NMDA Receptor Antagonists Reveal Age-Dependent Differences in the Properties of Visual Cortical Plasticity

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Submitted 21 February 2008; accepted in final form 22 July 2008

INTRODUCTION

N-methyl-D-aspartate receptor (NMDAR)-mediated synaptic plasticity is critical for learning and memory (Morris 1989; Moser et al. 1998) as well as experience-dependent modifications that have been particularly well defined in the visual cortex, such as ocular dominance plasticity and orientation selectivity (Bear et al. 1990; Ramoa et al. 2001; Roberts et al. 1998). Because of the heterogeneity of NMDAR subtypes (Cull-Candy et al. 2001; Dingledine et al. 1999), it has been suggested that different subpopulations of NMDARs may mediate unique aspects of synaptic plasticity. The primary visual cortex is an ideal model system to delineate the roles of NMDAR subunits because it undergoes a natural developmental transition in NMDAR expression (Quinlan et al. 1999a). These changes in NMDAR composition occur across a period of developmental (Jiang et al. 2007; Kirkwood et al. 1997) and experience-dependent (Kirkwood et al. 1996; Philpot et al. 2003, 2007) modifications in the properties of synaptic plasticity.

NMDAR composition is modulated over development in an experience-dependent manner (Quinlan et al. 1999b). While all NMDARs contain two obligatory NR1 subunits, these must dimerize with a combination of two NR2A-D or NR3A-B subunits to function as a receptor (Laube et al. 1998; Mayer and Westbrook 1987; McBain and Mayer 1994; Perez-Otano and Ehlers 2004). These secondary subunits confer distinct functional properties onto NMDARs by influencing current kinetics, glutamate affinity, and the milieu of intracellular signaling proteins proximal to the synapse (Barria and Malinow 2005; Chatterton et al. 2002; Flint et al. 1997; Vicini et al. 1998). The predominant NMDAR subtypes in the postnatal neocortex are NR2A and NR2B (Flint et al. 1997; Monyer et al. 1994; Watanabe et al. 1994). Over development, neocortical NMDARs transition from being primarily NR2B-containing (NR2B-type) to primarily NR2A-containing (NR2A-type) in an experience-dependent manner (Quinlan et al. 1999b). Because NR2B-type NMDARs have slower current kinetics than NR2A-type receptors, NMDAR currents become progressively faster with age (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992; Vicini et al. 1998). Changes in the composition and function of NMDARs have been tied to changes in the properties of synaptic plasticity (Carmignoto and Vicini 1992; Nase et al. 1999; Philpot et al. 2007), adding to the speculation that different NMDAR subunits contribute to distinct aspects of synaptic plasticity.

The suggestion that the induction of long-term potentiation and depression (LTP and LTD) were mediated, respectively, by NR2A and NR2B subtypes generated great excitement (Liu et al. 2004; Massey et al. 2004). However, while subsequent studies, using a variety of different stimulation protocols, have uncovered a similar subunit-specific trend (Fox et al. 2006; Gerkin et al. 2007), other studies have failed to do so (Bartlett et al. 2007; Berberich et al. 2005; Morishita et al. 2007; Toyoda et al. 2006; Weitlauf et al. 2005). Although the possibility of NR2-specific plasticity is appealing, we suggest that it must be considered in the context of several important factors. First, it is important to assess the specificity of subunit-specific...
antagonists used in these plasticity studies, as the selectivity of these drugs may change between brain regions due to differences in synaptic cleft glutamate (Frizzelle et al. 2006), the composition of NMDARs, or even the proportion of triheteromeric (NR1-NR2A-NR2B) NMDARs at the synapse (Kew et al. 1998; Neyton and Paoletti 2006). Because most regions of the brain, including the visual cortex, display a profound developmental shift in NMDAR subunit composition (Chen et al. 2000; Hestrin 1992; Quinlan et al. 1999a; Ramoa and McCormick 1994), characterizing the efficacy of an antagonist over development (within 1 region) can be a helpful way of discerning its specificity. Second, the effect of a subunit-specific antagonist should be compared with the effect of a global NMDAR antagonist that similarly attenuates NMDAR currents. Such an approach will help delineate whether deficiencies in plasticity can be attributed to a particular NMDAR subunit or to an overall reduction in NMDAR-mediated currents.

In an effort to elucidate whether visual cortical plasticity depends on NMDAR subunit-specific functions, our data reveal that the induction of LTD and LTP is not directed by distinct NMDAR subtypes. Instead our findings unexpectedly provide compelling evidence to suggest a developmental increase in the sensitivity of LTP to disruption by NMDAR antagonism. This developmental change in plasticity could also reconcile the wide range of results that have been reported using NVP-AAM007, a purported NR2A-selective antagonist.

METHODS

Animals

C57BL/6 mice (Charles River, Wilmington, MA) of both genders were used between postnatal days (P) 8–12 (young), P21-28 (juvenile), or P45-90 (adult). These age groups represent periods before, during, and after the classically defined critical period for ocular dominance plasticity in mice (Gordon and Stryker 1996) and are within a developmental period characterized by NR2A upregulation (Quinlan et al. 1999a). NR2A knockout mice were generously supplied by Dr. S. Nakanishi (Kadotani et al. 1996). These mice were rederived on a C57BL/6 background by Charles River Laboratories. All mice were raised on a 12-h light/dark cycle.

Cortical slice preparation

Mice were anesthetized with pentobarbital (40 mg/kg ip) and decapitated on disappearance of corneal reflexes, in compliance with the U.S. Department of Health and Human Services and the University of North Carolina guidelines. Brains were rapidly removed and kept at room temperature (composition in mM: 124 NaCl, 3 KCl, 1.25 Na2HPO4, 26 NaHCO3, 20 glucose, 4 MgCl2, 4 CaCl2, 0.001 glycine, 0.05 picrotoxin, and 0.02 6-cyano-7-nitroquinolxalene-2,3-dione (CNQX) or 6.7-dinitroquinolxaline-2,3-dione (DNQX). Cells were visualized using a Nikon E600FM microscope (Tokyo, Japan) equipped with infrared differential interference contrast (IR-DIC) optics. Patch pipettes were pulled from thick-walled borosilicate glass (P97, Sutter Instrument, Novato, CA). Open tip resistances were 3–6 MΩ when pipettes were filled with the internal solution containing (in mM): 102 cesium gluconate, 5 TEA-chloride, 3.7 NaCl, 20 HEPES, 0.3 sodium gluonosine triphosphate, 4 magnesium adenosine triphosphate, 0.2 EGTA, 10 bis-(ω-aminopropionophenoxy)-N,N,N’,N’-tetraacetic acid (BAPTA), and 5 QX-314 chloride (Alomone Labs, Israel) with pH adjusted to 7.2 and osmolarity adjusted to ~300 mmol/kg by addition of sucrose. Voltage-clamp recordings were performed at +40 mV in the whole cell configuration using a patch-clamp amplifier (Multiclamp 700A, Molecular Devices), and data were acquired and analyzed using pCLAMP 9.2 software (Molecular Devices). Cells were held at a depolarized potential to remove Mg2+ block, as opposed to recording in nominal Mg2+ at a resting membrane potential, to avoid polysynaptic responses. Pipette seal resistances were >1 GΩ and pipette capacitive transients were minimized prior to breakthrough. Changes in series resistance were monitored throughout the experiment by giving a test pulse and measuring the amplitude of the capacitive current. However, this method is susceptible to filtering error and overestimates series resistance by >30%. Therefore we used off-line measurements, employing a previously published approach that is highly insensitive to filtering (Santos-Sacchi 1993), to more accurately estimate series resistance, cell capacitance, and the fast membrane time constant. Drugs were infused into the recording chamber at a final concentration of 50 nM NVP-AAM077, 3 μM ifenprodil, or 1 μM APV and were applied for 20 min, with the last 10 min serving as the comparison to the pre-drug baseline. The concentrations for NVP-AAM077 and ifenprodil were chosen because previous reports demonstrate that they
provide the highest degree of specificity (Feng et al. 2004; Frizzelle et al. 2006; Williams 1993). The concentration of APV was empirically determined, using whole cell recordings, which is further described in RESULTS. NMDAR amplitudes and weighted time constants \( (\tau_w) \) of the receptor decay kinetics were compared before and after drug application. NMDAR excitatory postsynaptic current (EPSC) decays were well fit with a double exponential, as described previously (Rumbaugh and Vicini 1999), using the following equation: 

\[
I(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s)
\]

where \( I \) is the current amplitude, \( t \) is time, \( I_f \) and \( I_s \) are the peak amplitudes of the fast and slow components, respectively, and \( \tau_f \) and \( \tau_s \) are their respective time constants. NMDAR decay kinetics were quantified by a weighted time constant, which was calculated in milliseconds as: 

\[
\tau_w = \tau_f * [I_f/(I_f + I_s)] + \tau_s * [I_s/(I_f + I_s)].
\]

For cells to be included for analysis \( R_{input}, R_{series}^*, \) and \( I_{holding}^* \) had to fluctuate by \(<30\%\). The recorded series resistance averaged 25.9 \pm 1.5 MΩ. Input resistances recorded at \( +40 \) mV did not significantly differ between age groups (P12-P18; 182.1 \pm 32.0 MΩ; P21-P28; 152.2 \pm 13.1 MΩ; and P45-P90; 129.8 \pm 6.3) or drug applications (baseline, 151.2 \pm 10.0 MΩ; 50 nM NVP-AAM077, 122.1 \pm 11.1 MΩ; 3 µM ifenprodil, 162.7 \pm 18.9 MΩ; and 1 µM APV, 128.3 \pm 14.9 MΩ). EPSCs were evoked from a stimulating electrode (concentric bipolar; 200 µm tip separation) placed in L4, and stimulation was given for 200 µs every 15 s. EPSCs were recorded in L2/3 pyramidal cells.

Biochemical fractions

Each biochemical fraction was prepared using visual cortices pooled from 3–10 brains (brains per pooled sample; P8, n = 10; P16, n = 5; P26, n = 5; P62, n = 3), as previously described (Yashiro et al. 2005). Samples were homogenized in HEPES-buffered sucrose (4 mM HEPES, 0.32 M sucrose, pH 7.4) using a motor-driven dounce homogenizer. Postnuclear supernatant (PNS) fractions were prepared by centrifugation (150,000 g, 2 h) using a gradient consisting of 0.8, 3, 11.0, 15; P21-28, 131.3 \pm 16.0, n = 12; P45-90, 96.2 \pm 11.0, n = 16; \( P < 0.0001 \)).

By measuring NR2 expression and NMDAR current kinetics at different ages, we show \( I \) the expression of NR2A relative

Pharmacological agents

Unless otherwise noted, all chemicals were purchased from Sigma (St. Louis, MO).

Statistics

Data were expressed as means \pm SE. Statistical comparisons were performed using InStat3 software (GraphPad Software, San Diego, CA). For multiple group comparisons, analyses of variance (ANOVA) were first performed, followed by between-group comparisons with Student-Newman-Keuls (SNK) tests. Significance was placed at \( P < 0.05 \). All reported levels of statistical significance represent two-tailed values.

RESULTS

Developmental changes in NMDAR subunit expression and function in mouse visual cortex

To understand the roles of different NMDAR subunits in plasticity, we focused our studies on the primary visual cortex, a region that exhibits a developmental upregulation of NR2A-type relative to NR2B-type receptors. While this subunit transition in NMDARs has been well-described in several species (Chen et al. 2000; Quinlan et al. 1999a; Roberts and Ramoa 1999; Sheng et al. 1994), we wanted to characterize the specific nature of this trend in murine visual cortex. By enriching for proteins associated with the postsynaptic density (PSD), over a range of developmental time points, we were able to temporally reveal the graded expression of postsynaptic NMDAR subunits. Because this preparation enriches for postsynaptic proteins, it is well-suited to discern the composition of receptors mediating synaptically-evoked currents. Pools of visual cortex samples from P8, P16, P26, and P62 animals were run in triplicate, averaged, and compared. Consistent with observations in other species, we found a dramatic developmental increase in NR2A between P8 and P62, with only a modest upregulation of NR2B (Fig. 1, A and B; NR2A subunit levels relative to P8: P16, 3.66 \pm 1.13; P26, 7.97 \pm 0.92; P62, 9.79 \pm 0.81; \( P < 0.001 \)). NR2B subunit levels relative to P8: P16, 1.43 \pm 0.33; P26, 2.18 \pm 0.15; P62, 2.33 \pm 0.07; \( P < 0.01 \).

To further characterize the nature of this developmental subunit transition, we pharmacologically isolated whole cell NMDAR currents in layer (L) 2/3 pyramidal cells over the same age range. Because NR2A-type receptors have faster decay kinetics than NR2B-type receptors (Carmignoto and Vicini 1992; Flint et al. 1997; Hestrin 1992; Monyer et al. 1992; Stocca and Vicini 1998; Vicini et al. 1998), this allowed us to corroborate our biochemical data with a physiological measure. As expected, an ANOVA revealed a significant decrease in \( \tau_w \) over development (Fig. 1C), demonstrating that NMDAR currents become progressively faster with age (average: P12-18, 196.9 \pm 11.8, n = 15; P21-28, 131.3 \pm 16.0, n = 12; P45-90, 96.2 \pm 11.0, n = 16; \( P < 0.0001 \)).

Immunoblot analysis

PSD fractions (5 µg) were resolved by 7.5% SDS-PAGE and transferred to nitrocellulose membranes. Both blotting and imaging with the Odyssey system (LI-COR, NE) were carried out following the manufacturer’s protocols. The primary antibodies used were rabbit anti-NR2A (1:500, sc-9056, Santa Cruz, CA) and goat anti-NR2B (1:10,000, sc-1469, Santa Cruz, CA). The employed secondary antibodies were Alexa Fluor 680-labeled anti-goat IgG (1:5,000, Molecular Probes, OR) and IRDye 800-labeled anti-rabbit IgG (1:3,000, Rockland, PA). All protein quantification was based on the average of three separately loaded lanes. Because the PSD fraction is enriched for synaptic proteins, commonly employed loading controls that remain constant over the course of development are not available.

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By measuring NR2 expression and NMDAR current kinetics at different ages, we show \( I \) the expression of NR2A relative

\[ f_{exp}(t) = I_f \exp(-t/\tau_f) + I_s \exp(-t/\tau_s) \]

\[ \tau_w = \tau_f * [I_f/(I_f + I_s)] + \tau_s * [I_s/(I_f + I_s)] \]
to NR2B increases with development; 2) the most rapid up-regulation of synaptic NR2A occurs prior to P30; and 3) there is a functional correlate to changes in subunit expression at the synapse. With this information, we were able to evaluate synaptic plasticity at discrete developmental stages, using the natural process of NR2A upregulation as a mechanism to address the potentially unique roles of NR2A and NR2B-type NMDARs in synaptic plasticity.

**LFS-LTD is developmentally sensitive to NMDAR antagonism**

We began by evaluating whether LFS-LTD in the primary visual cortex was NR2B dependent, as had been reported previously in other regions of the brain (Liu et al. 2004; Massey et al. 2004). To isolate the roles of different NMDAR subunits, we conducted these experiments in the presence of ifenprodil, an NR2B-type antagonist (Williams et al. 1993), or NVP-AAM007, a low-affinity NR2A-type antagonist (Auberson et al. 2002; Feng et al. 2004). In young animals (P12-18), we found that neither ifenprodil nor NVP-AAM007 had an appreciable effect on the magnitude of LTD induced by 1-Hz stimulation (Philpot et al. 2003). Consistent with previous observations that 1-Hz LFS produces less robust LTD with age (Jiang et al. 2003, 2007; Kirkwood et al. 1997), we observed that the magnitude of 1-Hz LTD decreases substantially with age (Fig. 2C). Therefore, in adult (P45-90) animals, the small degree of plasticity evoked with 1-Hz stimulation (average: control, 95.4 ± 4.5%) precluded us from investigating the effect of antagonist application on this form of LTD. However, we do not suggest that LTD is absent in adult mice, as previous studies have used different stimulation protocols, such as a paired-pulse LFS stimulation, to induce LTD in the adult cortex (Jiang et al. 2003; Lee et al. 2003).

**NR2B-type NMDARs are not required for the expression of chemically induced LTD**

Because extrasynaptic NR2B-type NMDARs have been suggested to play a critical role in the induction of LTD (Massey et al. 2004), we chose to globally stimulate both synaptic and extrasynaptic NMDARs by bath application of NMDA. This protocol has been shown to induce a chemical form of LTD (chem-LTD) that is mediated by rapid dephosphorylation of AMPA receptors and is occluded by homosynaptic LTD, suggesting that chem-LTD and LFS-LTD share common under-lyings mechanisms (Lee et al. 1998). We found chem-LTD to decrease in magnitude with age, mirroring the developmental profile observed with LFS-LTD (Fig. 3A; average: P12-28, 44.7 ± 2.5%, n = 5; P45-90, 86.4 ± 3.9%, n = 5; P < 0.0001). We also confirmed that this form of LTD is NMDAR-dependent by completely blocking it with 100 μM D,L-APV (Fig. 3B; APV, 105%, n = 1). Yet chem-LTD is not attenuated by antagonizing ifenprodil-sensitive, presumably NR2B-type, NMDARs (Fig. 3B; average: control, 44.7 ± 2.5%, n = 5;...
Thus both our synaptic and extrasynaptic stimulation paradigms fail to suggest a critical role for NR2B-type NMDARs in the induction of LTD in the mouse visual cortex.

**NVP-AAM077 completely blocks LTP in adult, but not juvenile, animals**

In an attempt to investigate the discrete roles of NR2A- and NR2B-type NMDARs over development, we bath applied ifenprodil and NVP-AAM077 at different developmental time points. We observed that juvenile LTP was significantly attenuated, but not blocked, in the presence of either antagonist (Fig. 4A; average: control, 116.8 ± 3.1%, n = 12; 50 nM NVP, 108.0 ± 2.5%, n = 9; 3 μM ifenprodil, 105.7 ± 2.7% n = 7; P < 0.05). However, we were surprised that the induction of adult LTP was completely prevented by NVP-AAM077, while entirely unaffected by ifenprodil (Fig. 4B; average: control, 120.5 ± 3.7%, n = 6; 50 nM NVP, 101.4 ± 2.7%, n = 6; 3 μM ifenprodil, 116.9 ± 3.9%, n = 5; P < 0.01). To further examine why ifenprodil and NVP-AAM077 had different ef-

3 μM ifenprodil, 44.6 ± 4.5%, n = 5). Thus both our synaptic and extrasynaptic stimulation paradigms fail to suggest a critical role for NR2B-type NMDARs in the induction of LTD in the mouse visual cortex.

**Fig. 2.** NVP-AAM077 and ifenprodil have developmentally specific effects on the expression of low-frequency stimulation long-term depression (LFS-LTD) in the visual cortex. LTD was induced with 1-Hz stimulation (15 min) of L4 (△). Data represent averages ± SE. Representative waveforms show field potentials before and after 1-Hz stimulation under control conditions. A: in young animals (P12-18), LTD is insensitive to either 3 μM ifenprodil or 50 nM NVP-AAM077. B: in juvenile animals (P21-28), LTD is modestly attenuated by either 3 μM ifenprodil or 50 nM NVP-AAM077. C: in adult animals (P45-90), only a small level of LTD could be induced by 1-Hz stimulation under control conditions.

**Fig. 3.** Chemically induced LTD (chem-LTD) is insensitive to ifenprodil application. A: the magnitude of chem-LTD induced by brief application of NMDA (20 μM) is greater in the visual cortex of young (P12-18) animals than adult (P45-90) animals. Representative traces are shown from a P26 recording. B: ifenprodil (3 μM) fails to reduce the magnitude of chem-LTD, whereas 2-amino-5-phosphonovaleric acid (APV, 100 μM) prevents its induction.
Assumption that NVP-AAM077 is highly selective for NR2A-NR2A over NR2B (Auberson et al. 2002). To date, more than NVP-AAM077 had a 110-fold preference for human NR2A-containing NMDARs indicated that selective antagonist NVP-AAM077 has been the subject of examination (Williams et al. 1993), the recently developed NR2A-specific antagonist, ifenprodil, has been well characterized (Berberich et al. 2005, 2007; Fox et al. 2006; Hrabetova et al. 2003). Conversely, assuming a modest degree of selectivity, the development transition in synaptic NMDAR subunit expression by antagonizing significantly less NMDAR current in adult animals (Williams et al. 1993; Toyoda et al. 2006; Weitlauf et al. 2005). While the NR2B-specific antagonist, ifenprodil, has been well characterized (Williams et al. 1993), the recently developed NR2A-selective antagonist NVP-AAM077 has been the subject of controversy. Initial reports assessing the specificity of NVP-AAM077 for human NR2A-containing NMDARs indicated that NVP-AAM077 had a 110-fold preference for human NR2A over NR2B (Auberson et al. 2002). To date, more than 100 studies have been performed and interpreted under the assumption that NVP-AAM077 is highly selective for NR2A-containing NMDARs in rodents. However, several recent studies examining the specificity of NVP-AAM077 suggest that the drug has a much lower subunit preference in rodent compared with human NMDARs and that the drug cannot reliably distinguish between NR2A- and NR2B-type receptors (Feng et al. 2004; Frizelle et al. 2006; Neyton and Paoletti 2006).

Although NVP-AAM077 is reported to have only modest selectivity for rodent NR2A-type receptors (Feng et al. 2004; Frizelle et al. 2006), we wanted to test whether it was sensitive enough to discriminate the developmental upregulation of NR2A relative to NR2B that we had established both biochemically and electrophysiologically (Fig. 1, A–C). To probe the specificity of these antagonists, NMDAR current durations were quantified by a weighted time constant (see Methods) and measured before and after drug application. Because NR2B-type receptors have slower decay kinetics than NR2A-types, we reasoned that selective antagonism of NR2B-type receptors with ifenprodil should increase NMDAR decay kinetics and cause a decrease in current decay time (e.g., a decrease in \( \tau_w \)) relative to the pre-drug condition. We expected this effect would be most pronounced in young animals that express high levels of NR2B. Similarly, blockade of faster NR2A subtypes would be expected to prolong NMDAR decay kinetics, and this effect would be most profound during adulthood when NR2A-containing NMDARs predominate at the synapse.

As predicted, ifenprodil significantly shortened NMDAR decay kinetics in young but not adult animals (average in ms): P12-28 baseline, 171.2 ± 18.6; P12-28 3 \( \mu \)M ifenprodil, 119.1 ± 11.3, \( n = 13 \); P45-90 baseline, 98.4 ± 14.4; P45-90 3 \( \mu \)M ifenprodil 86.4 ± 15.4, \( n = 7 \), validating that the developmental change in relative NR2 expression can be pharmacologically discerned with a reliable subunit-specific antagonist (Fig. 5, A and B). However, we observed that NVP-AAM077 did not alter the duration of isolated NMDAR current kinetics at either developmental stage (Fig. 5, C and D); average in ms: P12-28 baseline, 171.2 ± 18.6; P12-28 50 nM NVP, 149.1 ± 11.3, \( n = 13 \); P45-90 baseline, 98.4 ± 14.4; P45-90 50 nM NVP 101.1 ± 14.9, \( n = 10 \). Thus NVP-AAM077 failed to selectively antagonist the fast NR2A component of the NMDAR current, even in adulthood when the relative levels of NR2A are highest. These data are summarized in Fig. 5E.

To provide an additional gauge of the subunit specificity of these drugs, we evaluated their degree of antagonism with consideration to the amount of subunit expression over development (Fig. 1, A and B). As shown previously, we expected ifenprodil-induced antagonism of NMDAR currents to be greatest in young animals that express relatively high levels of NR2B-type receptors (Vicini et al. 1998; Yoshimura et al. 2003). Conversely, assuming a modest degree of selectivity, NVP-AAM077 should attenuate a progressively greater proportion of NMDAR currents over development, as expression of the NR2A subunit increases. To evaluate the ability of NMDAR antagonists to block NMDAR-mediated currents, we measured their effects on current amplitude. As predicted, ifenprodil discriminated the developmental transition in synaptic NMDAR subunit expression by antagonizing significantly less NMDAR current in adult animals (Fig. 6A, % baseline amplitude: P21-28, 40.4 ± 3.5\% \( n = 6 \); P45-90, 69.5 ± 4.5\%, \( n = 5 \); \( P < 0.001 \). However, NVP-AAM077 failed to exhibit selectivity for NR2A subtypes, as it attenuates...
NMDAR EPSCs to a similar extent across development (Fig. 6B; % baseline amplitude: P21-28, 50.6 ± 5.3%, n = 4; P45-90, 52.0 ± 3.9%, n = 10; similar effects were seen on charge transfer, data not shown), suggesting that NVP-AAM077 largely lacks specificity for NR2A subtypes in mice. To additionally test whether NVP-AAM077 lacked selectivity for NR2A subtypes in the mouse visual cortex, we tested the effect of the drug on NMDAR currents from mice lacking NR2A. Further verifying that NVP-AAM077 lacks specificity for NR2A subtypes, the drug significantly blocked NMDAR currents in the visual cortex of NR2A knockout mice, across a broad developmental spectrum (Fig. 6B; % baseline: NR2A KO P12-90, 68.6 ± 4.3%, n = 8; P < 0.001).

In summary, we found evidence that NVP-AAM077 has limited specificity for NR2A-type receptors in mice. However, it is difficult to reconcile how a nonselective NMDAR antagonist can have discrete effects on plasticity at different developmental time points. The developmental effect of NVP-AAM077 cannot be attributed to increased NMDAR current antagonism because the drug blocks a comparable amount of NMDAR current at both ages (Fig. 6B). Therefore it seemed reasonable to postulate that NVP-AAM077 has a greater affect on adult LTP, not due to its action on NR2A-type receptors, but due to a developmental increase in the amount of NMDAR activation required for plasticity.

**Developmental differences in the requirements for LTP can explain why NVP-AAM077 has a more profound effect on adult LTP**

An age-dependent shift in the amount of NMDAR activation needed to induce LTP could underlie the developmental change in response to NVP-AAM077. That is, the induction of LTP in adult cortex may be more sensitive to disruption by a partial block of NMDAR currents than juvenile cortex.

To measure whether there are age-dependent differences in the ability to disrupt LTP with NMDAR antagonists, we first compared the amount of NMDAR current blocked by NVP-AAM077 during juvenile and adult stages of development (Fig. 6B). It is clear that the total amount of NMDAR current remaining after NVP-AAM077 application is comparable at both developmental stages. However, the same degree of NMDAR antagonism at these two developmental time points caused distinct effects on LTP (Fig. 4, A and B). These data
encouraged us to test whether other relatively nonsubunit selective NMDAR antagonists had similar age-dependent consequences on LTP induction.

Because the threshold for plasticity has been shown to vary with experience-dependent regulation of NMDAR current (Philpot et al. 2003), we postulated that there might also be age-dependent changes in NMDAR requirements for the induction of LTP. To investigate this possibility, we used a subsaturating concentration of APV, a pan-NMDAR blocker. While there are some indications that APV has a slight preference for recombinant NR2A-type receptors (Buller and Monaghan 1997), this has not been demonstrated in endogenously expressed receptors (Christie et al. 2000). In our preparation, 1 μM APV failed to attenuate more current with maturity and did not prolong NMDAR current kinetics (data not shown), as would have been expected if the drug had a higher affinity for NR2A-type receptors. These data are consistent with previous observations that APV cannot distinguish differences in the relative expression of NMDAR subtypes in rodent visual cortex (Quinlan et al. 1999b).

If the developmental effect of NVP-AAM077 on LTP could be attributed to a shift in NMDAR requirements needed to induce plasticity and not a subunit-specific effect, subsaturating concentrations of APV should mimic the age-dependent effects of NVP-AAM077. Through empirical observations, we found that 1 μM APV and 50 nM NVP-AAM077 antagonized a similar amount of NMDAR current in juvenile and adult animals (Fig. 7A; amplitude % baseline: P21–28: NVP, 50.6 ± 5.3, n = 4; APV, 53.9 ± 6.4, n = 6; P45–90: NVP, 52.0 ± 3.9, n = 10; APV 55.8 ± 9.2, n = 4). Excitingly, 1 μM APV failed to antagonize LTP in the juvenile visual cortex, whereas it strongly attenuated LTP in adults (Fig. 7, B and C; average (P21–28): control, 119.1 ± 4.1%, n = 8; 1 μM APV, 115.8 ± 5.5% n = 9; average (P45–90): control, 128.9 ± 3.6%, n = 4; 1 μM APV, 107.2 ± 1.5% n = 5; P = 0.001). Thus, much like NVP-AAM077, APV blocked a similar magnitude of the NMDAR current in juvenile and adult animals, yet differentially blocked the magnitude of LTP.

While the effect of NVP-AAM077 (Fig. 4, A and B) and APV (Fig. 7, B and C) are not identical, they both demonstrate the same developmental trend. This strongly suggests that the degree of NMDAR activation required for LTP increases with development. Although the induction of plasticity has been shown to be sensitive to partial NMDAR blockade (Berberich et al. 2007; Cummings et al. 1996; Nishiyama et al. 2000; Philpot et al. 2003), our study provides the first indication that LTP sensitivity to NMDAR antagonist changes with development.

The developmental effect of NMDAR antagonism on LTP could reflect a developmental shift in the postsynaptic response to the tetanizing LTP stimulus. This is particularly relevant because the neocortex undergoes a significant postnatal maturation of inhibitory networks (Hensch 2005; Rozas et al. 2001) and presynaptic release properties (Reyes and Sakmann 1999) that could influence short-term synaptic dynamics. To begin to address this, the relative field potential amplitude of the first 28 pulses in the 100-Hz tetanus were analyzed (Fig. 8, A–D). The rate of decay of the field response was quantified by fitting the normalized data with a triple exponential defined as \( I(t) = I_1 \exp(-t/\tau_1) + I_2 \exp(-t/\tau_2) + I_3 \exp(-t/\tau_3) + C \), where \( I_1 \) is the peak amplitude of the field response, \( \tau_1 \) is the corresponding decay time constant, and C is the plateau potential of the response. For quantification, the three decay constants were combined into a weighted time constant as follows: \( \tau_w = \tau_1 * [I_1/(I_1 + I_2 + I_3)] + \tau_2 * [I_2/(I_1 + I_2 + I_3)] + \tau_3 * [I_3/(I_1 + I_2 + I_3)] \). No significant differences were observed between the two ages of interest as both the weighted time constant and the plateau potential of the response decay during the 100-Hz tetanus were similar at both developmental stages (P21–P28 \( \tau_w = 13.0 \pm 1.4 \text{ ms}, \ P45–P90 \tau_w = 15.6 \pm 1.5 \text{ ms}, \ P = 0.22 \) P21–P28 \( C = 0.26 \pm 0.03, \ P45–P90 C = 0.20 \pm 0.03, \ P = 0.21 \). These findings are consistent with previous observations showing that there are minimal developmental changes in the L4-L2/3 paired-pulse response (Rozas et al. 2001), although there are much greater age-dependent effects on the L2/3 paired-pulse response when stimulation is evoked from white-matter (Ramoa and Sur...
In addition to observing a similar decay in the field potential response during the 100-Hz stimulation, we also observed that the half-maximal amplitude of the postsynaptic response was similar at both developmental ages (average fEPSP amplitude: P21-28, 1023.7 ± 118.4 μV, n = 10; P45-90, 1121.8 ± 149.0 μV, n = 6; P = 0.6166). In addition, using immunogold electron microscopy, it appears that the size of the postsynaptic density and the number of synaptic NMDARs is similar between juvenile and adult stages of development (R. J. Corlew and R. J. Weinberg, personal communications). These results, taken together, suggest that the increasing developmental sensitivity to NMDAR antagonism is not a reflection of a developmental change in our ability to drive postsynaptic L2/3 activity with L4 stimulation. Although our data suggest that the degree of NMDAR activation required for the expression of 100-Hz LTP is increased in adulthood at the L4-L2/3 synapse, we cannot rule out the possibility that age-dependent differences in the susceptibility to NMDAR antagonists might also arise from changes in the way extracellular stimulation recruits neocortical microcircuits.

FIG. 7. There is a developmental increase in the ability to disrupt LTP with a nonsubunit-selective NMDAR antagonist. A: in juvenile (P21-28) and adult (P45-P90) mice, subsaturating concentrations of APV (1 μM) and NVP-AAM077 (50 nM) block a similar degree of NMDAR current amplitude. NVP-AAM077 data are replotted from Fig. 6B for the purposes of comparison. B: subsaturating APV fails to attenuate the induction of LTP in the visual cortex of young mice (P21-28). C: subsaturating APV dramatically reduces the magnitude of LTP in adult mice (P45-90).

FIG. 8. There are no apparent differences between juveniles (P21-28) and adults (P45-90) in the L4-L2/3 field potential responses generated by 100-Hz stimulation. Example field potential traces from juvenile (A) and adult (B) stages of development. The first 28 pulses of a 100-Hz stimulus are depicted, and stimulus artifacts are clipped for clarity. C: the normalized field potential amplitude of the first 28 pulses of a 100-Hz stimulus does not differ between juvenile and adult animals.
AGE-DEPENDENT EFFECTS OF NMDAR ANTAGONISTS ON PLASTICITY

DISCUSSION

The work described here uses a pharmacological approach, over the course of development, to determine whether NMDAR subtypes have specific roles in synaptic plasticity. A major finding of our study is that ifenprodil-sensitive and NVP-AAM077-sensitive NMDARs have overlapping roles in synaptic plasticity. Both populations can contribute to the expression of LTD and LTP. Using the same NMDAR antagonists, our conclusions are in striking contrast to previous studies that suggest opposing roles for NR2A and NR2B in synaptic plasticity. We help resolve this controversy by showing it can be explained, at least in part, by the nonspecific effects of a purported NR2A-selective antagonist and by the age-dependent differences in the properties of synaptic plasticity. Specifically, we demonstrate that NVP-AAM077, a commonly employed antagonist, lacks strong selectivity for NR2A-containing NMDARs in rodents. This is highlighted by showing that the age-dependent effects of NVP-AAM077 on LTP can be largely replicated using a nonselective NMDAR antagonist (APV) at subsaturating concentrations. These studies also demonstrate that expression of synaptic plasticity is more easily disrupted by NMDAR antagonists in the adult, compared with the juvenile, cortex. The implications of these are not yet known, although we speculate that the more robust synaptic plasticity in early development is important for shaping cortical networks during early critical periods.

Activation of NMDARs is required for the induction of LTD in the visual cortex (Kirkwood and Bear 1994). However, this LTD is not critically mediated by synaptic or extrasynaptic NR2B-type receptors that are sensitive to ifenprodil. While these findings contrast with previous reports in the hippocampus and perirhinal cortex (Liu et al. 2004; Massey et al. 2004), our results are in agreement with a recent report in the hippocampus conducted across three separate laboratories (Morishita et al. 2007). Interestingly, we did observe a subtle, but significant, developmental increase in the disruption of LTD by NVP-AAM077 and ifenprodil. This suggests that there is an age-dependent increase in the degree of NMDAR activation required for the full expression of LTD. This observation may help explain why it is more difficult to induce LTD, if it can be induced at all, in many parts of the adult brain (Kirkwood et al. 1996, 1997) with a low frequency stimulation protocol.

NMDARs are also required for the induction of LTP in the visual cortex (Kirkwood and Bear 1995). Similar to what we observed for LTD, we show that neither NVP-AAM077 nor ifenprodil-sensitive populations of NMDARs are required for the induction of LTP. Interestingly, ifenprodil has no effect on adult LTP, although it attenuates LTP in juvenile mice. We suggest that this maturational change may simply be a consequence of the fact that ifenprodil blocks a greater proportion of the total NMDAR current in juvenile mice. In contrast to our observations using ifenprodil, we observed that NVP-AAM077 more effectively blocks LTD in adult cortex compared with juvenile cortex. This observation is in agreement with recent findings, at the single-cell level, in cortical neurons (Le Roux et al. 2007). While we initially hypothesized that the increasing ability of NVP-AAM077 to block LTD with age was due to an increasing role for NR2A in LTD with development, we discarded this notion for two reasons. First, we used convergent approaches to demonstrate that NVP-AAM077 lacks appreciable specificity for blocking NR2A-containing NMDARs. Second, because NVP-AAM077 blocks the same amount of NMDAR current at both developmental stages studied, we reasoned that a more plausible interpretation of our data is that the degree of NMDAR activation required for plasticity increases with age. In support of this idea, we show that a sub-saturating concentration of APV, a nonselective NMDAR antagonist, mimics the age-dependent consequences of NVP-AAM077 on LTD. Taken together, our data provide convincing evidence that the developmental effect observed with the NMDAR antagonists NVP-AAM077 and APV reflects an increase in the degree of NMDAR activation required for LTD.

The underlying mechanism regulating this developmental shift in the properties of LTP are likely multifold and are currently unknown. Although we demonstrate that NR2A and NR2B subtypes do not have polarized roles in synaptic plasticity, developmental changes in the properties of synaptic plasticity are likely to still be mediated, at least in part, by differences in the NMDAR signaling complex. For example, a developmental change in the relative proportion of diheteromeric (e.g., NR1-NR2B) and triheteromeric (NR1-NR2A-NR2B) NMDARs could alter the requirements for synaptic plasticity. In fact, our pharmacological data suggest that the relative amount of triheteromeric receptors may increase between juvenile and adult stages of development. While our findings show that the NR2A/NR2B ratio changes only modestly between P6 and P62, the NR2B antagonist ifenprodil blocks significantly more NMDAR current at the younger developmental time point. This is revealing because several studies have shown that ifenprodil can better antagonize diheteromeric NR2B-containing NMDARs than triheteromeric receptors, containing both NR2A and NR2B subunits (Kew et al. 1998; Tovar and Westbrook 1999). Therefore we postulate that the developmental decrease in ifenprodil sensitivity may reflect an increased presence of triheteromeric NMDARs, which have been documented in both the cortex and hippocampus (Al-Hallaq et al. 2007; Luo et al. 1997; Sheng et al. 1994). While a developmental change in the relative amount of triheteromeric receptors has not been demonstrated in hippocampus (Al-Hallaq et al. 2007), our data, and that of others (Kew et al. 1998), hint that there may be a developmental increase in cortical triheteromeric NMDARs. While the decay kinetics of triheteromeric NMDARs are intermediate to diheteromeric (NR1/NR2A and NR1/NR2B) NMDARs that contain only one type of NR2 subunit, it is unclear how these triheteromeric NMDARs influence plasticity. The intracellular signaling of triheteromeric receptors may differ from that of diheteromeric receptors because signaling cascades, linked to specific NR2 subunit, may be affected by oligomerization. However, the NR2B subunit, which is more likely to be in a diheteromeric form during juvenile stages of development, has several attributes that favor its involvement in the expression of LTP. First, NR2B-type receptors have been shown to allow more calcium influx per unit charge than NR2A-type receptors (Sobczak et al. 2005). In addition, NR2B subunits recruit CaMKII, a critical modulator of LTP, to the synapse (Barria and Malinow 2005; Lisman et al. 2002). Taken together, a developmental decrease in NR2B-like properties, which may parallel the increase in triheteromeric NMDARs, would be less permissive.
for calcium influx and NR2B-associated signaling cascades at the synapse. As such, LTP may be more easily induced, and less easily disrupted, when diheteromeric NR2B-type NMDAR expression is high (Philpot et al. 2007). Thus a developmental change in NMDAR subunit composition may be one factor, in addition to other developmental changes in the synaptic milieu (Berardi et al. 2004; Hensch 2005), that increases the sensitivity of LTP to NMDAR antagonism.

If the composition of NMDARs at young synapses allows LTP to occur at a lower threshold, thereby making LTP less easily disrupted by partial NMDAR blockade, it might be expected that the amplitude of LTP should be greater in young animals. However, previous findings indicate that LTP induction and expression may be regulated by separate mechanisms. Indeed synapses may compete for “plasticity factors” that limit the expression of LTP (Fonseca et al. 2004). Further support for this hypothesis comes from observations of plasticity in NR2A knockout animals, where the threshold for inducing LTP is lowered without an appreciable change in the magnitude of the LTP expressed (Philpot et al. 2007). Thus one set of “plasticity factors” might control the threshold for modifying synapses, whereas a second, likely overlapping, set of molecules might regulate the magnitude of synaptic modifications.

Parsuing out the mechanisms underlying the developmental increase in the degree of NMDAR activation required for plasticity will require further investigation. For example, it is already clear that the maturation of inhibitory circuitry is of profound importance (Corlew et al. 2007; Hensch and Fagiolini 2005; Huang et al. 1999; Maffei et al. 2006; Steele and Mauk 1999; Yoshimura et al. 2003), while its role in regulating the properties of synaptic plasticity continues to be more fully defined. Although we provide evidence that our ability to drive L4-L2/3 activity is similar between juvenile and adult stages of development, we cannot preclude the possibility that enhanced cortical inhibition, changes in presynaptic release probability, or differences in synaptic NMDAR content may play a role in shifting the ability of NMDAR antagonists to disrupt LTP over development.

Although the properties underlying the mechanisms of synaptic plasticity are complex, our work contributes several important findings that increase our understanding of developmentally regulated plasticity and distinct NMDAR subunit functions. First, our data indicate that ifenprodil-sensitive NR2B-type NMDARs are not critical for the induction of either LTD or LTP. Second, NVP-AAM077 is an unreliable NR2A-type antagonist in the mouse visual cortex. Thus the myriad of studies that have been interpreted with the assumption that NVP-AAM077 is a highly subunit-selective antagonist must be re-evaluated. Third, we reveal a developmental increase in the degree of NMDAR activation required for the induction of LTP. These observations not only elucidate an important mechanism that regulates plasticity over development, they may also reconcile discrepant results concerning NR2 subunit-specific roles in plasticity. The roles of NR2A and NR2B may be better understood in the future through careful consideration of the limitations of subunit-selective antagonists, the age of the animal, the contribution of triheteromeric NMDARs, and the brain region and species being examined.

ACKNOWLEDGMENTS

We thank Y. Auberson of the Novartis Institutes for Biomedical Research (Basel) for the generous gift of NVP-AAM077, and we are indebted to P. Maffei for useful suggestions and for assistance in generating off-line analyses. We also thank R. Corlew, R. Weinberg, and M. Henson for technical support and for editing of the manuscript.

GRANTS

This work was funded by a Helen Lyng White Fellowship and National Institutes of Health Grant T32-HD-40127 to A. C. Roberts and the Whitehall Foundation and National Institutes of Health Grant RO1 EY-018323 B. D. Philpot.

REFERENCES


Barria A, Malinow R. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMII. Neuron 48: 289–301, 2005.


J Neurophysiol • VOL 100 • OCTOBER 2008 • www.jn.org

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