

Distinct Populations of NMDA Receptors at Subcortical and Cortical Inputs to Principal Cells of the Lateral Amygdala

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Weisskopf, Marc G. and Joseph E. LeDoux. Distinct populations of NMDA receptors at subcortical and cortical inputs to principal cells of the lateral amygdala. *J. Neurophysiol.* 81: 930–934, 1999. Fear conditioning involves the transmission of sensory stimuli to the amygdala from the thalamus and cortex. These input synapses are prime candidates for sites of plasticity critical to the learning in fear conditioning. Because *N*-methyl-D-aspartate (NMDA)-dependent mechanisms have been implicated in fear learning, we investigated the contribution of NMDA receptors to synaptic transmission at putative cortical and thalamic inputs using visualized whole cell recording in amygdala brain slices. Whereas NMDA receptors are present at both of these pathways, differences were observed. First, the α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid-receptor-mediated component of the synaptic response, relative to the NMDA component, is smaller at thalamic than cortical input synapses. Second, thalamic NMDA responses are more sensitive to Mg^{2+} . These findings suggest that there are distinct populations of NMDA receptors at cortical and thalamic inputs to the lateral amygdala. Differences such as these might underlie unique contributions of the two pathways to fear conditioning.

INTRODUCTION

The amygdala is an essential component of the neural system involved in fear conditioning (Davis 1992; Kapp et al. 1992; LeDoux 1995; Maren and Fanselow 1996). During fear learning, the acoustic conditioned stimulus (CS) reaches the amygdala by way of inputs from both the auditory thalamus and cortex (LeDoux et al. 1990a,b, 1991; Romanski and LeDoux 1993). These two pathways converge onto single cells in the lateral nucleus of the amygdala (LA) (Li et al. 1996b), and the synapses formed by these undergo plasticity during fear conditioning (McKernan and Shinnick-Gallagher 1997; Quirk et al. 1995; Rogan et al. 1997). Much of what we know about synaptic processes in these pathways has been obtained from in vivo recording studies (e.g., Clugnet and LeDoux 1990; Clugnet et al. 1990; Li et al. 1995, 1996a,b). However, in vivo recordings are limited in the detail with which they can characterize synaptic transmission, and there are obvious advantages to pursuing such issues using an in vitro brain slice preparation.

In the present study, we performed whole cell recordings from principal cells in LA using differential interference contrast (DIC) optics to visualize the cells. The transmission properties of LA synapses activated by stimulation of two pathways that carry signals to LA were compared. Stimulation

sites were based on the results of tract tracing studies that show the trajectory of fibers from the auditory thalamus and cortex (LeDoux et al. 1990b; Romanski and LeDoux 1993). Specifically, thalamic fibers enter LA medially from the internal capsule, whereas cortical fibers enter laterally from the external capsule. By placing stimulating electrodes in the external capsule and in the striatum just medial to the internal capsule and just dorsal to the central nucleus of the amygdala, we targeted the path of fibers originating in the auditory cortex and thalamus, respectively. We cannot rule out a contribution of other fibers in these stimulation sites. However, findings to be described regarding the contribution of NMDA receptors to transmission in the two pathways are consistent with in vivo results based on directly stimulating the auditory cortex and auditory thalamus. In this paper, we therefore refer to the medial and lateral stimulation sites in terms of thalamic and cortical afferent pathways.

METHODS

Male Sprague-Dawley rats (3–5 wk) were anesthetized with halothane, and the brains were removed and transferred to ice cold artificial cerebrospinal fluid (ACSF). The ACSF contained (in mM) 119 NaCl, 2.5 KCl, 1 $MgSO_4$, 2 $CaCl_2$, 26 $NaHCO_3$, 1 NaH_2PO_4 , 5 lactic acid, and 10 glucose and was equilibrated with 95% O_2 –5% CO_2 . Unless otherwise noted, either 100 μM picrotoxin or 30 μM bicuculline was added to the ACSF. Coronal slices (400 μm), including the amygdala, were cut, and the cortex overlying the amygdala cut away with a scalpel to avoid cortical epileptic burst discharges. Slices were placed in a holding chamber at 32–34°C for 0.5 h and then at room temperature for at least another 0.5 h before recording. For recording, slices were transferred to a superfusion (1.5–2.5 ml/min flow rate) chamber (Warner Instruments) at room temperature and stabilized beneath a nylon net stretched over platinum wire. An upright microscope equipped with infrared differential interference contrast optics (IR-DIC, Olympus) was used to visualize cells. Voltage-clamp electrodes were filled with (in mM) 140 Cs-gluconate, 10 Cs-*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), 0.2 ethylene glycol-bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 2 Mg -ATP, 0.3 Na_3 -GTP, 8 NaCl, and 0.25–0.5% biocytin (pH 7.3, 290–300 mOsm). Current-clamp electrodes were filled with (in mM) 130 K-gluconate, 0.6 EGTA, 2 $MgCl_2$, 5 KCl, 10 HEPES, 2 Mg -ATP, 0.3 Na_3 -GTP, and 0.25–0.5% biocytin (pH 7.3, 290–300 mOsm). Membrane potentials recorded with voltage-clamp and current-clamp solutions were adjusted for 15- and 12-mV junction potentials, respectively. Electrodes had resistances of 4–8 $M\Omega$.

Bipolar stainless steel stimulating electrodes (Frederick Haer) were placed in such a way as to stimulate thalamic and cortical inputs to LA (Fig. 1A). Recordings were made using an AxoClamp 2B amplifier (Axon Instruments). Signals were filtered at 3 kHz and digitized at 5 kHz with a National Instruments A/D board. Data were stored and

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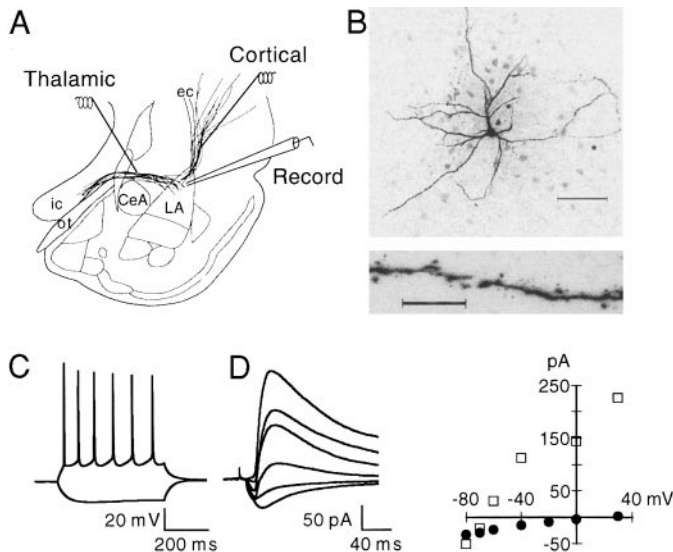


FIG. 1. Recordings in lateral nucleus of the amygdala (LA). *A*: schematic of stimulating and recording electrode placement. Thalamic fibers are depicted arriving medially and cortical afferents arriving laterally. CeA, central nucleus of the amygdala; ec, external capsule; ic, internal capsule; ot, optic tract. *B*: confocal image of a biocytin-filled cell recorded in LA. Scale bar is 100 μ m. A portion of the dendrite (*) is shown at higher magnification below. Note the presence of dendritic spines. Scale bar is 10 μ m. *C*: response of an LA cell to current injection of -0.17 and $+0.2$ nA. This cell had a resistance of 105 M Ω and a time constant of 32 ms. *D*: synaptic transmission into LA in the absence of γ -aminobutyric acid-A ($GABA_A$) antagonists. *Left*: responses of a neuron held at a series of membrane potentials between -80 and $+30$ mV to stimulation of thalamic afferents are shown. Traces are averages of 10 responses. *Right*: resulting current-voltage plot from the peak of the inward (●) and outward (□) current.

analyzed using software written with LabVIEW (National Instruments). Baseline responses were monitored at 0.05 Hz. Series resistance was monitored throughout experiments, and if it changed by $>15\%$, the data were discarded.

After recordings, slices were transferred to a 4% paraformaldehyde solution. Biocytin-filled cells were reacted for fluorescence by rinsing in phosphate-buffered saline (PBS) and then incubating in PBS with 0.2% Triton X-100 and avidin-Texas red (Vector) at 1:250 for ≥ 1 h at room temperature while shaking. Slices then were rinsed again with PBS, mounted on slides, and coverslipped with Permafluor (Lipshaw) for visualization under a fluorescent microscope. Reconstructions of some cells were done with a confocal scanning laser microscope (Zeiss).

Values are expressed as means \pm SE except where indicated. Drugs were applied by adding them to the superfusing ACSF. Drugs used were 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), D-2-amino-5-phosphonopentanoic acid (D-APV), bicuculline methiodide (Research Biochemicals International), and picrotoxin (Sigma). Animals were handled in accordance with National Institutes of Health guidelines.

RESULTS

Our recordings targeted the larger, presumably excitatory cells in LA. These cells had pyramid-like shapes, and their dendrites were covered with spines as is typical of excitatory projection neurons in LA (Lang and Paré 1998; McDonald 1992; Millhouse and DeOlmos 1983; Paré et al. 1995; Rainnie et al. 1993; Sugita et al. 1993; Washburn and Moises 1992) (Fig. 1*B*). Responses of LA cells to current pulses were examined in 85 cells. These showed various degrees of spike frequency adaptation (Fig. 1*C*), also typical of excitatory cells in

the amygdala (Paré et al. 1995; Rainnie et al. 1993; Washburn and Moises 1992). The average resting membrane potential, input resistance, and membrane time constant of the recorded cells were -69.6 ± 4.0 (SD) mV, 220 ± 80.9 (SD) M Ω , and 36.4 ± 10.0 (SD) ms, respectively. At negative holding potentials in the absence of γ -aminobutyric acid-A ($GABA_A$) antagonists, stimulation of either the thalamic or cortical input induced an inward postsynaptic current in isolation or an inward current followed by a slower outward current. An example is illustrated in Fig. 1*D*, where holding at a series of negative potentials showed that the outward current reversed at -61.7 mV, close to the chloride reversal potential, whereas the reversal for the inward current was near 0, typical of an excitatory synaptic current.

The non-NMDA [α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)] receptor antagonist CNQX (10 μ M) significantly reduced synaptic potentials recorded in current-clamp mode at resting membrane potentials and below elicited by stimulation of either thalamic or cortical fibers (Fig. 2*A*). However, a residual response sometimes remained in both pathways. These were blocked effectively by the NMDA-receptor antagonist D-APV (25 μ M) (Fig. 2*A*), indicating that NMDA receptors can contribute to synaptic responses in the amygdala even at these negative membrane potentials as also has been seen by others (Danover and Pape 1998).

In voltage clamp at negative holding potentials, cortically activated currents ($n = 19$) had a mean 10–90% rise time of 2.9 ± 1.3 (SD) ms and mean decay time constant of 11.3 ± 3.9 (SD) ms. Thalamically activated currents ($n = 20$) were similar [10–90% rise: 3.0 ± 1.1 (SD) ms; decay time constant: 11.9 ± 4.2 (SD) ms]. The current-voltage (I - V) relation for the peak of this response was linear and reversed near 0 mV (cortical: -0.1 ± 3.0 mV, $n = 9$; thalamic: 1.8 ± 3.6 mV, $n = 11$; Fig. 2*B*). At depolarized membrane potentials, a slower component to the synaptic current with a region of negative slope conductance between about -20 and -60 mV was seen at both inputs (Fig. 2*B*). The fast component of both synaptic responses was blocked by CNQX (10 μ M) and the slow component by D-APV (25 μ M), confirming that they were AMPA- and NMDA-mediated responses, respectively (Fig. 3*A*). There was no case at either synaptic input in which an NMDA-mediated response in voltage clamp was tested for and not found (cortical: $n = 28$; thalamic: $n = 29$). The kinetics of NMDA responses were similar at cortical and thalamic synapses [cortical, $n = 18$: 10–90% rise, 10.7 ± 3.3 (SD) ms; decay constant, 115 ± 37 (SD) ms; thalamic, $n = 16$: 10–90% rise, 11.1 ± 2.6 (SD) ms; decay constant, 125 ± 44 (SD) ms].

To determine the relative contribution of NMDA receptors at the two synapses, we recorded both cortical and thalamic excitatory synaptic responses onto the same cell. Cells were held at negative potentials to examine the AMPA component, and subsequently the NMDA component was examined in the presence of CNQX at positive potentials in the linear portion of the NMDA I - V curve. The ratio of these two components then could be compared at the two pathways. Because differences in electrotonic distance of synapses from the soma can differentially affect fast and slow currents (Spruston et al. 1993), we minimized such effects by including cesium in the patch electrode to reduce potassium currents and increase the space constant of the cell. The average 10–90% rise times were 3.3 ± 0.8 ms and 2.9 ± 0.5 ms, and the decay times were

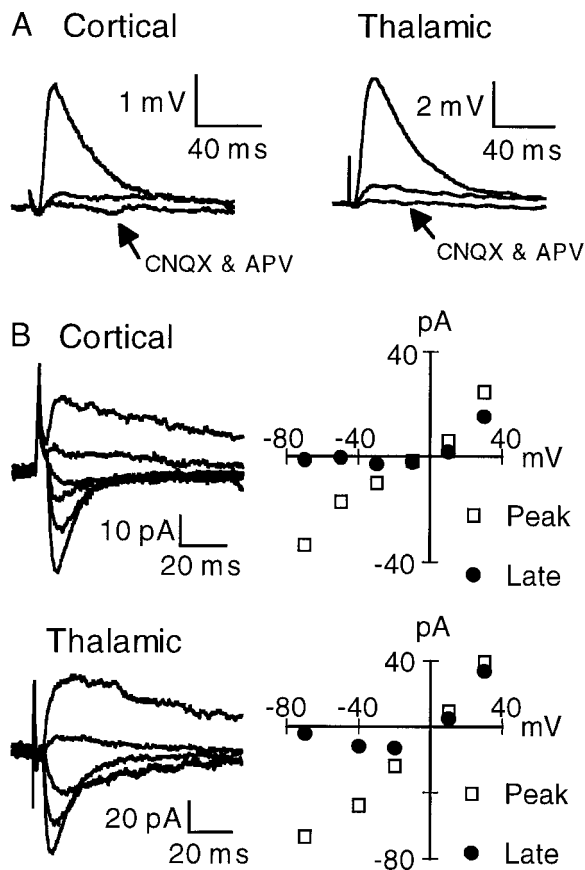


FIG. 2. Cortical and thalamic synaptic responses are mediated by α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and *N*-methyl-D-aspartate (NMDA) receptors. *A*: excitatory postsynaptic potentials (EPSPs) recorded in normal bath solution at -73 mV are superimposed on the reduced response remaining after addition of $10 \mu\text{M}$ 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX). This remaining response is abolished by subsequent addition of $25 \mu\text{M}$ D-2-amino-5-phosphonopentanoic acid (D-APV; CNQX & APV, \leftarrow). *B*: responses of 2 different cells to either cortical or thalamic stimulation at a series of holding potentials from -70 to $+30$ mV. *Right*: current-voltage plots from the peak of the fast (\square) and slow (measured 60 ms later; \bullet) components of the responses. Traces are averages of 10 responses.

11.5 ± 2.1 ms and 12.2 ± 3.0 ms for thalamic and cortical responses, respectively. The differences between pathways could be examined within individual cells, and they were not significant ($P \gg 0.05$, paired t -test, $n = 8$), suggesting that electrotonic distances were similar. The response amplitudes were 39.7 ± 15.6 pA and 37.1 ± 15.1 pA and latencies to peak were 11.4 ± 0.9 ms and 10.8 ± 0.8 ms at thalamic and cortical synapses, respectively, and these too, compared within cells, were not statistically different ($P \gg 0.05$, paired t -test, $n = 8$). To further guard against confounding by possible electrotonic distance differences, we calculated the AMPA to NMDA ratios using the total charge transfer of the synaptic responses by integrating over the excitatory postsynaptic currents (EPSCs); this is less sensitive to electrotonic distance than response amplitude (Bekkers and Stevens 1996). Figure 3, *A* and *B*, illustrates that the AMPA-to-NMDA ratio was significantly greater at cortical synapses than thalamic, suggesting a greater relative contribution of NMDA receptors at the thalamic synapses. However, distinct populations of NMDA receptors with different conductance levels also could contribute to this finding.

NMDA receptors expressing the NR2A or NR2B subunits have larger single-channel conductances than receptors expressing the NR2C or NR2D subunits (Stern et al. 1992; Wyllie et al. 1996), and the strength of the voltage-dependent block by Mg^{2+} is greater at receptors with the NR2A and NR2B subunits as well (Monyer et al. 1992, 1994). Therefore, we tested whether NMDA responses at cortical and thalamic synapses exhibit different sensitivity to Mg^{2+} . Cortical and thalamic NMDA synaptic responses were recorded in the same cell at -45 or -55 mV in the presence of CNQX ($10 \mu\text{M}$). Responses in control ACSF (1 mM Mg^{2+} ; thalamic: 10.4 ± 3.2 pA, cortical: 13.5 ± 4.7 pA) were compared with responses

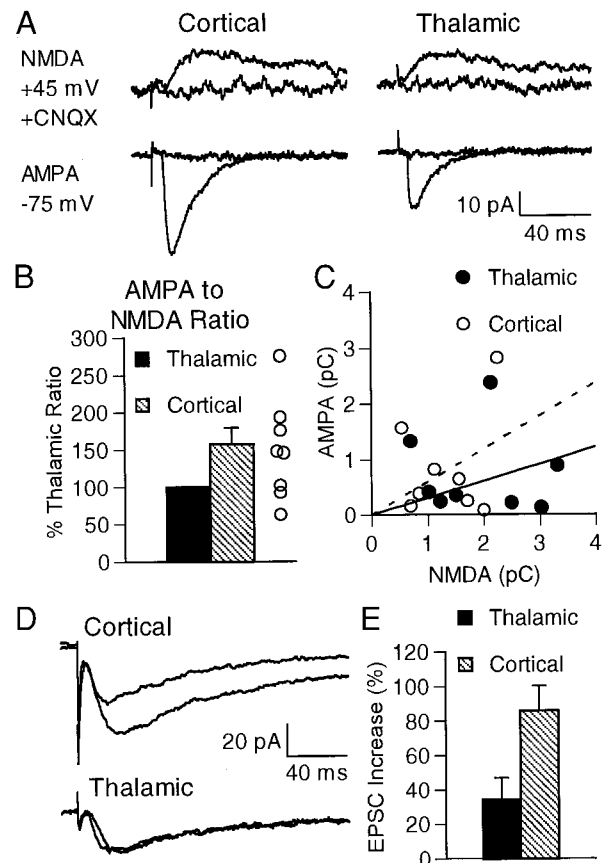


FIG. 3. Distinct populations of receptors at cortical and thalamic synapses. *A*: cortical and thalamic synaptic responses were monitored in the same neuron. AMPA component is seen at a holding potential of -75 mV where the NMDA receptors are blocked by Mg^{2+} . Responses after adding $10 \mu\text{M}$ CNQX to the bath are superimposed. In CNQX, the NMDA component is seen by holding at $+45$ mV to relieve the Mg^{2+} block. Responses after adding $25 \mu\text{M}$ D-APV are superimposed. AMPA to NMDA ratio is larger at the cortical synapse. *B*: summary of 8 similar experiments measuring the ratio of AMPA-to-NMDA response charge transfer. The cortical AMPA-to-NMDA ratio was $57.4 \pm 22.4\%$ greater than at thalamic synapses ($P < 0.05$, paired t -test; \circ , individual experiment results). *C*: AMPA total charge transfer (in picocoulombs, pC) is plotted against the NMDA charge transfer for the individual cortical (\bullet) and thalamic (\circ) cases. —, best fit to the cortical cases; - - -, best fit to the thalamic cases. Slope of the cortical best fit is greater than that of the thalamic, indicating a larger AMPA-to-NMDA ratio. *D*: sample cortical and thalamic synaptic NMDA responses evoked in another cell at -45 mV in the presence of $10 \mu\text{M}$ CNQX. When the external Mg^{2+} was switched from 1 to 0.1 mM , the cortical response increased more than the thalamic. *E*: summary from 6 cells of the increase in NMDA excitatory postsynaptic current on switching to 0.1 mM Mg^{2+} . For within-cell comparisons, on average the cortical response increased $41.7 \pm 14.7\%$ more than the thalamic ($P < 0.05$, paired t -test). All traces are averages of 10 responses.

in 0.1 mM external Mg^{2+} (thalamic: 15.4 ± 4.2 pA, cortical: 24.4 ± 6.0 pA; Fig. 3C). The increase in the NMDA response in 0.1 mM Mg^{2+} compared with 1 mM Mg^{2+} was significantly greater in the cortical than in the thalamic pathway, indicating that the thalamic responses were more sensitive to blockade by Mg^{2+} (Fig. 3D).

DISCUSSION

Although the auditory thalamus was not included within the amygdala brain slice and only the rostral-most part of the auditory cortex remained, it was possible, on the basis of tract tracing findings (LeDoux et al. 1990b; Romanski and LeDoux 1993), to stimulate regions in the slice through which thalamic and cortical fibers traversed. Specifically, thalamic fibers en route to LA course medially, entering LA from the internal capsule, whereas cortical fibers come in laterally, descending from the external capsule (see Fig. 1). However, these stimulation sites may well have also activated other fibers, for example, external capsule stimulation could antidromically activate collaterals of amygdalocortical projections. The fact that physiological differences exist between the two implies that two different sets were stimulated. Furthermore, these physiological differences are consistent with findings obtained from in vivo studies where the electrodes actually were placed in the auditory thalamus and cortex (Li et al. 1996b). These data suggest that we at least in part activated thalamic and cortical auditory input fibers in vitro even though other fibers may have contributed to the responses.

The kinetics of the AMPA EPSCs we observed in these putative thalamic and cortical input pathways to the amygdala are similar to those found in the hippocampus (Hestrin et al. 1990). This component did not show rectification, suggesting that these receptors are not permeable to calcium (Hollmann et al. 1991; Washburn et al. 1997). Although we found that NMDA and AMPA receptors contribute to synaptic responses in both pathways, the AMPA-to-NMDA ratio of EPSCs on average was smaller at thalamic than cortical input synapses. The similar response kinetics in our comparison experiments suggests that differences in electrotonic distance from the soma did not contribute to the observed differences between the two pathways. However, the actual dendritic location of the synapses of these two pathways is not known. If differences do exist in synaptic location along the dendrites, this could contribute in vivo to an augmentation or diminution—depending on the direction of the difference—of the effects we report here. The simplest explanation for these results is that these input synapses to LA actually express different ratios of NMDA and AMPA receptors. However, the thalamic NMDA responses are more sensitive to Mg^{2+} than cortical responses, suggesting distinct populations of NMDA receptors at these two synapses. By analogy with NMDA receptor properties seen in recombinant expression systems (Monyer et al. 1992, 1994), this would suggest that NMDA receptors at thalamic inputs contain either the NR2A or NR2B subunit, whereas at the cortical input they contain the NR2C or NR2D subunit. In this case, the thalamic NMDA receptors also would have greater single-channel conductances than cortical (Stern et al. 1992; Wyllie et al. 1996). Thus it is also possible that the receptor distributions are similar, but the thalamic NMDA response is greater because the population of NMDA channels

at this synapse pass more current. Distinct populations of NMDA receptors within single cells have been reported (Gottmann et al. 1997; Kew et al. 1998), and, indeed, in culture the type of presynaptic input can dictate the subunit composition (Gottmann et al. 1997). It also has been shown that NMDA receptors expressing both NR2A and NR2B subunits are expressed in the brain (Luo et al. 1997; Sheng et al. 1994). However, the physiological characteristics of these receptors are not known, therefore it cannot be determined whether they play a role in the differences seen in the present results.

The presence of NMDA receptor transmission in both input pathways to the LA studied here even at resting membrane potentials adds to concerns (Li et al. 1995, 1996b; Maren et al. 1996) about behavioral studies showing that infusion of an antagonist of NMDA receptors into the lateral and basal amygdala interferes with the acquisition of fear conditioning (Gewirtz and Davis 1997; Maren et al. 1996; Miserendino et al. 1990). The possibility of effects on routine transmission must be considered, particularly given that APV infusion in the amygdala has been found to block the expression of fear (Lee and Kim 1998; Maren et al. 1996).

It is intriguing to speculate that the differences seen here between cortical and thalamic NMDA responses may lead to different contributions to plasticity and fear conditioning. In the rodent sensory cortex, long-term potentiation (LTP) susceptibility closely parallels the critical period for behavioral modification of the whisker representation (Crair and Malenka 1995). Furthermore, the thalamocortical synapses show a decrease in NMDA receptor-mediated synaptic currents with age that is accompanied by a reduced capacity to generate LTP (Crair and Malenka 1995). By extension, if a similar situation exists in the amygdala, the reduced NMDA-receptor-mediated synaptic current at cortical compared with thalamic inputs might suggest that the cortical input would show a lesser capacity for LTP than the thalamic input. This issue could be addressed in LTP experiments either in vitro or in vivo.

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