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NMDA GluN2A and GluN2B receptors play separate roles in the induction of LTP and LTD in the amygdala and in the acquisition and extinction of conditioned fear

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ABSTRACT

Synaptic plasticity mediated by NMDA glutamate receptors is thought to be a primary mechanism underlying the formation of new memories. Activation of GluN2A NMDA receptor subunits may induce long-term potentiation (LTP), whereas low-frequency stimulation of GluN2B receptors induces long-term depression (LTD). In the present study, we show that blockade of GluN2A, but not GluN2B receptors with NVP-AAM077 and Ro25-6981 respectively, prevented LTP of auditory thalamic inputs to the lateral amygdala. Conversely, LTD induction in this pathway was prevented by blockade of GluN2B, but not GluN2A receptors. As this pathway plays a critical role in the acquisition, retrieval and extinction of a learned auditory-cue fear association, we next examined the effects of blockade of GluN2A and GluN2B receptors on the development and retention of a conditioned fear response. Administration of NVP-AAM077, but not Ro25-6981, prior to conditioning disrupted the expression of conditioned fear 24 h later. Conversely, Ro25-6981 but not NVP-AAM077 impaired extinction of the conditioned fear response. These data expand on previous work showing that LTP/D in the thalamic-lateral amygdala pathway is dependent on NMDA receptors, by demonstrating selective roles for GluN2A and GluN2B NMDA receptor subunits in LTP and LTD respectively. Furthermore, GluN2A receptor activation and associated LTP may be involved specifically in the initial formation and/or stabilization of a learned fear response, whereas GluN2B receptor activation and associated LTD may facilitate the suppression of Pavlovian fear responses during extinction.

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1. Introduction

Changes in the strength of synaptic transmission provide a fundamental mechanism through which learning and memory occurs in the mammalian brain. Long-term potentiation (LTP) and depression (LTD) of synaptic activity are currently the best characterized forms of synaptic plasticity and are suggested as physiological substrates of learning and memory (Bliss and Collingridge, 1993; Bear and Malenka, 1994; Rioult-Pedotti et al., 1998; Whitlock et al., 2006).

Fear conditioning is a simple form of associative learning that entails an association between a previously neutral conditioned stimulus (CS) with the onset/occurrence of an aversive unconditioned stimulus (US). After repeated pairings, presentation of the CS alone elicits behavioral and physiological responses consistent with fear or anxiety (Fendt and Fanselow, 1999; Gewirtz and Davis, 2000; LeDoux, 2000; Nijsen et al., 1998). LTP-like changes have been observed following the formation of a CS/US association (McKernan and Shinnick-Gallagher, 1997; Rogan and LeDoux, 1995; Rogan et al., 1997) and treatments that block the induction of LTP *in vitro* prevent the formation of a Pavlovian fear response in rats (Bauer et al., 2002). Although the mechanisms through which Pavlovian fear is extinguished are still debated, recent evidence suggests that a reversal of learning-induced potentiation and/or LTD may play an important role (Dalton et al., 2008; Kim et al., 2007). In a seminal study, Quirk et al. (1995) demonstrated that neurons of the lateral amygdala

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(LA) showed a significant increase in firing in response to a tone CS following pairing with a footshock US. This LTP-like response was attenuated following extinction training such that firing of these neurons resembled pre-conditioning levels once extinction was achieved. Subsequently, Lin et al. (2003) demonstrated that depotentiation of LA neurons with low-frequency stimulation administered after fear conditioning blocked the expression of conditioned fear, in a manner resembling extinction.

Recent work from our laboratory has examined the role of LTD on fear learning and extinction. Administration of an interference peptide (Tat-GluR2$_{3Y}$), that blocks AMPA receptor endocytosis and LTD *in vivo* (Brebner et al., 2005; Fox et al., 2007; Wong et al., 2007), selectively blocked the extinction of a learned fear response (Dalton et al., 2008). This effect is particularly interesting given that the same peptide had no effect on the acquisition of a learned fear response in these animals. These results were replicated by Kim et al. (2007) using intra-amygdala infusions of the same peptide. These data suggest that LTD may be a primary mechanism through which inappropriate responses are suppressed while mechanisms responsible for the acquisition of new learning remain intact. This hypothesis is supported by a recent report that the selective GluN2B *N*-methyl-D-aspartate (NMDA) receptor subunit antagonist Ro25-6981 not only blocked the induction of LTD but also increased perseveration during reversal learning in a water maze (Duffy et al., 2008), increased premature responding in a 5-choice serial reaction time task (Higgins et al., 2003), increased perseveration while shifting strategies in a set-shifting paradigm (Dalton et al., 2011), and induced a similarly selective impairment in extinction learning to Tat-GluR2$_{3Y}$ (Dalton et al., 2008). These data are consistent with a role for LTD in the inhibition of a previously learned response.

Collectively, these findings support the conjecture that LTP-like processes play a critical role in the acquisition of a new response (such as learned fear) whereas LTD-like processes may contribute to the suppression of a previously acquired but no longer appropriate response (as occurs during extinction). Until recently, appropriate pharmacological means with which to selectively target each of these processes were lacking. Recent electrophysiological data suggest a functional dissociation between the activation of a given NMDA receptor subunit and the induction of LTP as distinct from LTD. Under certain experimental conditions, GluN2A subunit activation results in the induction of an LTP-like response, whereas activation of receptors containing the GluN2B subunit mediates the generation of LTD (Izumi et al., 2006; Liu et al., 2004; Woo et al., 2005; Yang et al., 2005; but see Hendricson et al., 2002; Morishita et al., 2007). Thus, in the present study, we investigated the potential roles of GluN2A or GluN2B receptors in 1) the induction of LTP or LTD in the LA, a nucleus critical for fear memories and 2) the acquisition and extinction of associative fear memories.

2. Materials and methods

2.1. Experiment 1: relative contribution of GluN2A and GluN2B receptors to induction of LTP and LTD in the LA

Male Sprague Dawley rats (17–24 days old; Charles River Laboratories) were placed under deep anesthesia and decapitated. The brain was rapidly removed into ice-cold slicing solution containing (in mM): 87 NaCl, 2.5 KCl, 7 $MgCl_2$, 0.5 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 25 glucose and 75 sucrose that infused continuously with carbogen (95% O_2 and 5% CO_2) to adjust pH to 7.4. Coronal slices of 400 μm thickness containing the amygdala were produced using a vibrating blade microtome and recovered in an incubation chamber with carbogenated artificial cerebrospinal fluid (ACSF) containing (in mM): 125 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 2.0 $CaCl_2$, 1.25 NaH_2PO_4, 26 $NaHCO_3$ and 25 glucose for 30 min at 34 °C, and were then returned to room temperature (22 °C) for at least 30 min before recording. All experiments were carried out at room temperature.

A single slice was transferred to a recording chamber, perfused with carbogenated ACSF at a flow rate of 1.5–2.5 ml/min and held beneath a platinum wire. Whole–cell patch clamp recordings were performed using the "blind" method from neurons in the dorsal part of the LA. Recording pipettes were filled with pipette solution containing (in mM): 122.5 Cs-gluconate, 17.5 CsCl, 2 $MgCl_2$, 10 HEPES, 0.5 EGTA, 4K-ATP and 5 QX-314, with pH adjusted to 7.2 by CsOH, (290–300 mOsm). To isolate NMDA-mediated component of EPSCs, perfusing solution was replaced by Mg^{2+} free ACSF containing AMPA antagonist NBQX (5 μM), and bicuculline methiodide (10 μM). Once stable, EPSCs were obtained, Ro25-6981 (3 μM) and NVP-AAM007 (0.4 μM) were applied sequentially to assess the GluN2B- and GluN2A-components of EPSCs. The resistance of electrodes was typically 4–8 MΩ. Immediately after obtaining the whole cell configuration, current clamp was used to identify the firing pattern of the cells.

After cell characterization, the membrane potential was held at −70 mV. Excitatory postsynaptic currents (EPSCs) were evoked by stimulating the synaptic inputs from the medial geniculate nucleus of the thalamus, which processes auditory stimuli (Weisskopf et al., 1999; Yu et al., 2008) via a constant current pulse (0.05 mS) delivered through a tungsten bipolar electrode and recorded through a MultiClamp 700B amplifier (Axon Instruments). In addition, 10 μM bicuculline methiodide was included in the ACSF. Synaptic responses were evoked at 0.05 Hz except during the induction of LTP and LTD. After obtaining a stable EPSC baseline, either LTP or LTD was induced by applying either 200 pulses at 2 Hz while depolarizing the cell to −5 mV (LTP), or 480 pulses at 1 Hz while holding the cell at −50 mV (LTD). The stimulation intensity of induction was the same as that used during baseline recording. The induction of LTP and LTD was performed within 10 min after the establishment of a whole cell configuration to avoid washout of intracellular contents.

The drugs used in all electrophysiological experiments were made up in stock solution and diluted 1000 times into the perfusion ACSF on the day of recording. NBQX, and the selective NMDA GluN2B antagonist (+/−)-(R*,S*)-alpha-(4-hydroxyphenyl)-beta-methyl-4-(phenylmethyl)-1-piperidine propanol (Ro25-6981) were obtained form Tocris Biosciences (Ellisville, Missouri). The selective GluN2A antagonist NVP-AAM007 (NVP) was obtained from Novartis Pharma AG, Base (Switzerland). All other chemicals were obtained from Sigma (St. Louis, MO).

2.2. Experiment 2: roles of GluN2A and GluN2B receptors in the acquisition, recall and extinction of conditioned fear

2.2.1. Subjects

Two hundred and thirty-seven male Sprague Dawley rats (280–350 g; Charles River Laboratories) were used. Rats were pair-housed and maintained on a 12 h light/dark cycle with free access to standard laboratory chow and water. Rats were given 7–8 days to acclimatize to the colony before behavioral procedures began. All experiments were conducted in accordance with the standards of the Canadian Council on Animal Care and were approved by the Committee on Animal Care, University of British Columbia.

2.2.2. Apparatus

All training and testing occurred in four identical observation chambers (30.5 × 24 × 21 cm; Med-Associates, St. Albans, Vt., USA) enclosed in sound-attenuating boxes. The chambers were constructed of aluminum (two side walls) and Plexiglas (rear wall, ceiling, and hinged front door). Each chamber was illuminated by a single 100-mA house light located in the top-center of one wall. Auditory stimuli were delivered via a speaker connected to a programmable audio generator (ANL-926, Med-Associates) located in the top-left corner of the same wall as the houselights. Two identical 100-mA stimulus lights, 2.5 cm in diameter and 7 cm above the floor, were located on the wall opposite the houselight and speaker. The floor of each chamber consisted of 19 stainless steel rods spaced 1.5 cm apart. The rods were wired to a shock source and solid-state grid scrambler for the delivery of footshock. A video camera connected to a VHS video recorder was mounted above each of the chambers to permit video scoring of the animals' behavior.

Each chamber rested on a load-cell platform that recorded chamber displacement in response to each rat's motor activity (Med Associates). To ensure inter-chamber reliability, each load cell amplifier was calibrated to a fixed chamber displacement. Load cell output of each chamber was set to a gain (vernier knob, 8); optimized for detecting freezing behavior. During both conditioning and extinction, each rat's activity was monitored continuously. Load cell amplifier output from each chamber was digitized and acquired on-line using Threshold Activity software (MED-Associates). Activity was digitized at 5 Hz, yielding one observation per rat every 200 ms (300 observations per rat per minute). Freezing was quantified by computing the number of observations for each rat that had a value less than the *freezing threshold*. The freezing threshold was determined in a separate group of pilot animals by comparing load cell output with an observer's ratings of freezing behavior. To achieve a sensitive freezing threshold, the load cell gain for all chambers was set to yield a freezing threshold that correlated with the observer's ratings of freezing behavior. Thus, movements such as grooming, head turning, and sniffing produced load cell output that exceeded the freezing threshold. The freezing threshold was absolute and used for each rat and experiment in the present study. To avoid counting momentary inactivity as freezing, an observation was only scored as freezing if it fell within a contiguous group of at least five observations that were all less than the freezing threshold. Thus, freezing was only scored if the rat was immobile for at least 1 sec (see Maren, 1998). We verified the threshold output of freezing behavior with video scoring of the animals' response; defined as the

cessation of all movement with the exception of respiration-related movement and non-awake or rest body posture (Sotres-Bayon et al., 2007).

2.2.3. Drugs

The GluN2A-preferring antagonist NVP and GluN2B antagonist Ro25-6981 were dissolved in 1 part DMSO: 2 parts physiological saline. NVP (1.2 mg/kg) was injected 45-min and Ro25-6981 (6 mg/kg) was injected 30-min before testing began. The broad-spectrum competitive NMDA antagonist CPP (10 mg/kg) was dissolved in 0.9% NaCl and was given 60-min before testing. All drugs were administered i.p. at a volume of 1 ml/kg. Dose and route of administration of NVP and Ro 25-6981 was chosen with reference to Fox et al. (2006). Dose and route of CPP was chosen with reference to Goosens and Maren (2004). In each of these studies, administration of these compounds had been reported to disrupt the acquisition and/or extinction of a conditioned freezing response or electrophysiologically induced LTP or LTD *in vivo*. Ro25-6981 was obtained form Tocris Biosciences (Ellisville, Missouri), NVP was obtained from Novartis Pharma AG, Base (Switzerland), CPP was obtained from Sigma (St. Louis, MO).

2.2.4. Fear conditioning and testing

2.2.4.1. Experiment 2A: systemic blockade of GluN2A and GluN2B receptors and the acquisition of conditioned fear. In this experiment, we sought to compare directly the effects of the GluN2A-preferring antagonist NVP and the GluN2B antagonist Ro25-6981 on the acquisition of a conditioned fear response. Rats treated with the broad-spectrum NMDA antagonist CPP were also included as a positive control. Given the inherent pharmacokinetic differences between these drugs, each experiment included drug-treated and vehicle-treated sub-groups. Thus, each drug-treated group was compared to its corresponding vehicle-treated cohort for statistical analyses.

The fear acquisition experiments were conducted over 3 days. *Day 1 – Habituation*: Rats were exposed to the chambers for 10 min. *Day 2 – Conditioning:* rats were given five presentations of the tone CS (4 kHz, 80dB, 20 sec) each co-terminating with a 0.8 mA footshock lasting 0.5-s (US). The first CS-US pairing was presented 120 s into the session and the inter-trial interval (ITI) between CS-US presentations was 105 s on average (range 90–120). Conditioning sessions lasted 11 min. Immediately after conditioning, rats were removed from the chamber and returned to their home cage. *Day 3 – Fear recall tests*: Twenty-four hrs following the acquisition training, rats were given five 20-s CS presentations in the absence of the US. The first tone was presented 120 s into the session and the inter-trial interval (ITI) between CS-US presentations was 105 s on average (range 90–120). Percent time spent freezing was measured during each tone. Recall test sessions lasted 11 min. CPP, NVP, Ro 25-6981 or their respective vehicles were administered once before conditioning (Day 2).

2.2.4.2. Experiment 2B: systemic blockade of GluN2A and GluN2B receptors on the extinction of conditioned fear. The fear extinction experiments were conducted over 4 days: Days 1 and 2 (Habituation and Conditioning) were identical to those described above for Experiment 2A. As we were particularly interested in effects on the progression and recall of extinction training, rats went through an extended extinction training session on day 3. Thus, during *Day 3 – Extinction Training:* rats were exposed to 20 presentations of the CS in the absence of the US (mean ITI 180 s, range 120–240 s). *Day 4 – Extinction Recall Test:* 10 presentations of the CS only (mean ITI 180 s, range 120–240 s; see Fig 3A).

This experiment tested the effects of each drug on either the acquisition or recall of extinction learning. Thus, in separate groups of rats, Ro 25-6981, NVP or vehicle were administered prior to Extinction Training (Day 3) or Extinction Recall test (Day 4) making a total of 6 groups. Freezing behavior was recorded during each presentation of the tone CS and is expressed as average percent time spent freezing per block of two tones (1 CS trial).

2.2.5. Data analysis

Freezing data from Experiment 2A were analyzed using a three way ANOVA with Antagonist (NVP, Ro25-6981 and CPP) and Treatment (drug or vehicle) as two between-subjects factors and Tone as a within-subjects factor. Data from each drug-group were compared only to that drug's respective vehicle control. For Experiment 2B, the average percent time spent freezing during each tone CS was expressed in blocks of two tones (1 CS trial = average of 2 tones) and compared with a two-way between/within subjects factorial ANOVA, with drug treatment as the between subjects factor and CS trial as the within subjects factor. All significant main effects and interactions were further analyzed using Dunnett's comparisons.

3. Results

3.1. Electrophysiology

3.1.1. Experiment 1: differential roles of GluN2A- and GluN2B-containing NMDA receptors in the LTP and LTD at thalamic input synapses in the LA

We employed standard pairing protocols to induce LTP and LTD in pyramidal neurons of the LA. Our initial experiments on synaptic plasticity within this region revealed that both LTP and LTD were blocked by the non-specific NMDA-receptor antagonist, APV (Yu et al., 2008). Subsequent experiments that used identical procedures and are described here were designed to ascertain the relative contribution of different NMDA receptor subtypes to these forms of plasticity.

As shown in Fig. 1, stimulation of the auditory thalamic pathway to the LA with a train of 200 stimuli at 2 Hz, while the postsynaptic neuron was held at −5 mV, induced robust LTP in the LA (150.1 ± 18% of control EPSC amplitude 35 min after the pairing protocol; $n = 8$, Fig. 1A), whereas stimulation of the same pathway with a train of 480 pulses at 1 Hz while holding the cell at −50 mV resulted in stable LTD (the amplitude of EPSCs being 60.7 ± 9% of the control levels 35 min after LTD induction; $n = 9$, Fig. 1C). As mentioned above, both LTP and LTD were found to be NMDA receptor-dependent, as they were blocked by the non-specific NMDA antagonist APV (Yu et al., 2008). To determine the relative contribution of NMDA-receptor subtypes to these opposing forms of synaptic plasticity in this system, subsequent experiments utilized the GluN2A-prefering antagonist NVP and the GluN2B antagonist Ro25-6981. Using the same preparations and recording conditions, we performed sequential applications of NVP and Ro25-6981, and found that these two drugs have preferential antagonisms at GluN2A and GluN2B receptors, respectively (Yu et al., 2010). Specifically, NVP (0.4 μM) blocked ~65% of the NMDAR-mediated currents, with the residual currents largely blocked by subsequent application of Ro25-6981. Conversely, Ro25-6981, suppressed the magnitude of NMDAR-mediated EPSCs by ~45% (Yu et al., 2010), and the residual currents were nearly completely blocked by subsequent application of NVP. These results demonstrated that both GluN2A and GluN2B subunit-containing NMDA receptors are functionally expressed at thalamo-amygdala synapses, and can be at least partially isolated using these two drugs. Therefore, we tested the effects of these NMDA subunit antagonists on the formation of LTP/LTD in LA neurons.

Application of the GluN2A-preferring antagonist NVP (0.4 μM) prevented LTP induction (93.9 ± 8% of the control EPSCs 35 min after the paring protocol; $n = 8$, Fig. 1A) while having little effect on LTD induction (61.8 ± 19% of the control 35 min after LTD induction; $n = 8$, Fig. 1D). In contrast, application of the GluN2B selective antagonist Ro25-6981 (3 μM) failed to affect LTP induction (153.2 ± 25% of the control EPSCs 40 min after the induction stimulation; $n = 8$, Fig. 1B), but did prevent LTD induction (the normalized EPSC amplitude was 90.1 ± 6% of the control 35 min after LTD induction; $n = 9$, Fig. 1C). These results indicate that GluN2A- and GluN2B-containing NMDARs in the LA may be differentially required for the production of LTP and LTD. Thus, under our experimental conditions, NVP and Ro25-6981 appear to inhibit the induction of LTP and LTD of auditory thalamic inputs to LA neurons, respectively.

3.2. Behavior

3.2.1. Experiment 2A: blockade of NMDA GluN2A but not GluN2B receptors impairs acquisition of conditioned fear

Six separate groups of rats were given injections of CPP (10 mg/kg, $n = 10$), Ro25-6981 (6 mg/kg, $n = 10$), NVP (1.2 mg/kg, $n = 13$) or the respective vehicles (n's = 8, 9 and 10, respectively) prior to a fear conditioning session. By the end of the conditioning session, all rats in all groups displayed a robust, but not asymptotic freezing response during the last two presentations of the CS (~60% freezing during CS presentation, see Fig. 2). Notably, across all groups, there were no differences between drug vs. vehicle controls on this measure (all Fs < 0.53, n.s.). This confirms that none of the NMDA receptor antagonists used here impaired the short term

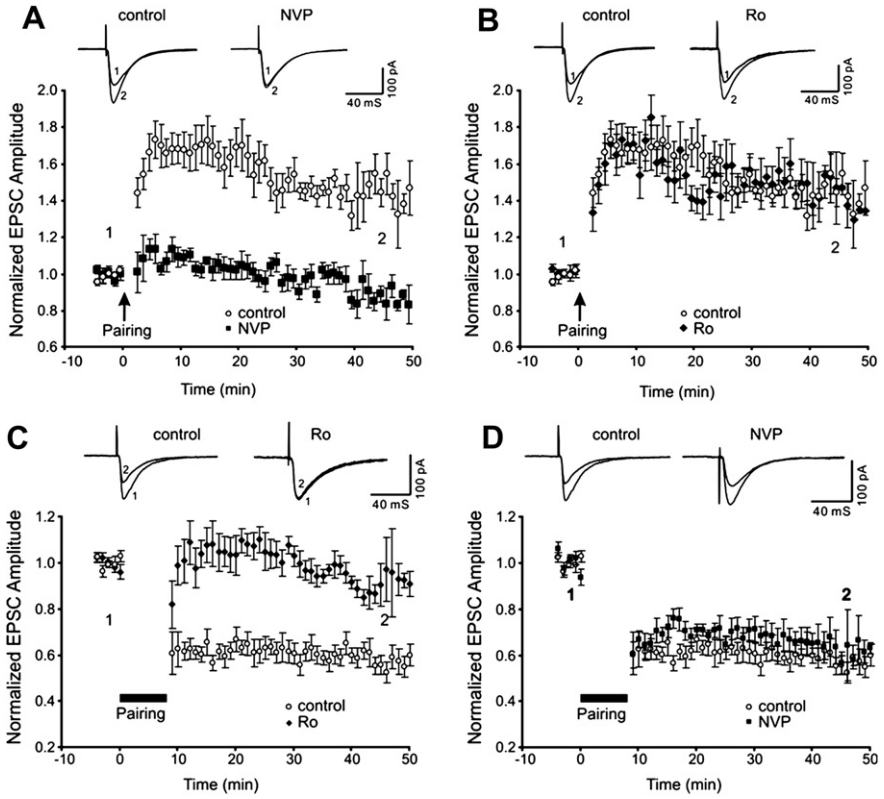

Fig. 1. *Top:* NR2A receptor activation is required for the induction of LTP in the auditory thalamic pathway within the LA. A) LTP in LA neurons was reliably induced by pairing presynaptic stimulation (2 Hz, 200 pulses) with postsynaptic depolarization to −5 mV. Bath application of NVP-AAM077 (0.4 mM) prevented LTP induction ($n = 8$ for each group). B) Bath application of Ro25-6981 (3 mM) did not prevent LTP induction ($n = 8$ and 5, respectively for control and Ro25-6981 groups) The amplitude of individual EPSCs was normalized to the averaged amplitude of EPSCs during the 5 min baseline recordings just before LTP induction. Representative traces on the top of panel A and B are averaged EPSCs from three consecutive responses taken before (1) and 35 min after (2) LTP induction. *Bottom:* NR2B receptor activation is required for the induction of LTD at the auditory thalamic pathway in the LA. C) LTD in LA neurons was reliably induced by pairing presynaptic stimulation (1 Hz, 480 pulses) with postsynaptic depolarization to −50 mV. Bath application of Ro25-6981 (3 mM) prevented LTD induction ($n = 9$ and 7, respectively for control and Ro25-6981 groups). D) Bath application of NVP-AAM077 (0.4 mM) failed to prevent LTD induction. Representative traces on the top of panel A and B are averaged EPSCs from three consecutive responses taken before (1) and 35 min after (2) LTP induction.

acquisition of a conditioned fear response, nor did these treatments alter general activity.

A fear recall test was conducted drug-free 24-h later during which total percent time spent freezing to 5 presentations of the tone CS was measured (Fig. 2A–D). Analysis of these data revealed a significant drug group × treatment (drug or vehicle) interaction ($F(2,54) = 4.081$, $p < 0.05$). There were no significant interactions with CS presentation and either of the between subjects factors (all F's < 1.5, *ns*.). Simple main effects analyses revealed that administration of Ro25-6981 had no effect on percent time spent freezing to the tone CS. In contrast, rats that had received injections of either the non-selective NMDA receptor antagonist CPP or the GluN2A antagonist NVP prior to the conditioning session showed a significant decrease in the amount of freezing elicited by the tones during the subsequent drug-free recall test 24 h later ($p < 0.05$ for both). However, the nature of the disruption in the fear response was different between the two compounds. Fig. 2A and B depicts the percentage of time drug- or vehicle-treated rats spent freezing during each of the 5 tone presentations during the recall test. Rats receiving CPP prior to conditioning displayed lower levels of freezing during presentation of the first tone, and this remained consistent over the session. In contrast, animals treated with NVP displayed comparable levels of freezing during the first two tones, but then displayed a significant reduction in freezing ($p < 0.05$) during the remaining 3 tones relative to controls. Thus, administration a broad-spectrum or preferential GluN2A (but *not* GluN2B) NMDA antagonist prior to fear conditioning resulted in a blunted fear response during the recall test, but the manner in which this blunted effect was expressed differed between drugs.

Following completion of this experiment, an additional experiment was added in which we attempted to disrupt the acquisition of a fear response using a higher dose of Ro25-6981 (12 mg/kg, $n = 8$) or vehicle ($n = 8$) given 30 min prior to conditioning. This experiment was conducted exactly as described for other drug treatments in experiment 2A. Administration of this dose of Ro25-6981 during conditioning did not affect expression of fear during the subsequent recall test conducted 24 h later (all Fs < 1.0, *n.s*; Fig. 3C).

3.2.2. Experiment 2B: blockade of GluN2B, but not GluN2A receptors impairs the acquisition of extinction of conditioned fear and its recall

3.2.2.1. GluN2A receptor antagonism. NVP (1.2 mg/kg) was administered i.p. 45-min before either extinction training or extinction recall. Conditioning, extinction training and extinction recall were all separated by 24-h. Extinction training consisted of 20 presentations of the CS alone (Fig. 3A). Administration of NVP prior to the extinction training session on day 2 did not affect expression of conditioned fear as assessed from freezing levels during the first CS trial (tones 1 and 2; $F(1,32) = 2.143$, *ns*, Fig. 3B). In addition, expression of extinction training was unaffected by pre-treatment with NVP (all Fs < 1.3, *ns*). Thus, NVP-treated rats ($n = 18$) displayed an extinction curve that did not differ from control rats ($n = 19$). The absence of a significant difference between groups

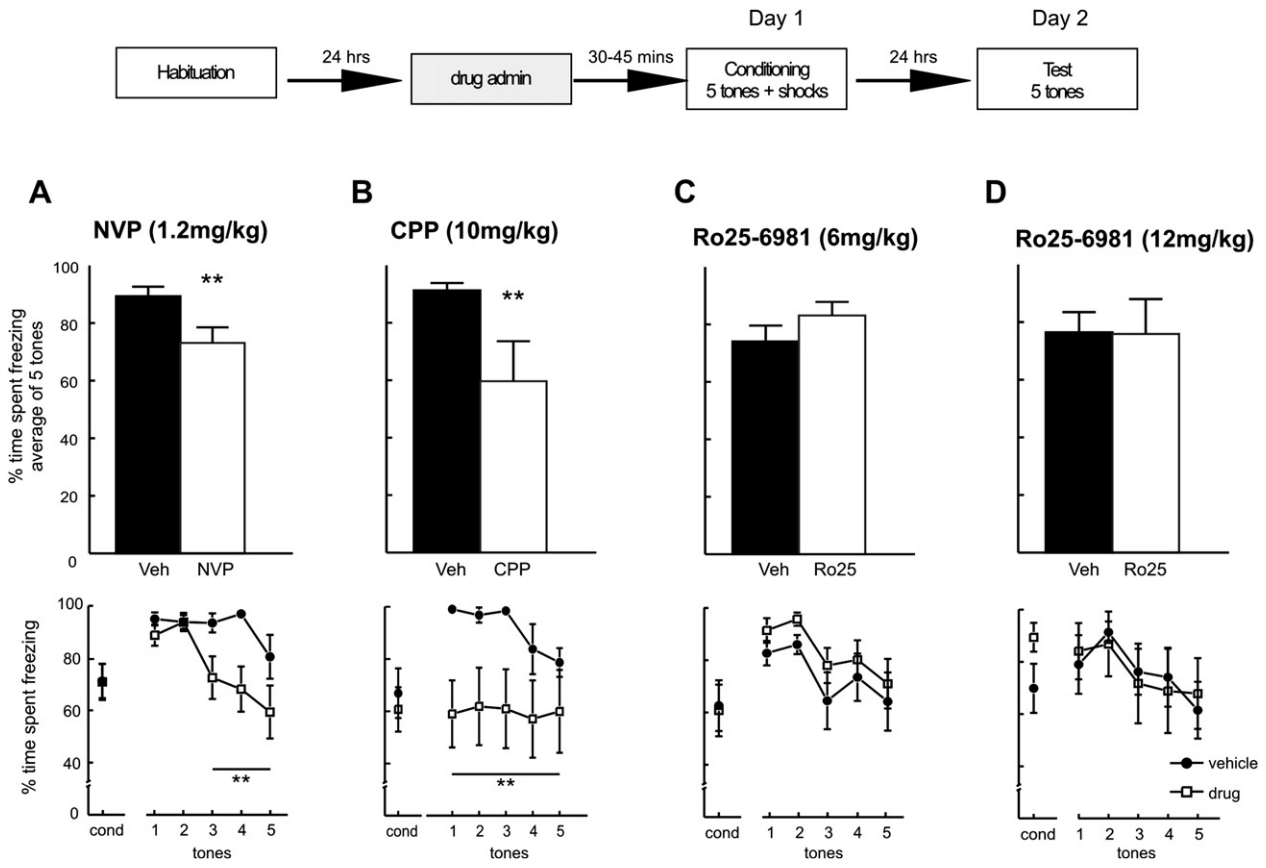

Fig. 2. Acquisition of conditioned fear is impaired by pre-conditioning administration of drugs that block NMDA NR2A but not NR2B receptor subunit activation. *Top:* Schematic describing the experimental time line. *Bottom:* Bar (upper panel) and line graphs (lower panel) showing freezing behavior during the first 5 tones of the fear recall test for rats given either a) the selective NMDA NR2A receptor antagonist NVP-AAM077 (1.2 mg/kg); b) the competitive, non-selective NMDA receptor antagonist CPP (10 mg/kg); c) the selective NMDA NR2B receptor antagonist Ro25-6981 (6 mg/kg); d) a higher dose of Ro25-6981 (12 mg/kg) or their respective vehicles. Bar graphs show the average percent time spent freezing during the 5 test tones of the fear recall test. Line graphs show expression of conditioned fear during the last two tones of conditioning (cond) and during each of the 5 tone CS presentations during the fear recall test. Data expressed as mean (±S.E.M.) percent time spent freezing during the tone CS. Stars denote $p < 0.05$.

during the extinction training session indicates that NVP does not affect the expression of conditioned fear or that of extinction learning in fear-conditioned animals. This conclusion is further emphasized by our observation that rats given NVP during extinction training displayed normal recall of that extinction training in an extinction recall test conducted 24-h later.

Administration of NVP prior to the extinction recall test ($n = 18$) did not affect the recall of extinction, the overall levels of freezing during the session or the rates of extinction between groups (all Fs < 1.3, *ns*; Fig. 3B).

3.2.2.2. GluN2B receptor antagonism. In a separate group of rats, Ro25-6981 was given either 30-min before extinction training or 30-min before an extinction recall test. In contrast to the lack of effect of GluN2A receptor blockade, administration of Ro25-6981 before extinction training retarded short-term, within-session extinction learning (Fig. 3C). The ANOVA of these data revealed that animals given Ro25-6981 ($n = 12$) displayed significantly higher levels of freezing to the tone CS compared to vehicle-treated ($n = 10$) controls (tone × group interaction; $F(9,180) = 2.02$, $p < 0.05$). Post-hoc analyses revealed no significant difference in freezing levels between vehicle and Ro25-6981-treated rats during the first 4 trials indicating that Ro25-6981 did not impair recall or expression of conditioned fear. However, Ro25-6981-treated rats displayed significantly more freezing during extinction training trials 4 through 9 ($p < 0.01$). Ro25-6981-treated rats showed a trend toward higher freezing levels during the last extinction training trial but this difference did no reach significance.

Analysis of freezing levels during the extinction recall test conducted 24-hrs after extinction training showed a main effect of group ($F(2,30) = 7.5$, $p < 0.01$; Fig. 3C). Post-hoc analysis revealed that only rats given Ro25-6981 before the extinction recall test ($n = 11$) displayed an impaired recall of extinction training ($p < 0.01$), whereas rats whose extinction training had been affected by Ro25-6981 on the previous day showed no detrimental effects of this disruption. Rats given Ro25-6981 before extinction training ($n = 12$) did not differ significantly from control ($n = 10$) rats in freezing levels during the extinction recall test. Collectively, these data show that blockade of GluN2B, but not GluN2A-subunit containing NMDA receptors, retards the extinction of conditioned fear.

4. Discussion

The major findings of this study concern important differences between the effects of GluN2A and GluN2B receptor blockade on both the induction of LTP or LTD and acquisition and extinction of conditioned fear. It has been well established that synaptic plasticity in this region is critically dependant on NMDA receptor activity. Here we show for the first time that NVP, a GluN2A receptor-preferring antagonist, disrupted both LTP in amygdala slices and also compromised the acquisition of a Pavlovian

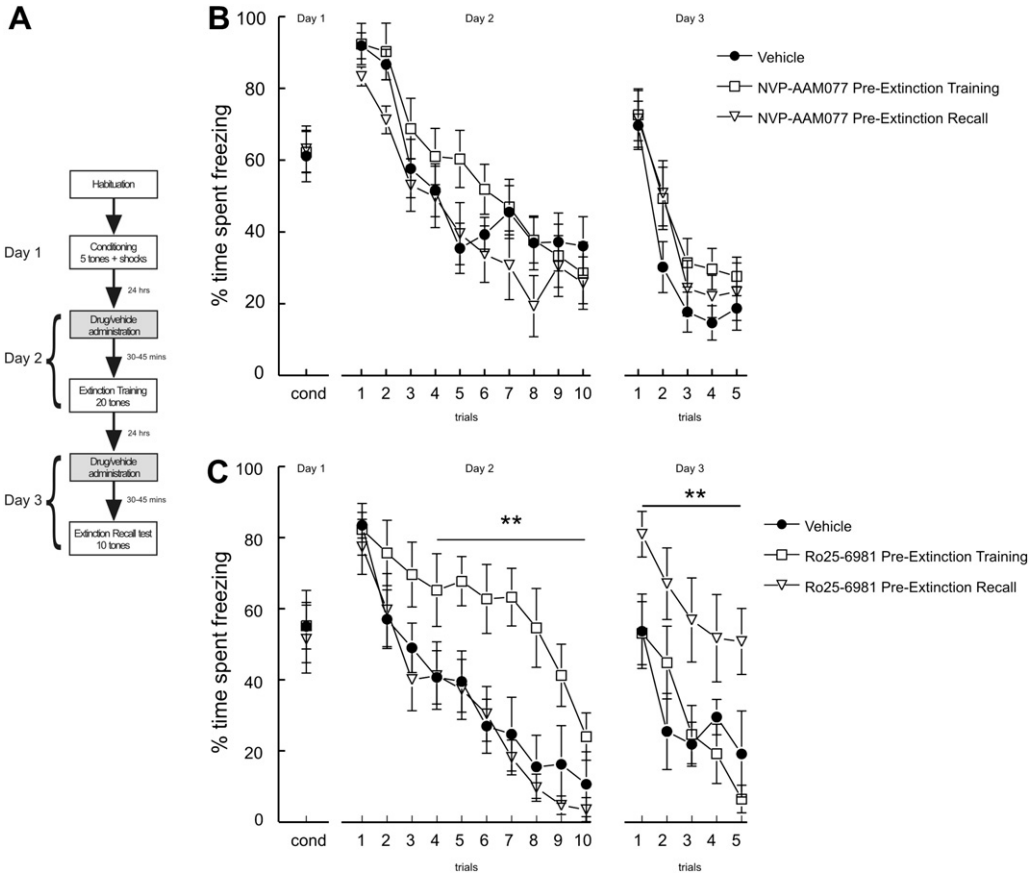

Fig. 3. Effects of NVP-AAM077 or Ro25-6981 on the suppression of a learned fear response during extinction training (day 2) or during an extinction training recall test (day 3). A) Schematic describing the experimental time line. B and C) Administration of either NVP-AAM077 (B) or Ro25-6981 (C) before testing on day 2 did not affect expression of a learned fear response (trials 1and 2, day 2). Rats given Ro25-6981 prior to extinction training (open squares) showed a significant impairment in the suppression of learned fear during extinction training but did not differ from controls during the extinction recall test. Administration of Ro25-6981 prior to the extinction recall test (open triangles) induced a significant impairment in recall of prior extinction training. NVP-AAM077 had no effect on performance during either extinction training or extinction recall. Data expressed as mean (±S.E.M.) percent time spent freezing during 2 tone CS's (trials). Stars denote $p < 0.05$.

conditioned fear response. However, NVP did not affect LTD or the extinction of conditioned fear. In contrast, blockade of GluN2B receptors with Ro25-6981 impaired LTD in the LA and extinction learning, while having no effect on induction of LTP or the acquisition of learned fear. The fact that compounds which exert preferential blockade of different NMDA receptor subtypes selectively blocked increases or decreases in synaptic strength within the lateral amygdala and also impaired acquisition or extinction of conditioned fear mediated by this nucleus provide novel insight into the roles these receptors play in these aspects of associative learning.

4.1. GluN2 receptor blockade and synaptic plasticity

Experiment 1 revealed differences between NMDA receptor subunit activation and the nature of synaptic plasticity observed in the LA, findings that are entirely consistent with previous observations in other brain regions. Liu et al. (2004) showed that blocking GluN2B receptors using Ro25-6981 abolished the induction of LTD, but not LTP, in hippocampal slices. In the same study, NVP prevented the induction of LTP without affecting LTD. More recently, similar results with these compounds have been observed in the hippocampus *in vivo* (Fox et al., 2006; Ge et al., 2010). Likewise, Kohr et al. (2003) have demonstrated reduced hippocampal LTP in mutant mice lacking the C-terminal domain of the GluN2A receptor subunit, and Brigman et al. (2010) have reported impaired hippocampal LTD in mice whose GluN2B subunits in the hippocampus and cortex were specifically deleted. It is important to note that this selective gating of LTP/LTD by GluN2A/B receptors has not been observed in other brain regions (de Marchena et al., 2008; Morishita et al., 2007). Nevertheless, the present data suggest that within the amygdala, GluN2A and GluN2B receptors can make dissociable contributions to the induction of LTP and LTD-like processes.

Although our electrophysiological data regarding GluN2 subunit activation and the direction of synaptic plasticity are consistent with many other studies (Izumi et al., 2006; Liu et al., 2004; Massey et al., 2004; Woo et al., 2005; Yang et al., 2005), there are challenges to this interpretation (Hendricson et al., 2002; Morishita et al., 2007). Many factors, including differences in brain region and developmental stage of the slice preparations, as well as concentrations of the drugs used, may account for these discrepancies. In particular, some of the controversy has been attributed to the variations in the concentration of NVP used in these studies. In one of our previous studies (Liu et al., 2004), we found that NVP is ~100 times more selective toward recombinant GluN2A NMDARs expressed in oocytes and that at 0.4 μM concentrations, it can fully block GluN2A receptors, with little effect on GluN2B receptors. However, one recent study using rat GluN2A and GluN2B recombinant receptors over-expressed in HEK cells reports that NVP has only <10 times selectivity for GluN2A over GluN2B, and the selectivity can only be maintained at a concentration of 0.1 μM

(Berberich et al., 2005). Differences in concentrations for selectivity of NVP have also been observed for native NMDARs in slice preparations. Thus, while Berberich and colleagues found that GluN2A selectivity for NVP may only be achieved at the concentration below 0.1 μM, Wu et al. (2007) revealed that a full blockade of GluN2A containing NMDA receptors by NVP could not be achieved at a concentration of 0.1 μM, requiring instead a concentration of NVP at 0.4 μM in acute cortical brain slices. Importantly, they found that at this higher concentration, NVP has little effect on blocking GluN2B-containing NMDARs (Wu et al., 2007). These discrepancies highlight the importance of determining the subunit specificity of these pharmacological agents under the experimental conditions and the need to interpret pharmacological results with caution. Of specific relevance to LA neurons in a slice preparation under our recording condition, recent work by our group has characterized the relevant specificity of NVP and Ro25-6981 as preferential antagonists for GluN2A and 2B respectively using a sequential drug application protocol (Yu et al., 2010). As reported in previous studies using other neuronal preparations (Tigaret et al., 2006; Wu et al., 2007), NVP at the concentration used in the present study has a GluN2A subunit preference with only a small (about 10%) contaminant blockade of GluN2B component. These results provide evidence that NVP and Ro25-6981, represent useful antagonists to differentiate roles of GluN2A and GluN2B in mediating LTP and LTD in the LA neurons under our experimental conditions. Indeed, we found that in this LA slice preparation, NVP specifically prevents the induction of LTP without affecting LTD. The lack of effect of this drug on LTD not only argues that GluN2A-containing receptors have little, if any, role in the induction of LTD, but also indicates that small degree of contaminant blockade of GluN2B-containing receptors by NVP (Yu et al., 2010) is not sufficient to prevent its induction. Thus, in contrast to the non-specific NMDAR antagonist APV that blocks both LTP and LTD in LA neurons (Yu et al., 2008), NVP can be used to selectively prevent LA LTP under these experimental conditions. On the other hand, prevention of LTD by Ro 25-9681 strongly argues for an essential role of GluN2B-containing receptors in LTD induction. Note that the selective contribution of GluN2A/2B-containing receptors in mediating LTP/LTD of auditory thalamic inputs to the LA differs from observations following stimulation of local (non-specific) or cortical (external capsule) fibers to the LA, where both forms of synaptic plasticity are attenuated by antagonist for either receptor (Müller et al., 2009). Therefore, the differential blockade of LTP and LTD by NVP and Ro25-6981 in this particular pathway suggests that these two antagonists may serve useful in probing roles of LTP and LTD in the LA in the context of auditory fear conditioning.

4.2. LTP and the acquisition of fear memories

Our electrophysiological data are complemented by our behavioral findings in that the acquisition of a learned fear response can be distinguished from it's extinction on a mechanistic level. The notion that LTP-like processes are the primary mechanism underlying the acquisition of new associative memories is well accepted (Malenka and Nicoll, 1993, 1999; McKernan and Shinnick-Gallagher, 1997; Milner et al., 1998; Rogan et al., 1997). Treatments that block LTP, such as NMDA receptor antagonists and protein synthesis inhibitors, disrupt the acquisition of a conditioned fear response (Bailey et al., 1999; Fanselow and Kim, 1994; Frey et al., 1996; Kim et al., 1991; Miserendino et al., 1990; Schafe and LeDoux, 2000). More specifically, a role for GluN2A subunits in LTP and learning has been suggested previously using both transgenic and pharmacological methods. Mutant mice lacking the GluN2A subunit or its C-terminal domain exhibit reduced hippocampal LTP (Kiyama et al., 1998; Kohr et al., 2003; Sakimura et al., 1995) and impairments in contextual fear conditioning (Kiyama et al., 1998; Sprengel et al., 1998) and spatial learning (Bannerman et al., 2008; Sakimura et al., 1995). Pre-conditioning infusions of NVP into the amygdala also impair recall of conditioned fear (Walker and Davis, 2008; Zhang et al., 2008). Our observation that NVP (which selectively blocked LTP in the LA) prior to conditioning did not affect normal levels of freezing at the end of the conditioning session yet did compromise a freezing response to a CS 24 h after conditioning suggests that LTP in the LA (putatively mediated by GluN2A receptors) contributes to the consolidation of fear memories.

It is interesting to note that the NVP-induced impairment in conditioned freezing on the 24 h recall test was observed only at the later stage of the testing period. NVP-treated rats showed normal freezing during the first CS presentation, but significantly less freezing with subsequent CS presentations relative to vehicle-treated controls. The accelerated extinction suggests that NVP did not abolish the acquisition of fear memory, but instead, influenced the stability of this memory making it more susceptible to modification by unreinforced CS presentations. This behavioral profile stands in contrast to CPP-treated rats that showed reduced freezing throughout the test session. While it is possible that a higher dose of NVP may disrupt freezing through-out the test session, it is important to note that systemic administration of this dose of NVP has been shown to significantly attenuate intra-hippocampal LTP *in vivo* (Fox et al., 2006). Unfortunately, the results from an experiment using a higher dose of NVP would be confounded by the lack of selectivity of this drug at higher doses (see Walker and Davis, 2008). Until a more selective means of blocking GluN2A receptor function becomes available this interpretation remains speculative.

The behavioral pattern observed in NVP-treated rats raises the possibility that other forms of plasticity, independent of GluN2A receptor activity also contribute to fear learning. Thus, changes in synaptic strengths mediated by GluN2A activation may not play as great a role in the formation of a novel fear memory, but instead help to be stabilize these associations rendering them more robust. Together, our combined neurophysiological and behavioral data suggest that GluN2A-mediated LTP is not only involved in the formation of a fear memory on its own, but more importantly, also in facilitating retention of memory that is recalled frequently.

In direct contrast to its effects on the acquisition of a fear response, NVP did not affect the recall of conditioned fear when administered prior to an extinction test session, nor did these treatments disrupt subsequent fear extinction. These data suggest that disrupting LTP with this GluN2A-preferring antagonist does not compromise the extinction of learned fear.

4.3. GluN2B receptors, LTD and the extinction of fear memories

Antagonism of GluN2B receptors with Ro25-6981 completely blocked amygdalar LTD. In addition, and in contrast to GluN2A receptor antagonism, blockade of GluN2B receptors disrupted fear extinction while leaving acquisition and expression intact. There is growing interest in the role that GluN2B receptors play in learning and memory formation, with a number of studies postulating that these receptors are involved in the acquisition of a fear response (e.g. Rodrigues et al., 2001). However, the lack of selectivity of the GluN2B receptor antagonist used in those studies (ifenprodil), which also has a high affinity for adrenergic receptors, makes interpretation of those data problematic. The GluN2B antagonist used here (Ro25-6981) is approximately 25-fold more potent than ifenprodil as an antagonist at GluN2B subunit-containing NMDA receptors (Fischer et al., 1997; see also Lynch et al., 2001, Mutel et al., 1998, Pinard et al., 2001).

In the present study, Ro25-6981 did not block the induction of amygdalar LTP, nor did it affect the acquisition of a learned fear

response. These findings are consistent with reports for other brain regions (hippocampus, Liu et al., 2004; perihinal cortex, Massey et al., 2004) and other fear conditioning paradigms (Mathur et al., 2009, Zhang et al., 2008). These results provide compelling evidence against a role for GluN2B receptor activation in memory formation and suggest that the effects of compounds such as ifenprodil and CPP on the acquisition of learned fear may not be attributed solely to GluN2B receptor activation.

Pre-treatment with Ro25-6981 had pronounced effects on both the induction of LTD and extinction of learned fear. The induction of LTD was completely blocked by Ro25-6981. Likewise, when Ro25-6981 was administered before testing, both within-session extinction of learned fear and the retrieval of learned extinction were significantly disrupted. Interestingly, rats treated with Ro25-6981 prior to extinction training did not show enhanced freezing during the recall test given 24 h later, consistent with the absence of effect of this compound on the consolidation of extinction memories. These effects of Ro25-6981 differ from those induced by broad-spectrum NMDA antagonists (e.g.; CPP) which do not disrupt within-session extinction but instead impair consolidation of these memories (Santini et al., 2001). The main difference in the effect of CPP vs. Ro25-6981 in the present study is that rats given Ro25-6981 prior to extinction training attain levels of extinction comparable to controls by end of the training session. This effect may reflect the clearance of Ro25-6981 from the brain. Thus the lack of effect of GluN2B- receptor blockade on consolidation of extinction memories may simply reflect the fact that rats eventually extinguished freezing, albeit at a substantially slower rate. This finding, in combination with previous studies, highlights the dissociable nature of mechanisms underlying acquisition, consolidation and retrieval of extinction memories (Santini et al., 2001).

The higher levels of freezing observed during extinction training and recall after treatment with Ro25-6981 does not appear to reflect a reduction in general activity as similar doses of Ro25-6981 do not affect locomotor activity in rodents (Dalton et al., 2011; Iijima et al., 2010; Tallaksen-Greene et al., 2010). Moreover, Ro25-6981 did not enhance freezing during fear conditioning in Experiment 2A (see Fig. 2C). In addition, rats given Ro25-6981 before conditioning did not differ from controls during the drug-free fear recall test. Likewise, rats that were conditioned drug-free and treated with Ro25-6981 prior to extinction training did not show disrupted recall of the fear memory at the beginning of the extinction session. This lack of effect on fear learning or recall of conditioned fear suggests that the effects of this compound on extinction learning and recall are unlikely to be attributable to non-specific state-dependent learning effects.

When LTD is prevented by blockade of GluN2B subunits as in the present study, or by inhibition of AMPA receptor endocytosis, using either systemic or intracerebral administration of an interference peptide, within-session extinction of a learned fear response is disrupted (Dalton et al., 2008; Kim et al., 2007). These findings are consistent with the hypothesis that GluN2B-mediated LTD plays a central role in response inhibition and behavioral flexibility. Higgins et al. (2003) reported increased premature responding on a differential reinforcement of low rate task following antagonism of GluN2B subunits. More recently, treatment with Ro25-6981 has been reported to disrupt reversal learning in a Morris water maze (Duffy et al., 2008) and to selectively disrupt strategy set-shifting while leaving initial cue-discrimination learning intact (Dalton et al., 2011). Thus, mounting evidence suggests that GluN2B receptor-mediated LTD plays a specific role in an animal's ability to adapt behavior in the face of changing environmental contingencies.

The lack of effect of Ro25-6981 on the acquisition of fear conditioning (experiment 2A) contrasts with recent reports that intra-amygdala infusion of either Ro25-6981 (Zhang et al., 2008) or the selective GluN2B antagonist CP101-606 (Walker and Davis, 2008) prior to conditioning impaired subsequent expression of conditioned fear. Although procedural differences both between these studies and our own make comparisons difficult, we did attempt to resolve this discrepancy with a higher dose of Ro25-6981 (12 mg/kg) given prior to conditioning, with recall tested 24 h later. Ro25-6981 again failed to impair either acquisition or expression of a Pavlovian fear response (Fig. 3D). Other methodological issues that may explain this discrepancy include conditioning strength (number of CS/US pairings as well as US intensity), the role of the particular fear paradigm used (fear potentiated startle vs conditioned freezing), the length of time between conditioning and retrieval test (48 vs 24 h) and the impact of separating contextual and cue-induced fear. Moreover, the unusual dose-response effect of CP101,606 reported by Walker and Davis (2008) suggests that this particular compound may be affecting other neurochemical systems at different doses.

The GluN2A/B mediated increases/decreases in synaptic strength within the amygdala reported here are likely mediated in part by alterations in AMPA receptor trafficking. Thus, expression of LTP is prevented by post-synaptic blockade of vesicle-mediated exocytosis whereas LTD is prevented by interference with AMPA receptor endocytosis (Yu et al., 2008). Likewise, extinction of conditioned fear is blocked by disruption of AMPA receptor endocytosis (Kim et al., 2007; Dalton et al., 2008). However, the possibility remains that acquisition and extinction of aversive memories mediated by GluN2A/B receptors may in turn modify activity of these receptors as well. Future studies investigating alterations in these NMDA receptor currents induced by acquisition and extinction of associative memories may provide further insight to this issue.

To summarize, the present study provides evidence that GluN2A receptor subunit activation is central to both the induction of LTP in the LA and the acquisition of a stable learned fear response, whereas activation of GluN2B receptor subunits is necessary for the induction of LTD and the suppression of previously-acquired Pavlovian responses in the face of changing contingencies. A better understanding of the mechanisms, and potential pharmacological targets involved in both the acquisition and suppression/extinction of learned fear responses may prove invaluable in attempts to devise novel treatments for pathological anxiety, OCD and the phobias (Myers et al., 2011).

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