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Amygdala infusions of an NR2B-selective or an NR2A-preferring NMDA receptor antagonist differentially influence fear conditioning and expression in the fear-potentiated startle test

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Within the amygdala, most N-methyl-D-aspartate (NMDA) receptors consist of NR1 subunits in combination with either NR2A or NR2B subunits. Because the particular subunit composition greatly influences the receptors’ properties, we investigated the contribution of both subtypes to fear conditioning and expression. To do so, we infused the NR1/NR2B receptor antagonist CP101,606 (0.5, 1.5, or 4.5 µg/amygdala) or the NR1/NR2A-preferring antagonist NVP-AAM077 (0.075, 0.25, 0.75, or 2.5 µg/amygdala) into the amygdala prior to either fear conditioning (i.e., light-shock pairings) or fear-potentiated startle testing. CP101,606 nonmonotonically disrupted fear conditioning but did not disrupt fear expression. NVP-AAM077 dose-dependently disrupted fear conditioning as well as fear expression. The results suggest that amygdala NR1/NR2B receptors play a special role in fear memory formation, whereas NR1/NR2A receptors participate more generally in synaptic transmission.

The N-methyl-D-aspartic acid (NMDA) receptor antagonist AP5 prevents fear conditioning when infused into the amygdala prior to light-shock (Miserecondino et al. 1990; Walker and Davis 2000), tone-shock (Campeau et al. 1992), or odor-shock (Walker et al. 2005) pairings. In the studies noted above, AP5 did not disrupt fear expression when infused prior to testing. These dissociations suggest a special role for amygdala NMDA receptors in fear memory formation (c.f., Walker and Davis 2002b) but have not been universally observed. Indeed, several groups have found that pretest intra-amygdala AP5 infusions do disrupt fear expression (Maren et al. 1996; Fendt 2001; Lee et al. 2001; Jasnow et al. 2004).

NMDA receptors are heterimeric complexes, requiring the presence of both NR1 and NR2 subunits (c.f., Cull-Candy et al. 2001; Prybyloowski and Wenthold 2004). There are several NR2 subtypes, of which two—NR2A and NR2B—occur in the amygdala (e.g., Monyer et al. 1992; Lopez de Armentia and Sah 2003). For several reasons, NR2B subunits have attracted special attention vis-à-vis learning. It has been shown, for example, that they confer to NMDA receptors relatively long-duration excitatory postsynaptic potentials (EPSPs) (e.g., Monyer et al. 1994; Vicini et al. 1998) that may be especially important for associative conditioning (c.f., Tsien 2000). They are also the dominant subtype during development—a time when neural mechanisms of plasticity are especially active (e.g., Laurie et al. 1997; Lopez de Armentia and Sah 2003; for review, see Loftis and Janowsky 2003). Interestingly, binding of the plasticity-associated enzyme calcium/calmodulin-dependent kinase II (CaMKII) to the NR2B subunit renders CaMKII constitutively active (Bayer et al. 2001), and recent results suggest that this interaction is a major determinant of the magnitude of hippocampal long-term potentiation (LTP) (Barria and Malinow 2005). Also, Shin et al. (2006) have reported that whereas the NR2A-preferring antagonist NVP-AAM077 (0.5 µM) produces a much greater inhibition of synaptic transmission in cortico-amygdala and thalamo-amygdala pathways than does the NR2B-selective antagonist ifenprodil (10 µM) (~75% and 25% reduction, respectively), only ifenprodil blocks spike-timing-dependent LTP in the same pathways. Given then what may be a special role for NR2B-containing receptors in synaptic plasticity, we wondered whether antagonists of these receptors might preferentially disrupt fear conditioning versus fear expression with greater reliability than has previously been found for nonselective NMDA receptor antagonists or for selective antagonists to other NMDA receptor subtypes.

Unfortunately, analyses of the relative contributions of these different NMDA receptor subtypes have been greatly hindered by the lack of subtype-selective antagonists. Importantly, Rodrigues et al. (2001) found that pretraining systemic or intraamygdala ifenprodil infusions disrupted fear conditioning to a tone (see also Blair et al. 2005) and also to a context conditioned stimulus (CS) but had no effect on conditioned freezing when infused prior to testing. More recently, similar effects of ifenprodil were reported for fear extinction learning (Sotres-Bayon et al. 2007). These results are particularly relevant because ifenprodil is highly selective for NR2B- versus NR2A-containing receptors (Williams 1993). However, ifenprodil also acts as an α1-adrenergic (e.g., Chenard et al. 1991) and 5-HT1A (McCool and Lovinger 1995) receptor antagonist, binds to 5-HT1A (Chenard et al. 1991) and 5-HT1D (Karbon et al. 1990; Chenard et al. 1991) receptors, and modestly reduces NMDA-evoked EPSCs even in mice lacking NR2B subunits (Tovar et al. 2000). Genetic manipulation techniques might allow for more selective targeting and, indeed, Tang et al. (1999) have reported that mutant mice overexpressing the NR2B subunit show increased conditioned freezing relative to wild-type controls and improved performance in several other learning tasks as well. More recently, Nakazawa et al. (2006) reported that knock-in mutations that interfere with NR2B phosphorylation also interfere with conditioned freezing (and disrupt amygdala LTP). Although these behavioral effects can be attributed with confidence to influences on NR2B function or number,
it is unclear whether they reflect primary influences on learning or performance, or increased expression within the amygdala.

The primary goals of this study then were threefold: first, to evaluate the contribution of amygdala NR1/NR2B receptors to fear conditioning and expression using the putatively more selective (i.e., versus ifenprodil) antagonist CP101,606 (Chenard et al. 1995; Chazot et al. 2002; Nagy et al. 2004); second, to assess the generality of the Rodrigues et al. (2001) finding by using a different CS modality (visual) and behavioral measure (fear-potentiated startle); and third, to evaluate for the first time the contribution of amygdala NR2A-containing receptors to fear memory formation and expression using preconditioning and pretest infusions of the NR1/NR2A-prefering antagonist NVP-AAM077 (Feng et al. 2004; Weitlauf et al. 2005; Neyton and Paoletti 2006).

For these studies, rats were chronically implanted with cannulae aimed at the amygdala. After a recovery period of 8–10 d, they received infusions of either CP101,606 or NVP-AAM077 prior to either fear conditioning or fear-potentiated startle testing (i.e., 2 × 2 design). Conditioning consisted of either 1 or 2 d of 10 light-shock pairings each. We typically train animals using 2 consecutive sessions because this produces very reliable conditioning. We included the 1-d conditioning protocol in this study because we were concerned that weak drug effects might go undetected in rats that received the stronger conditioning procedure.

Results

Cannulae placements

Placements for rats that met the inclusion criteria (i.e., both cannulae within 0.5 mm of the basolateral amygdala complex or central amygdala nucleus; N = 298) are shown in Figure 1.

CP101,606 effect on fear conditioning

CP101,606 disrupted fear conditioning when infused into the amygdala prior to each of either 1 or 2 conditioning sessions. ANOVA indicated a significant Dose effect, $F_{3,125} = 3.98$, $P = 0.009$, but not a significant effect of the number of training days, hence the data from animals that received 1 or 2 d of training are combined for the following analyses. As shown in Figure 2A, footshock reactions in the control group were significantly different from those in the CP101,606 group (Dunnett’s $t$ analysis indicated that footshock reactions in the control group were significantly different from those in the 1.5 mg/amygdala group ($P = 0.001$) but were not significantly different from those in the 4.5 mg/amygdala group ($P = 0.71$). Thus, the lower footshock reactions occurred in a group of rats that showed normal footshock reactivity (i.e., in rats that received vehicle or CP101,606 infusions prior to each of 2 conditioning sessions) as assessed with either Pearson’s (i.e., parametric; $P = 0.52$) or Spearman’s (i.e., nonparametric; $P = 0.36$) correlation analyses. Thus, the relatively modest differences in footshock reactivity were unrelated to and could not account for the effect of CP101,606 on fear conditioning.

CP101,606 effect on fear expression

CP101,606 administered prior to testing did not significantly influence fear-potentiated startle (Fig. 2B). Statistically, there was neither a significant Dose effect nor a significant effect of the
number of Training Days. Baseline startle amplitude was similarly unaffected (data not shown).

**NVP-AAM077 effect on fear conditioning**

NVP-AAM077 disrupted fear conditioning when infused into the amygdala prior to each of either 1 or 2 conditioning sessions (Fig. 2C). ANOVA indicated a significant effect of Dose, F(0.001) = 10.07, P < 0.001, but not of the number of Training days. Hence, the data for animals that received 1 or 2 conditioning sessions were combined for the following analyses. Post-hoc Dunnett’s t comparisons indicated significant differences between the control group and the groups that received 0.25 (P < 0.001) and 0.75 (P = 0.002) µg/amygdala, and a nearly significant difference versus rats that received 0.075 (P = 0.058) µg/amygdala. As with the CP101,606 data, the dose-response curve was nominally U-shaped, although this was less pronounced than for the CP101,606-infused rats. There were no significant effects on baseline startle amplitude or on footshock reactivity as assessed with parallel ANOVAs (data not shown).

**Discussion**

The primary findings are that intra-amygdala infusions of the NR1/NR2B receptor antagonist CP101,606 disrupted fear conditioning but not fear expression, whereas intra-amygdala infusions of the NR1/NR2A-prefering antagonist NVP-AAM077 disrupted both. The disruptive effect of pretraining CP101,606 infusions, as assessed with fear-potentiated startle, is similar to that of pretraining ifenprodil infusions as assessed with freezing (Rodrigues et al. 2001; Blair et al. 2005). Importantly, neither disrupted fear expression when infused prior to testing. Together with our observation that pretest NVP-AAM077 infusions did disrupt fear-potentiated startle, the results suggest that previously observed disruptions by pretest AP5 (Lee et al. 1988, 2001; Maren et al. 1996; Fendt 2001; Jasnow et al. 2004; Lindquist and Brown 2004)—a broad spectrum NMDA receptor antagonist (e.g., Buller et al. 1994)—are more likely to have resulted from NR1/NR2A rather than NR1/NR2B blockade. This view is compatible with recent behavioral observations that social defeat-induced anxiety in Syrian hamsters is prevented by predefeat intra-amygdala infusions of AP5 or ifenprodil but that only AP5 disrupts defeat-induced induced anxiety when infused prior to testing (Jasnow et al. 2004; D.E. Day, M.A. Cooper, and K.L. Huhman, unpubl.), and with recent electrophysiological findings that the induction of thalamo-amygdala LTP is also disrupted by AP5 and ifenprodil but that only AP5 disrupts synaptic transmission (Chapman and Bellavance 1992; Maren and Fanselow 1995; Bauer et al. 2002).

Most importantly, these results strongly suggest that amygdala NR2B-containing NMDA receptors participate directly in fear learning, rather than indirectly via an involvement in synaptic transmission. If the latter were the case, then CP101,606 would also be expected to disrupt fear-potentiated startle when infused prior to testing, as shown previously for other compounds, such as ifenprodil and AP5. However, neither disrupted fear-potentiated startle in rats given 2 d of training, we did not also examine its effect in rats trained with the weaker conditioning procedure.
as the AMPA receptor antagonists NBQX or CNQX, which do reduce excitatory neurotransmission (c.f., Walker and Davis 2002b).

Recent findings from Matus-Amat et al. (2007) suggest that nonspecific effects, unrelated to NMDA receptors, might also contribute to the effects on fear expression of intra-amygdala APS infusions. They found that L, D-AP5 (the most commonly used form of AP5) and L-AP5 both disrupted conditioned freezing when infused prior to testing but that D-AP5, at equimolar concentrations shown in the same study to disrupt fear potentiation, did not. Because the D but not the L isomer is an NMDA receptor antagonist, these results again suggest a special role for amygdala NMDA receptors in fear learning rather than expression. Given that pretest NVP-AAM077 infusions did disrupt fear-potentiated startle and that AP5 disrupts NR2A- and NR2B-subunit-containing receptors with nearly equal efficacy (Beaton et al. 1992; Priestley et al. 1995), it is unclear why disruptive effects of pretest AP5 have not been consistently observed. One possibility is that the disruptive effect of NR1/NR2A blockade is opposed by facilitatory effects of NR1/NR2B blockade. Indeed, we found in this study that fear-potentiated startle was markedly elevated in several groups that received pretest CP101,606 infusions (see Fig. 2B). Nonetheless, disruptive effects of intra-amygdala APS infusions have been observed in other studies, not only in other laboratories but also, more recently, in our own. For us, this change in the effect of AP5 infusions seems to have coincided with our move from Yale University to Emory University and was accompanied by several other, mostly inconsequential, changes in the behavioral responses of our animals as well. For example, the unconditioned effect of a brief light pulse, which had previously been slightly inhibitory, has now become slightly facilitatory (<20% in either direction), whereas the unconditioned increase in startle produced by a longer duration light has decreased in magnitude.

One source of interlaboratory variability may be variability in the composition of NMDA receptors themselves. Many studies have now shown that the ratio of NR2A to NR2B protein or message is dynamic—changing in response to LTP induction (Thomas et al. 1996; Williams et al. 1998), stress (Bartanusz et al. 1995), synaptic activity (Eihlers 2003), NMDA receptor blockade (Bartanusz et al. 1995; Fujisawa and Aoki 2003), development (Laurie et al. 1997; Lopez de Armentia and Sah 2003), and several other manipulations (Lu et al. 2005; Wu et al. 2005). Thus, differences in the relative proportions of NR2A to NR2B subunits in rats from different laboratories or suppliers or both may contribute to the variable effects on fear expression of nonselective NMDA receptor antagonists, and perhaps also to variable assessments of the contribution of NMDA receptors to synaptic transmission in amygdala pathways (Li et al. 1996; Mahanty and Sah 1999; Weisskopf and LeDoux 1999; Szinyei et al. 2003).

A surprising aspect of our results was the nonmonotonic dose-response curve observed with pretraining CP101,606 infusions (and to a lesser degree with NVP-AAM077). We are unaware of any precedent for this, although LaLumiere et al. (2004) did report that intra-amygdala APS infusions at a single dose either impaired or enhanced inhibitory avoidance retention depending on footshock intensity (see also Kojima et al. 2005 who found that systemic injections of ifenprodil reversed freezing deficits observed in Fyn-transgenic mice which show increased NR2B subunit phosphorylation). Although U- and inverted U-shaped dose-response curves are not uncommon in learning and memory research, their underlying cause remains largely uncertain. With respect to our data, there are several possible, albeit purely conjectural, possibilities. It may be relevant, for example, that NR2B receptor antagonists increase the affinity of glutamate agonists for NMDA receptors, even while simultaneously interfering with channel function (Mott et al. 1998) and that, at low agonist concentrations, the summed effect on NMDA-evoked currents is facilitatory (Kew et al. 1996; Zhang et al. 2000). Insofar as stimulation of presynaptic NMDA receptors, including NR1/NR2B receptors, increases glutamate release (Woodhall et al. 2001), it is possible that the ascending arm of the nonmonotonic curve reflects increased glutamate binding to presynaptic NR2B-containing receptors (assuming that these receptors normally see low concentrations of glutamate), which increases the release of glutamate, which would then be free to bind to postsynaptic NR2A-containing receptors and possibly compensate for the behavioral effect of blocking postsynaptic NR2B-containing receptors.

More generally, the overall effect on behavior of any pharmacological manipulation may reflect the summation of multiple effects on neural function—some of which may be opposite in sign and which may occur at different thresholds. For example, amygdala NR1/NR2B receptors mediate transmission not only onto excitatory projection neurons but also onto inhibitory interneurons (Danzer et al. 2000; Sah and Lopez De Armentia 2003; Szinyei et al. 2003). Amygdala NMDA receptors of indeterminate subtype are located not only postsynaptically but also presynaptically (Farb et al. 1995), where they are required for the induction of certain types of LTP (Humeau et al. 2003; Samson and Pare 2005) and may regulate neurotransmitter release as well. In other brain areas, and perhaps the amygdala also, NR2B-containing receptors participate not only in LTP but also in long-term depression (e.g., Liu et al. 2004; Massey et al. 2004; Fox et al. 2006; Bartlett et al. 2007). It is possible therefore that the disruptive effects of an intermediate dose of CP101,606 are attenuated or overcome at higher doses by facilitatory effects that occur via different mechanisms.

As previously indicated, we also examined the effect on fear conditioning of the NR1/NR2A-prefering antagonist NVP-AAM077. As with CP101,606, pretraining NVP-AAM077 infusions also disrupted conditioning. However, the finding that pretest NVP-AAM077 infusions disrupted fear expression as well suggests that NVP-AAM077 might influence fear conditioning simply by disrupting synaptic transmission. Interestingly, however, fear conditioning appeared to be somewhat more sensitive to NVP-AAM077 than fear expression. For instance, comparable effects (i.e., a reduction of fear-potentiated startle of ~50% rela-

![Figure 3](image-url)
Rats were trained and tested in four identical 8-20-cm (depth × width × height) polycarbonate cages, each suspended between compression springs within a steel frame located within a custom-designed sound-attenuating chamber. The floor of each cage consisted of four 6.0-mm diameter stainless steel bars spaced 18 mm apart.

Startle responses were evoked by 50-msec 95-dB white-noise bursts (5-msec rise-decay time, 0–22 kHz) generated by a Macintosh G3 computer sound file, amplified by a Radio Shack amplifier (Model MPA-200; Tandy), and delivered through Radio Shack Supertweeter speakers located 4 cm in front of each cage. Background noise (60-dB wideband) was produced by an ACO Pacific white-noise generator (Model 3024) and was delivered through the same speakers as those used to provide background noise.

Surgery
Rats were anesthetized with sodium pentobarbital (50 mg/kg i.p.), injected with atropine sulphate (10 mg/kg i.p) to reduce postoperative discomfort. Once unresponsive to tailpinch, rats were placed in a Kopf Instruments stereotaxic frame whereupon they received 10 light-shock pairings. The first 200 msec of the startle-eliciting white-noise burst. Footshock reactions were measured in the same way that noise-evoked startle responses were measured with the exception that the accelerometers’ output was sampled for 500 rather than 200 msec.

The presentation and sequencing of all stimuli were under the control of the Macintosh G3 computer using custom-designed software (The Experimenter; Glassbeads Inc.).
later, rats in different experiments received either 1 or 2 fear conditioning sessions. Twenty-four hours later, rats that were to receive pretest infusions received a brief drug-free pretest. The data from this test were used to assign rats to different treatment groups such that the mean level of fear-potentiated startle, prior to drug treatment, was comparable in each. For this test, rats were placed into the test chamber where, after 5 min, they received 20 startle-eliciting noise bursts (ISI = 30 sec). Five of the final 10 noise bursts were presented 3.2 sec after onset of the visual CS (light-noise trial type) and the other 5 were presented in the absence of the CS (i.e., noise alone trial type). Using these final 10 startle responses, a fear-potentiated startle score was calculated for each rat as described in the “Statistical Analyses” section. For each rat, the mean startle amplitude on noise alone and on light-noise test trials (i.e., the 30 light-noise and 30 noise alone trials of the full 60-trial test session) was calculated (i.e., intermixed) noise alone test trials—a period during which fear-potentiated startle scores showed no systematic change.

The scores for all animals were analyzed using ANOVA with Dose and Training (i.e., 1 versus 2 conditioning sessions) as between-group factors. Separate ANOVAs were performed for the effect of CP101,606 and for NVP-AAM077 using data from the same PBS-infused animals as a control. Post-hoc comparisons were made using Dunnett’s t-tests (two-tailed) for multiple comparisons with a control. Also, single-sample t-tests were used to determine if the fear-potentiated startle scores for any individual group were significantly different from 0% potentiation. Similar analyses were performed on baseline startle amplitude (i.e., from the noise alone test trials), and also on the rats’ footshock reactions. Inferential statistics were performed using SPSS software (version 13.0.0; SPSS, Inc., Chicago, IL).

**Histology**

Rats were sacrificed by chloral hydrate overdose and perfused intracardially with 0.9% (wt/vol) saline followed by 10% (vol/vol) formalin. The brains were removed and immersed in a 30% (wt/vol) sucrose-formalin solution for at least 3 d, after which 40-µm coronal sections were cut through the area of interest. Every fourth section was mounted and stained with cresyl violet. Cannulae placements and the determination as to whether the cannula was within or sufficiently near the intended target to be scored as a hit (within 0.5 mm of the central amygdala nucleus or in the lateral amygdala complex but not in a ventricle and not lateral to an intact external capsule) were judged by a scorer blind to the animal’s group assignment and behavioral data.

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**References**


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