOVA demonstrated a significant interaction of treatment and pre-exposure ($F_{(1,36)} = 5.61$). LI of the PE group was obvious and significant in the final test in the vehicle-treated animals (V-PE vs. V-NPE, $t = 3.70$, $p < 0.01$). Nifedipine treatment before pre-exposure blocked LI completely (N-PE vs. N-NPE, $t = 0.07$, $p > 0.05$). Thus, latent inhibition, like extinction, appears to be dependent on LVGCCs.

One substantial concern in any experiment of this kind is that learning, including LI learning, may be state-dependent (Bouton et al. 1990). State-dependent learning can only be expressed when the same drug is present during testing that was present during acquisition. We therefore performed an experiment to demonstrate that there was no state-dependent latent inhibition during testing in the presence of nifedipine, using the same overall protocol as above (Fig. 2). In this experiment, we injected all animals before each phase of the experiment with either vehicle or nifedipine. As controls for state-dependent acquisition of latent inhibition, we injected nifedipine before both pre-exposure and the final test, and also before all three phases of the experiment. Because freezing in the NPE animals treated with nifedipine was near ceiling in Experiment 1, and because we have seen no evidence that injection of nifedipine during acquisition or testing increases expression of freezing (Cain et al. 2002), we did not include NPE groups receiving all patterns of injections. ANOVA revealed a significant effect ($F_{(3,28)} = 7.216$, $p < 0.01$). As expected, there was a significant LI of freezing at final test in

Figure 1 Nifedipine treatment during preexposure blocks latent inhibition. (A) Schema of protocol. (B) One day after fear conditioning, when tested in Context B with three CS presentations, significant latent inhibition was apparent in the V-PE group. LI was completely blocked by nifedipine.

Figure 2 The nifedipine block of latent inhibition does not reflect state-dependent learning. (A) Protocol schema. (B) When tested on day 3 in the pre-exposure Context B, only the VVV-PE group showed significant latent inhibition. There was no evidence for state-dependent recall of latent inhibition in the nifedipine-treated groups. (*) $p < 0.01$, VVV-PE versus all other groups.

VVV-PE group treated with vehicle at all three stages compared to VVV-NPE control (also treated with vehicle at all stages; $p < 0.01$). LI was significantly blocked by treatment with nifedipine before both PE and testing (NVN-PE vs. VVV-PE, $p < 0.01$), or before all three stages of the experiment (NNN-PE vs. VVV-PE, $p < 0.01$). Thus, there is no evidence of any state-dependent LI learning in the presence of nifedipine.

Conceivably, blockade of latent inhibition by nifedipine might have nothing to do with its nominal targets, the LVGCCs, but rather might be caused by some nonspecific effect of this drug. We therefore used a second LVGCC blocker, diltiazem, in a replication of this experiment (Fig. 3). Whereas nifedipine is a dihydropyridine, diltiazem belongs to a different pharmacological class, the benzothiazepines, and thus should show little overlap in non-LVGCC-specific effects. For this experiment we used the same protocol and gave injections of diltiazem or vehicle before each stage of the experiment. ANOVA revealed a significant effect in the experiment ($F_{(3,28)} = 3.893$, $p < 0.05$). Mice treated with vehicle before pre-exposure and both subsequent phases of the experiment showed significant LI on the final test (VVV-PE vs. VVV-NPE, $p < 0.05$). Diltiazem blocked LI with no evidence for state-dependent recall (Fig. 3B; VVV-PE vs. DVV-PE, $p < 0.05$; VVV-PE vs. DDD-PE, $p < 0.05$). Thus, like nifedipine, diltiazem blocks LI in a state-independent fashion.

These results provide substantial evidence that LI, like extinction, depends on LVGCCs (Figs. 1–3; Cain et al. 2002). The data support the hypothesis that LVGCCs may be important for CS-alone learning, because both extinction and LI learning appear to be blocked by LVGCC inhibitors administered during presentations of the CS alone. They support an argument against the alternate hypothesis, which we considered in the introduction, that LVGCCs play a role during extinction because of the previous association of CS and US. Other protocols involving learning about unpaired CSs also appear to depend on LVGCCs (C.K. Cain, A.M. Blouin, and M. Barad, unpubl.), which may suggest a common theme for the involvement of these channels

Figure 3 Diltiazem blocks latent inhibition in a non-state-dependent manner. (A) Schema of protocol. (B) When tested 1 d after fear conditioning, only the VVV-PE group showed significant latent inhibition compared with all the other groups. Diltiazem blocked latent inhibition in a non-state-dependent manner. (*) $p < 0.05$, VVV-PE versus all other groups.

in a variety of learning protocols. Why might this be so? As suggested above, because these protocols do not involve the pairing of a CS with a salient US, they may not engage mechanisms of synaptic plasticity that depend on the NMDA receptor, which requires coincidence of glutamatergic activation and postsynaptic depolarization to open. The LVGCCs require only a single signal, depolarization, to open, and thus may map better onto learning protocols that involve only a single salient stimulus.

In addition, this unusual shared molecular mechanism between extinction and LI may suggest that the large literature of LI can be mined for testable hypotheses about the anatomical and molecular bases of extinction. More generally, LVGCC blockers, like nifedipine and diltiazem, may be used as tools to assess whether or not different learning protocols recruit a common cellular mechanism, providing a means of classifying learning protocols besides purely psychological methods.

MATERIALS AND METHODS

Subjects

Naive male mice, 12–20 wk old, C57/b16, were housed four/cage with unrestricted access to food and water. Mice were tested during the light phase of a 12:12 light/dark schedule according to the UCLA Institutional Animal Care and Use Committee protocol. All groups had $n = 8$ –12 mice.

Drugs

Nifedipine (40 mg/kg) and diltiazem (20 mg/kg) were suspended in 10% Cremophor EL/PBS vehicle. Subcutaneous injections of 10 mL/kg volume were performed 50 min (nifedipine) or 30 min (diltiazem) prior to testing.

Latent Inhibition Protocol

Pre-exposure

Conditioning boxes with dimensions $30.5 \times 24.1 \times 21$ cm (Context B) were used for pre-exposure. These boxes had transparent front and back walls and white acrylic floors. Boxes were

wiped with 10% methanol prior to pre-exposure. Pre-exposure consisted of a 2-min acclimation period followed by 60 2-min, 80-dB white noise CS presentations, each separated by a 5-sec stimulus-free period. Non-pre-exposure (NPE) controls were injected and placed in the box for an equivalent duration (2 h, 7 min) but received no CS presentations.

Acquisition

Shuttle box compartments with dimensions $20.3 \times 15.9 \times 21.3$ cm (Context A) with transparent front and back walls and stainless steel grid floors were used, and compartments were wiped down with 10% ethanol prior to acquisition. Acquisition consisted of two pairings of a 2-min, 80-dB white noise tone coterminating with a 2-sec, 0.7-mA scrambled footshock. A 2-min stimulus-free period preceded, separated, and followed the pairings.

Testing

Testing in the pre-exposure context consisted of a 2-min acclimation period followed by three 2-min 80-dB white noise presentations separated by 5-sec stimulus-free periods.

General Methods and Statistics

All 80-dB white noise presentations were delivered through speakers on the inside chamber wall, and a PC, Med-PC software, and a SmartCTL Interface System were used for stimulus control and delivery. A 62-dB white noise was used for background throughout all behavioral experiments. Pre-exposure, acquisition, and testing were videotaped using individual video cameras mounted on each chamber ceiling and connected to a standard monitor and VCR. All freezing was rated by an experienced observer using a 5-sec instantaneous time sampling technique (12 observations/min). The observer was blinded to the drug treatment of the mice. Experiment 1 was analyzed by a mixed ANOVA with Bonferroni posttests. Experiments 2 and 3 were analyzed with one-way ANOVA and planned post hoc Dunnett tests using the V-PE group as the defined control to allow comparison to both the V-NPE, to demonstrate LI, and to the drug groups, to demonstrate blockade of LI. Differences were considered significant if $p < 0.05$.

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