NR2A \(^{-/-}\) Mice Lack Long-Term Potentiation But Retain NMDA Receptor and L-Type Ca\(^{2+}\) Channel-Dependent Long-Term Depression in the Juvenile Superior Colliculus

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Whether the subunit composition of NMDA receptors (NMDARs) controls the direction of long-term plasticity is currently disputed. In the visual layers of NR2A \(^{-/-}\) juvenile superior colliculus (SC), synapses lose miniature NMDAR currents, leaving NR2B-rich receptors in extrasynaptic regions. Compared with wild type (WT), evoked NMDAR currents in mutant neurons have slower rise and decay times and lower NMDAR/AMPAR current ratios. Moreover, NMDAR and L-type Ca\(^{2+}\) are missing in LTP, whereas extrasynaptic or NR2B-rich NMDARs are necessary for LLF-LTD. However, synaptic NMDARs as well as the NR2A subunit are missing in NR2A \(^{-/-}\) mice. Therefore, NR2 subunit-specific ligand binding/channel properties and/or separate signaling pathways interacting with NMDARs at synaptic versus extrasynaptic receptors could underlie these results.

Key words: NR2A \(^{-/-}\); synaptic NMDAR; extrasynaptic NMDAR; LTP; LTD; L-type Ca\(^{2+}\) channel

Introduction
NMDA receptor (NMDAR) activity can strengthen or weaken brain synapses, but whether current through NR2A- or NR2B-subunit-containing NMDARs dictates long-term potentiation (LTP) versus depression (LTD) is subject to much debate (Kohr, 2006). The visual cortex of NR2A \(^{-/-}\) mice are reported to show reduced LTP in the hippocampus (Sakimura et al., 1995; Ito et al., 1996; Kiyama et al., 1998) but not in barrel cortex or the nucleus of the stria terminals (Lu et al., 2001a; Weitlauf et al., 2005). The visual cortex of NR2A \(^{-/-}\) mice shows LTP of field EPSPs but lacks both metaplasticity (Phlipot et al., 2007) and orientation tuning (Fagiolini et al., 2003). In hippocampal CA1, the NR2A antagonist NVP-AAM077 ((\((\text{R})\)-(\((\text{S})\)-1-(4-bromo-phenyl)-ethylamino)\)-(2,3-dioxo-1,2,3,4-tetrahydroquinolin-5-yl)-methyl]-phosphonic acid) appears to block LTP, whereas NR2B blockade disrupts LTD (Liu et al., 2004). However, in this same region, calcium/calmodulin-dependent kinase II binding to the NR2B tail appears critical to LTD (Barria and Malinow, 2005).

The distribution of NMDARs is also important to their ability to induce LTP versus LTD. NR2A-rich receptors concentrate at subsynaptic sites, whereas NR2B-rich receptors are prominent in perisynaptic or extrasynaptic regions (Tovar and Westbrook, 1999; van Zundert et al., 2004; Thomas et al., 2006). Activation of subsynaptic NMDARs in hippocampal cultures induces LTP, whereas activation of extrasynaptic NMDARs produces LTD (Lu et al., 2001b). Pairing low-frequency stimulation with postsynaptic depolarization produces hippocampal LTP regardless of NR2A or NR2B activation (Berberich et al., 2005). However, pairing synaptic stimulation with pulses of depolarizing current is also reported to produce LTP even when NMDARs are blocked with AP-5 (Kullman et al., 1992).

In visual superior colliculus (SC) synapses of the NR2A \(^{-/-}\) mouse, miniature NMDAR (mNMDAR) currents disappear before eye opening [postnatal day 13 (P13)], whereas extrasynaptic NR2B-NMDARs remain and mediate evoked NMDAR currents with prolonged rise times (Townsend et al., 2003). Mutant mice lacking the NR2A cytoplasmic domain show a similar decrease in synaptic NMDARs in hippocampal CA1 (Steigerwald et al., 2000). The NR2A cytoplasmic domain therefore appears necessary to bind NMDARs to the synapse, and their loss from that position in NR2A \(^{-/-}\) mutants is consistent with the relatively poor binding of the remaining NR2B cytoplasmic tail to the mature scaffold postsynaptic density-95 (PSD-95) (Townsend et al., 2003). In rat SC, L-type Ca\(^{2+}\) channels and NMDARs act synergistically to induce LTP (Zhao et al., 2006). Here we show that juvenile NR2A \(^{-/-}\) mice (P15–P17) lack synaptic NMDARs and show no mNMDAR current in both WT and NR2A \(^{-/-}\) neurons. The data suggest that synaptic NMDARs are critical to SC...
LTP, whereas extrasynaptic NMDARs are critical to LLF-LTD but they cannot fully address the question of NMDAR subunit specificity in these responses.

Materials and Methods

Slice preparation. Wild-type (WT) C57BL/6 mice and NR2A−/− mice (Sakimura et al., 1995) (a gift from M. Mishina, University of Tokyo School of Medicine, Tokyo, Japan) with a C57BL/6 background were bred and maintained in Massachusetts Institute of Technology facilities. All animal procedures were in accord with approved Massachusetts Institute of Technology Animal Care and Use Committee protocols. Parasagittal SC slices were prepared from these mice at P15–P17 as described previously (Zhao et al., 2006).

Electrophysiology. All recordings used whole-cell patch clamping from narrow field vertical neurons in the stratum griseum superficiale of the SC. When plasticity was examined the artificial CSF (ACSF) and pipette solutions were as in the study of Zhao et al. (2006). For miniature and evoked current recording the pipette solution contained the following (in mM): 122.5 Cs-glucuronate, 17.5 CsCl, 10 HEPES, 0.2 NaEGTA, 4 ATP-Mg, 0.4 GTP-Na, and 8 NaCl, pH adjusted to 7.3 with CsOH.

Miniature EPSCs (mEPSCs) were recorded at −70 mV in the presence of 1 µM tetrodotoxin (Sigma, St. Louis, MO), 10 µM bicuculline methiodide (BMI) (GABA_A receptor antagonist; Sigma), 5 µM glycine, and 0 mM Mg2+. All mEPSCs more than two times baseline noise (2.5 pA) in a 1–3 min period (≥360 events) were averaged.

Evoked AMPAR and NMDAR currents (eAMPARcs and eNMDARcs) were obtained at −70 and +40 mV, respectively, with 20 µM BMI. eNMDARcs were recorded after eAMPARcs with 20 µM 2,3-dihydroxy-6-nitro-7-sulfonfyl-benzof[1]quinoxaline (NBQX) (selective AMPA/kainate receptor antagonist; Tocris Bioscience, Ballwin, MO). Constant current stimuli (0.05 Hz, 0.2 ms duration) were delivered through a bipolar tungsten electrode in the stratum opticum of SC slices.

Decay time (90–37% peak amplitude for minis and 0.37 peak amplitude for evoked events), rise time (10–90% peak amplitude), and amplitude of averaged mEPSCs, eAMPARcs, and eNMDARcs were measured with Mini Analysis (Synaptosoft, Decatur, GA). Stimulation intensity for evoked currents was defined relative to the threshold for inducing a response. Paired-pulse ratios (PPRs) used 100 ms separation and were calculated as second eAMPARc divided by the first eAMPARc (Zhao et al., 2006).

Results

Juvenile NR2A−/− SC neurons lose mNMDARcs
Previous work documented loss of mNMDARcs in the NR2A−/− SC by P13 (Townsend et al., 2003). This difference from WT was retained in the P15–P17 pups used here. We recorded mEPSCs...
from WT and NR2A\(^{-/-}\) neurons in the absence and presence of 50 µM AP-5 (Fig. 1A). In WT, the ratios of decay time with AP-5 without AP-5 were significantly lower than in NR2A\(^{-/-}\) (Fig. 1B), indicating a significant contribution of mNMDARcs in WT compared with NR2A\(^{-/-}\). Neither mEPSC rise time (Fig. 1C) nor amplitude ratios (Fig. 1D) differed between the two strains.

**Different evoked NMDAR/AMPAR current ratios between NR2A\(^{-/-}\) and WT neurons**

Townsend et al. (2003) also demonstrated at P11–P13 slower rise and decay time eNMDARcs in NR2A\(^{-/-}\) SC neurons compared with WT, with no difference in AMPARc rise or decay times. We corroborated this after eye opening [eNMDARc rise times in NR2A knock-out NR2AKO\(^{-/-}\) slower than eNMDARc rise times in WT, \(p < 0.001\); eAMPARc rise times in NR2A\(^{-/-}\) not different from eAMPARcs in WT, \(p = 0.66\); recorded at +40 mV with NBQX and at \(-70\) mV, respectively]. eNMDARc/eAMPARc ratios for both WT and mutant neurons were then compared at multiple stimulation intensities (Fig. 2A). Evoked NMDARc/AMPARc rise time and decay time ratios were significantly higher in NR2A\(^{-/-}\) than that in WT neurons, confirming respectively slower rise time eNMDARcs and more NR2B-rich NMDARs in NR2A\(^{-/-}\) neurons. Amplitude ratios were significantly lower in NR2A\(^{-/-}\) SC, indicating fewer total NMDARs in the mutant. The ratios remained constant for both strains over the full range of stimulating intensities. Differences between WT and NR2A\(^{-/-}\) NMDAR currents were not caused by altered presynaptic release because PPRs did not differ between genotypes (Fig. 2B).

**NR2A\(^{-/-}\) mice lack SC LTP**

As in rat SC neurons, LTP could be induced with 20 Hz stimulation for 20 s at intensity of 30–50% spike threshold (ST) in WT but not in NR2A\(^{-/-}\) neurons (Fig. 3A,B,E). Increasing induction intensity produced a small but significant depression in WT but not in NR2A\(^{-/-}\) neurons (Fig. 3A,B,E). Stimulating frequencies of 10 Hz at 30–50% ST produced no change in both WT and NR2A\(^{-/-}\) neurons (Fig. 3E), whereas 50 Hz stimulation produced a significant LTD in both WT and NR2A\(^{-/-}\) neurons (Fig. 3E). SC LTP shows similar frequency sensitivity in rats (Zhao et al., 2006). Using the effective 20 Hz, 30–50% ST protocol in WT neurons, bath application of AP-5 or AP-5 plus Nim prevented plasticity, Nim or Nim plus Ro 25 resulted in LTD (Fig. 3C,E), Nim applied after stimulation blocked change in EPSP slopes (Fig. 3D,E), and LTP switched to LTD with Nim during preinduction (Fig. 3D,E). Therefore, L-type Ca\(^{2+}\) channel activity is necessary for both the induction and maintenance of SC LTP in WT.

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**Figure 2.** WT and NR2A\(^{-/-}\) SC neurons show different NMDAR/AMPAR evoked current ratios but similar presynaptic release probabilities. A. Top, Sample average recordings of eAMPARcs at \(-70\) mV and eNMDARcs at \(+40\) mV in WT and NR2A\(^{-/-}\) SC neurons over a range of stimulation intensities. Each average is of 10 evoked currents. Calibration: 50 pA, 100 ms. Bottom, Summary plots showing averaged eNMDARc/eAMPARc ratios for rise time, decay time, and amplitude (\(n = 6\) and 2 for each genotype). Genotype had a significant effect on rise time ratio (\(F = 3.7, df = 7, p < 0.0001\)), decay time ratio (\(F = 5.7, df = 7, p < 0.0001\)), and amplitude ratio (\(F = 4.9, df = 7, p < 0.0001\)). NR2A\(^{-/-}\) neurons had higher rise and decay time ratios and lower amplitude ratios than WT neurons. Stimulus intensity had no effect (\(p > 0.05\)) on any ratio, and there was no significant interaction between stimulus intensity and genotype (\(p > 0.05\)) (two-factor ANOVA). B. PPR sample traces (left; calibration: 20 pA, 50 ms) and summary graph (right) showing no difference between WT and NR2A\(^{-/-}\) neurons (WT, 0.95 ± 0.06, \(n = 14\) and 3; NR2A\(^{-/-}\), 0.95 ± 0.07, \(n = 14\) and 3; \(p = 0.97\)). Each sample trace is the average of 10 paired-pulse responses. T, Threshold. White diamonds represent individual experiments, and black diamonds are means of all the experiments in that group.
NR2A\textsuperscript{−/−} cells, 20 Hz, 30–50% ST did not change EPSP slopes in the presence of Nim (Fig. 3E). Nim alone did not change the baseline activation in either genotype (data not shown). The mechanisms of the LTD induced by high-frequency stimuli or L-type Ca\textsuperscript{2+} channel blockade remain unknown. As suggested by Zhao et al. (2006), they could involve fatigue of presynaptic terminals and postsynaptic conductance that change excitability or entrainment of metabotropic glutamate receptor-mediated LTP. Nim versus Nim plus Ro 25 in WT suggest that NR2A-containing receptors are involved in the LTD induced by 20 Hz. Consistently, no LTD is induced in NR2A\textsuperscript{−/−} mice in the presence of Nim. In contrast, LTD induced by 50 Hz appears to be NR2A independent.

**LLF-LTD is unaltered in WT and NR2A\textsuperscript{−/−} SC neurons**

Significant LTD was induced with 900 stimuli at 30–50% ST presented at 1 Hz (LLF stimulation) in WT (Fig. 4A, E) and NR2A\textsuperscript{−/−} (Fig. 4C, E) neurons. In both mouse strains, application of AP-5 (Fig. 4A, C, E), or Nim (Fig. 4B, D, E) or Ro 25 (Fig. 4B, D, E) alone eliminated this depression. Therefore, blockade of either NR2B-NMDARs or L-type Ca\textsuperscript{2+} channels is sufficient to eliminate SC LTD whether or not NR2A subunits are present.

**Discussion**

Evoked neurotransmitter release can activate extrasynaptic NMDARs even in neurons that show no evidence of mNMDAR currents (Chen and Diamond, 2002; Clark and Cull-Candy, 2002). Thus, the presence or absence of synaptic NMDARs can be determined only when miniature currents are recorded. Steigerwald et al. (2000), Townsend et al. (2003), and Fu et al. (2005) all report the importance of the NR2A subunit or its cytoplasmic domain for maintaining mNMDAR currents as the brain matures. In all reports, AMPAR-mediated miniature and evoked currents remain. Nevertheless, adult NR2A\textsuperscript{−/−} mice or mice with truncated NR2A lack field potential LTP in CA1 in response to a single tetanization but can show an NR2B-dependent LTP of comparable magnitude with WT LTP when induction consists of multiple tetanic stimuli (Kiyama et al., 1998; Kohr et al., 2003). However, the contribution of L-type Ca\textsuperscript{2+} channels to this high-threshold LTP remains unknown, although L-type Ca\textsuperscript{2+} channels have been reported to contribute to CA1 LTP particularly in response to intense stimulation (Cavus and Teyler, 1996).
The present results are consistent with previous work in several preparations (Liu et al., 2004; Massey et al., 2004; Lu et al., 2001b): Namely, synaptic, NR2A-rich NMDARs are obligatory for the induction of SC LTD, and extrasynaptic NR2B-rich NMDARs are critical to SC LTD induced by low, low-frequency stimulation. However, three questions critical to the NR2A/NR2B debate remain unanswered. Are there unique attributes of NR2A subunits that require a concentration of this protein at synapses to initiate NMDAR-dependent LTP? At P15–P17, PSD-95 is the dominant scaffold for synaptic SC NMDARs (van Zundert et al., 2004). Are the cytoplasmic signaling molecules specifically associated with PSD-95 critical to LTP? Do NR2B-rich NMDARs selectively drive depression or is their requirement in SC LLF-LTD attributable to a different signaling complex that they specifically associate with as a result of being predominantly extrasynaptic? Answers to these questions are important. They should reveal how one receptor produces two diametrically opposite effects on synaptic plasticity and brain function.

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