Experimental Down-Regulation of the NMDA Channel Associated With Synapse Pruning

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Hickmott, Peter W. and Martha Constantine-Paton. Experimental down-regulation of the NMDA channel associated with synapse pruning. J. Neurophysiol. 78: 1096–1107, 1997. The N-methyl-D-aspartate (NMDA) receptor has been implicated in activity-dependent synapse stabilization, but its role as a detector of correlated activity during development is debated. In the amphibian retinotectal system, synaptic sorting and stabilization occur throughout larval life, and map refinement is dependent on continuous NMDA receptor function. Moreover, tadpole tecta chronically treated with NMDA selectively fail to maintain retinal synapses wherever their activity correlations are lowest. To determine whether this synapse elimination is associated with a specific down-regulation of NMDA receptor function, whole cell voltage-clamp recordings were made from single neurons in tectal slices. After chronic NMDA treatment, decreases in the magnitude of NMDA currents were detected in glutamatergic synaptic currents, in agonist-evoked currents, and in single-channel currents activated by NMDA. The results suggest that the efficacy of NMDA receptors on tectal neurons determines the amount of correlation required to stabilize sets of tectal inputs during formation of the retinotectal projection.

INTRODUCTION

Patterned activity is necessary for the development of precise connections between neurons and their postsynaptic targets (for reviews see Fields and Nelson 1992; Goodman and Shatz 1993). In many cases, it appears that a Hebb-like rule applies: synapses from afferents with correlated firing patterns (i.e., coactive inputs), which strongly depolarize the postsynaptic target cell through temporal summation, are stabilized, whereas less effective synapses are weakened and eventually lost (Hebb 1949; Stent 1973). The N-methyl-D-aspartate (NMDA) subtype of glutamate receptor has been proposed to be a critical component of the mechanism that is responsible for detecting and stabilizing coactive inputs during development (Constantine-Paton 1990; Constantine-Paton et al. 1990; Fox and Daw 1993). The NMDA receptor’s unique properties, particularly its ligand and voltage gating requirements, its Ca2+ permeability, and its long-duration current (Mayer and Westbrook 1987), could generate a second-messenger signal that is proportional to the amount of activity that converges on target dendrites or somata. This signal would then initiate processes to stabilize synapses that were active. For similar reasons, this receptor has also been theorized to be important in Hebbian models of learning and memory (for review see Brown et al. 1990). Furthermore, experiments in a number of regions of the CNS, including various mammalian visual and somatosensory nuclei (Bear et al. 1990; Hahn et al. 1991; Lewin et al. 1994; Li et al. 1994; Simon et al. 1992), climbing fiber–Purkinje cell synapses (Rabacchi et al. 1992), and the optic tecta of amphibians and goldfish (Cline et al. 1987; Scherer and Udin 1989; Schmidt 1990) have shown that chronically antagonizing or eliminating the NMDA receptor blocks the activity-dependent refinement of afferents. However, particularly in young animals, the NMDA receptor carries a large proportion of excitatory current (e.g., Fox et al. 1989; Kwon et al. 1991; Wu et al. 1996). Thus it is unclear whether the results of these studies in which the NMDA receptor is blocked reflect a critical role for the NMDA receptor as a detector of correlated afferent activity during development or whether decreasing current flow through the receptor simply causes a general decrease in activity, which could retard the differentiation of the neuropil (Kali et al. 1986).

Resolving this issue requires a central projection where changes in the efficacy of NMDA receptors can be produced and associated with detectable and graded changes in the ability of the system to stabilize coactive inputs. Specifically, if current flow through the NMDA receptor plays the initiating role in the detection and stabilization of coactive inputs, then a decrease in the receptor’s efficacy should increase the amount of nearly coincident activity necessary to drive the detection/stabilization mechanism. This reasoning predicts that receptor down-regulation should result in the loss of afferents with the lowest activity correlations, whereas synapses on afferents whose activity is well correlated with neighboring afferents should be stabilized normally. Chronic NMDA treatment of the tadpole tectum provides a preparation in which this prediction can be tested. During treatment, NMDA is released at low levels from a plastic sheet applied to the pial surface of the tectum. After chronic NMDA treatment, a long-lasting decrease in the responsiveness of neurons to acutely applied NMDA has been detected in tadpole tecta, even though the number of receptors appears to remain unchanged (Debski et al. 1991). Previous studies have shown that the treatment sharpens the borders of the eye-specific stripes of retinal ganglion cell (RGC) terminals that form in doubly innervated tecta of three-eyed frogs and tadpoles (Cline and Constantine-Paton 1990; Cline et al. 1987). In addition, the RGC terminal arbors within the treated regions of these tecta have significantly reduced numbers of distal branches (Cline and Constantine-Paton 1990). The synapses normally supported by these distal branches are also lost (Yen et al. 1995). These structural effects are only present in doubly innervated tecta, where the degree of affer-
ent activity correlation per unit volume of neuropil is lower than in singly innervated tecta (Cline and Constantine-Paton 1990; Constantine-Paton and Ferrari-Eastman 1987; Law and Constantine-Paton 1981; Norden and Constantine-Paton 1994). Retinal synapses and arbor morphologies are not altered by NMDA treatment of singly innervated tecta, and therefore the changes observed with chronic treatment of doubly innervated tecta are unlikely to reflect a direct effect of NMDA on the retinal afferents (Yen et al. 1995). In short, NMDA treatment only causes a loss of synapses in the regions of tectal neuropil where activity correlations among RGC afferents are relatively low: in doubly innervated tecta; at stripe boundaries where uncorrelated inputs from two eyes mix; and in the distal regions of RGC terminals, where the new synapses formed by random sprouting (O’Rourke and Fraser 1990) are unlikely to encounter well-correlated neighbors.

The previous physiological study on the effects of chronic NMDA treatment was performed with the use of extracellular recording techniques and thus could not identify the cellular bases for the decreased responsiveness of tectal neurons to acutely applied NMDA (Debski et al. 1991). To support the hypothesis that the NMDA receptor is central to the detection and stabilization of affерents with correlated activity patterns, the locus of the decrease in NMDA effectiveness in tecta must be at the level of the NMDA receptor or channel. In this report, we use whole cell voltage-clamp techniques in slices of tecta treated for several weeks with NMDA to demonstrate a decrease in neurotransmitter effectiveness that is selective for NMDA current, occurs at synaptic and extrasynaptic receptors, and is detectable at the level of single NMDA receptor channels. Some of these data have appeared previously in abstract form (Hickmott and Constantine-Paton 1991, 1993a).

METHODS

Chronic treatment

For chronic treatment of tecta, Elvax plastic polymer (gift of DuPont) was infiltrated with 10^{-4} M NMDA as detailed previously (Cline and Constantine-Paton 1989; Silberstein and Daniel 1982). Briefly, Elvax beads were thoroughly washed in 95% ethanol and 100 mg of beads were dissolved in 1 ml of methylene chloride for >1 h. Ten microliters of 10^{-5} M NMDA (Sigma) dissolved in water, or 10 μl of water for sham treatment, and 30 μl of fast green (1 mg/ml in dimethyl sulfoxide) were added to the Elvax and vortexed to suspend the solution evenly throughout the Elvax. The Elvax was then rapidly frozen to −100°C and left overnight, then desiccated overnight at −20°C and placed under vacuum for 2 days to remove all methylene chloride and solidify the Elvax. The Elvax was subsequently cut into 30-μm-thick slices on a cryostat and stored for <2 wk at −20°C until implantation into tadpoles.

Elvax slabs were implanted over the tecta of Taylor and Kollros stage XV–XVII tadpoles in the following manner. Tadpoles were anesthetized by immersion in 0.05% ethyl m-amino benzoate (MS-222, Sigma). The tecta were then exposed, the dura was slit along the midline, and the pia was removed from the rostral half of the dorsal tectum. Elvax pieces were trimmed to fit over the exposed region of the tectum and were placed on the tectal surface and held in place by the dura and skull. The skull and skin were replaced, and the wound was closed with Vetbond (3M). Animals recovered from surgery in oxygenated dilute Instant Ocean. Sham Elvax was prepared and implanted in an identical manner, but without added NMDA, to control for possible effects of Elvax and surgery. Recordings were performed 4–6 wk after surgery and the position of the Elvax was verified before the brain was sliced; any animals with displaced Elvax were not used.

Slice preparation

Slices of optic tectum and diencephalon were obtained as detailed previously (Hickmott and Constantine-Paton 1993). Briefly, brains were rapidly removed from anesthetized (0.1% MS-222) late larval Rana pipiens (Taylor and Kollros stage XVI–XXV+). The brain was embedded in solidifying low-gelling-temperature agar (Sigma Type 7, 4.3%) and 500-μm-thick slices were cut and maintained in cold (16–18°C) buffer (composition, in mM: 112 NaCl, 2 KCl, 17 NaHCO3, 3 MgCl2, 3 CaCl2, and 12.2 dextrose, pH 7.3–7.4) saturated with 95% O2–5% CO2. Slices were left in buffer for >1 h before being stabilized in the bottom of a low-volume recording chamber by a thrombin clot (Blanton et al. 1989). The alternating fiber and cell layers of the tectum were clearly visible at low power (×40) in these slices with side illumination. Currents were recorded with an Axopatch 1D amplifier (Axon Instruments) from neurons in tectal layers 6 or 8 with the use of the blind technique developed by Blanton et al. (1989). A buffer similar to that detailed above was used for recording, but no Mg2+ was added, so that NMDA currents would not be blocked. The electrode filling solution (composition, in mM: 100 CsCl, 10 ethylene glycol-bis(β-aminopropyl ether) N,N,N’,N’-tetraacetic acid, 20 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, 0 MgCl2, 1 NaCl, and 2 MgATP, pH 7.1–7.3) yielded tip resistances of 5–12 MΩ. Relatively high-resistance electrodes were needed to record from these small (generally ~10 μm soma diameter) neurons. The series resistance was examined periodically throughout the experiment and any neurons in which it changed significantly were not used. Amplified currents were filtered (Bessel low-pass filter at 2 kHz), digitized at 0.25–10 kHz, and stored for offline analysis on an Epson Equity II computer with the use of RapidSystems Data Acquisition System or on a Macintosh IICX computer with the use of custom-written software for a Lab-NB acquisition board (National Instruments). Appropriate compensation for electrode and membrane capacitance and series resistance were determined and examined periodically throughout the experiment. The approximate input resistance of the cell was calculated by examining the current that resulted from a −100-mV, 300-ms square pulse. To avoid cells that were damaged or may have poorly sealed to the electrode, only cells with resting potential between −70 and −50 mV with input resistance >250 MΩ were used.

Spontaneous excitatory postsynaptic current analysis

Eight untreated, seven NMDA/Elvax-, and six sham/Elvax- treated animals were used for the spontaneous excitatory postsynaptic current (sEPSC) analysis. sEPSCs were recorded in the presence of 25 μM (-)bicuculline methiodide to block spontaneous inhibitory postsynaptic currents. Neurons were held at −100 mV to improve signal-to-noise ratio and there was no Mg2+ in the bathing saline. Currents were digitized at 5 or 10 kHz. Events were classed as sEPSCs if they 1) exceeded −4 pA from baseline and 2) had relatively rapid time-to-peak (<6 ms). At least 30 events were acquired for baseline. Then, to determine the relative contribution of NMDA current to these sEPSCs, 100 μM (+)-2-amino-5-phosphonovaleric acid (APV) was bath applied to the slice for ~15 min, which was sufficient for maximal effect, and ≥30 sEPSCs were acquired. To be assured of reversibility, another 30 sEPSCs were acquired after 30–40 min of bath-applied normal buffer. Only neurons in which sEPSCs were obtained both during APV application and after washout were used for subsequent analysis.
For each condition (i.e., control, APV, and washout) in a given neuron, the initial part of the rising phase of each sEPSC was used to align the sEPSCs with each other, and then a 50-ms time window from that point was averaged across 30–50 sEPSCs. Note that sEPSCs were not used if there was a subsequent sEPSC within this 50-ms time window. Three parameters were determined for each averaged sEPSC. 1) Rise time was measured from the baseline to the peak of the averaged current. 2) Peak amplitude was measured from baseline to peak of the sEPSC. 3) Fall time was measured by determining the τ value for a single-exponential fit to the falling phase of the sEPSC (see Fig. 1B). Standard least-squares minimization (Microlab Origin software) was used to fit these exponentials. Note that all averaged sEPSCs were well fit by a single exponential (r > 0.9), so no attempt was made to fit higher-order exponentials to the falling phase. Data from neurons in which these averages did not return to within 20% of their original responses after APV washout were discarded. The averaged parameters from sEPSCs before APV application and after washout were not significantly different (paired Student’s t-test) and were averaged together and are referred to as “control” responses. Control and APV averages were then compared with the use of planned, paired two-tailed Student’s t-tests. P < 0.05 was considered to be significant for these and all subsequent statistical tests. Values are expressed as means ± SE.

The magnitudes of the contributions of NMDA currents to compound sEPSCs were estimated by subtracting the averaged sEPSCs for a neuron in the presence of APV from the average of the control sEPSCs for that neuron. These averages were aligned such that the start of their rising phases were in register. The average time-to-peak of the current in control sEPSCs averaged across all treatment groups was 2 ms (see Fig. 2); therefore the amplitude at 2 ms was used as a measure of the fast, presumably non-NMDA-mediated component of the sEPSC. The peak in the difference current averages across all treatment groups occurred at an average latency of 7.5 ms (Fig. 3); therefore the amplitude at 7.5 ms was used as a measure of the NMDA-mediated component of the sEPSC. The amplitudes of the difference currents for each neuron at each of these two latencies were compared across treatment groups with the use of a one-way analysis of variance (ANOVA). A significant difference was found across treatment groups at the 7.5-ms latency, and comparisons of the 7.5-ms amplitudes between pairs of treatments used two-tailed Student’s t-tests. To test the specific hypothesis that the 7.5-ms-latency currents were larger in the sham group versus the untreated neurons, a one-tailed t-test was employed.

### Agonist iontophoresis

Ten untreated, seven NMDA-treated, and six sham-treated tadpoles were used for this analysis. (±)-α-Amino-3-hydroxy-5-methylisoxazole-4-propionic acid (AMPA) and NMDA currents were evoked in slices bathed in buffer that contained 0.5–1.0 μM tetrodotoxin (Sigma) to prevent responses from evoked action potentials in the afferents and to facilitate measuring currents. Further, the buffer contained no added Mg²⁺ to prevent block of NMDA currents. Iontophoresis electrodes were pulled from four-barrel filament glass (AM Systems), the tip of which was broken back under a microscope to a diameter of ~4–6 μm. Two barrels were filled with 50 mM AMPA-HBr (RBI) and the other two with 50 mM NMDA. Each barrel was connected to a constant-current source (Neuro Data Instruments, Model IP-X5), and the resistance through each channel was determined; these resistances ranged from 20 to 100 MΩ and were similar among the four barrels of a given electrode. A 50- to 100-nA retaining current was continuously applied to each barrel.

The electrode was placed in the slice relatively close to the voltage-clamped cell, and 1-s pulses of −0.8 to −1.0 μA in amplitude were used to evoke AMPA currents in the cell. The iontophoresis electrode was then repositioned to optimize the AMPA response. The ejection currents for each agonist were then increased or decreased until a maximal amplitude current was obtained for AMPA and NMDA. If it was impossible to achieve saturation of the current, the iontophoresis electrode was moved until it was possible; if this never occurred, the cell was not used for quantitative analysis. The maximal agonist-evoked currents were then elicited with an interstimulus interval of 90 s and alternating AMPA and NMDA presentation at various holding potentials, typically between −80 and −20 mV. These currents were digitized at 25–100 Hz and stored on computer hard disk for later analysis.

For quantitative analysis, a current-voltage (I-V) plot was generated for the peak amplitudes of the maximal AMPA and NMDA currents for each neuron. Because there was no added Mg²⁺ in the bathing buffer, both AMPA and NMDA plots were relatively linear, and the standard linear regression line was determined for each plot. The slope of each of these regression lines was used as a measure of the overall magnitude of the appropriate current. The ratio of the NMDA slope to the AMPA slope (N/A ratio) was used as a measure of the relative amount of NMDA current for a neuron in the presence of APV from the average of the control sEPSCs for a particular neuron (Hille 1992). This measure was used to minimize variability in the data, particularly in electrode position relative to the soma of the neuron. The mean slopes and N/A ratios were then compared across treatment groups with the use of a two-way ANOVA, followed by planned comparisons between the groups with the use of a Student’s t-test. Values are expressed as means ± SE.

### Single-channel recording

Six untreated, four NMDA-treated, and three sham-treated tadpoles were used for this analysis. Outside-out patches were obtained by pulling the recording electrode off the neurons after the whole cell configuration was obtained; channels were recorded in ~20% of these. All data are from patches held at −120 mV with no
Mg$_2^+$ added to the buffer. NMDA (100 mM) was iontophoretically applied to these patches in 1-s pulses (−0.5 to −1.0 μA, retaining current 50 nA). Amplitudes and open times of ≥100 channel opening events were used to determine the mean amplitude, open time, and probability of opening for each neuron. Channels were considered to be open until the current returned to within 90% of baseline; small changes in the channel current were thus not taken to be closings. Mean amplitude values for each neuron were obtained from the median of the single Gaussian least-squares fit to the amplitude distribution of each neuron. Mean open times were obtained from the fall time τ of the least-squares single-exponential fit to the open-time frequency distribution of each neuron. For both, individual means were averaged to yield overall mean values. These mean values were compared with the use of a one-way ANOVA, followed by planned two-tailed Student’s t-tests. Values are expressed as means ± SE.

Note that for all three of these assays, data from individual neurons were pooled across animals rather than mean data from each animal being pooled. This procedure was justifiable, because in the majority of cases (59%) only a single neuron was obtained from a given animal. Furthermore, in the remainder of the animals, the mean intra-animal variance of the data was not significantly different from the mean interanimal variance in the appropriate group of animals.

**RESULTS**

Three groups of *Rana pipiens* tadpoles were used in each of these studies: normal tadpoles, stage-matched tadpoles treated for 4–6 wk with slabs of Elvax plastic implanted over the dorsal tectum, and stage-matched tadpoles in which the Elvax was infiltrated with 10$^{-4}$ M NMDA. All neurons studied were in tectal layers 6–8 and were from the rostral-dorsal region of the tectum, which was directly below the Elvax implant. The mean resting potential, input resistance, and series resistance of neurons did not differ significantly among the three treatment groups (planned, 2-tailed Student’s t-test). The pooled mean values for these parameters were $-56 \pm 1.7$ (SE) mV, 3.63 ± 0.38 GΩ, and 13.3 ± 1 MΩ, respectively.

**Analysis of sEPSCs**

The NMDA-mediated current contributes predominantly to later portions of glutamatergic synaptic events. At these same latencies there is often a significant contribution from various polysynaptic currents. In our previous studies, in which the tectal slice preparation was used, we found that a polysynaptic component could not be unambiguously eliminated from EPSCs evoked by electrical stimulation of the optic tract, even at minimal stimulation intensities (Hickmott and Constantine-Paton 1993b). Largely for these reasons, in this study we have used sEPSCs, which definitely reflect monosynaptic activity, although from a heterogeneous population of retinotectal, intratectal, and thalamotectal contacts, to assay changes in the NMDA receptor contribution to synaptic events following chronic treatments. In addition, previous data obtained from electron microscopic analyses of tectal synapses after similar treatments revealed that the anatomic effects on inputs in the retinotectal neuropil are not restricted to RGC terminals (Yen et al. 1993). Thus analyses of sEPSCs also provide an effective method of broadly sampling the tectal synaptic population for functional changes that might be the basis of these anatomic responses.

sEPSCs were recorded from neurons in slices bathed in 25 μM (−)bicuculline methiodide to block inhibitory synaptic currents and in 0-Mg$_2^+$ to maximize NMDA receptor permeability. In the presence of the AMPA receptor blocker 6-cyano-7-nitroquinolinic acid-2,3-dione (CNQX, 10 μM), purely NMDA-mediated sEPSCs could be detected (data not shown), but their small size made it impossible to obtain
an unbiased sample of sEPSC parameters. Moreover, for the majority of events, decay kinetics could not be measured accurately, because the bulk of the decay was masked by noise. Attempts to use changes in the holding potential to enhance these small currents were also unsuccessful. Consequently, to maximize unbiased sampling, 100 μM APV was applied to each slice to block the NMDA receptor after an initial series of sEPSCs had been recorded from a cell. A second series of sEPSCs was then recorded in the presence of APV, followed by antagonist washout and a final series of sEPSC recordings. Comparisons of the events recorded in the presence and in the absence of active NMDA channels were used to determine the contribution of NMDA-mediated current to the sEPSC. As can be seen in Fig. 1A, there is significant variation in the sEPSCs recorded from a given neuron, probably because there is a heterogeneous population of synapses contributing to the sEPSCs detected. Consequently, the sEPSCs recorded with and without APV from a single neuron were averaged and compared (Fig. 1B). The solid traces in Fig. 1 illustrate the averaged sEPSCs in the presence or absence of APV, and the dotted traces show the single exponential that best fits the falling phase of each sEPSC average. In the untreated neuron, the main effect of APV is to reduce the falling time of the sEPSC, with little effect on its amplitude or rise time. The estimate of the fall time, measured from the single-exponential fit, for this sEPSC changed from 5.35 to 3.35 ms with the addition of APV. For the neuron in the NMDA-treated tectum, however, APV had little effect on the sEPSC. Fall time τ was 4.7 ms before APV and 4.4 ms during APV application.

These effects of APV were quantified for neurons from all three treatment groups (Fig. 2). Each set of histograms represents one of the treatment groups and compares the values for the rise time (R), the fall time (τ), and the peak amplitude (A) of the averages of sEPSCs from each neuron under control (black bars) and APV-bathed (hatched bars) conditions. Across the three treatment groups, there was no significant difference in either sEPSC rise time or peak amplitude in the presence or absence of APV. However, APV application did cause a significant decrease in τ (P < 0.05, 2-tailed paired t-test) in both the untreated (Fig. 2, left) and sham-treated (Fig. 2, right) groups, indicating that NMDA-mediated current contributed significantly to the falling phase of the compound sEPSC. By contrast, in neurons from NMDA-treated animals, τ was unaffected by APV.

To examine the putative NMDA current directly, the averaged sEPSCs in the presence of APV were subtracted from the averaged control sEPSCs for each neuron, referred to as the “difference current.” Examples from an untreated (top) and NMDA-treated (bottom) neuron are shown in Fig. 3A. This difference current thus corresponded to the APV-sensitive current quantified in Fig. 2. As would be expected from the data presented in Fig. 2, there is a significant difference current in neurons from untreated animals that is not observed in neurons from NMDA-treated animals. Unfortunately, the variability in the difference current waveforms was high, making it impossible to analyze the kinetics of the difference currents in detail. Consequently, to compare the currents across treatment groups quantitatively, we measured the amplitude of the difference currents at 2 and 7.5 ms (Fig. 3B). Two milliseconds corresponds to the mean rise time of control sEPSCs (Figs. 1B and 2), and thus amplitudes measured at this latency estimate the fast, predominantly non-NMDA component of the response not factored out by the subtraction. Seven and one-half milliseconds is the average time-to-peak of the difference currents from neurons in untreated and sham-treated tecta, and thus amplitudes measured at this latency should reflect the later, predominantly NMDA-receptor-mediated, component of the response. Seven and one-half milliseconds also corresponds closely to the fall time measured for sEPSCs (Fig. 2). The difference currents of the averaged sEPSCs measured at 2 ms were small and so highly variable that they were not significantly different from zero in any treatment group (1-group t-tests). Thus there was no apparent effect of APV on the rapid, primarily non-NMDA-mediated region of the sEPSC. In untreated and sham-treated neurons the difference currents measured at 7.5 ms were significantly larger than zero (1-group t-test) and less variable than those measured at 2 ms. Moreover, comparison among the treatment groups of the amplitudes measured at 7.5 ms indicated that NMDA-treated neurons had currents that were much smaller and differed significantly from the amplitudes measured at 7.5 ms in neurons from both the untreated (P < 0.05, 2-tailed t-test) and the sham-treated (P < 0.005, 2-tailed t-test) tecta. Figure 3B also indicates that the difference current amplitudes measured at 7.5 ms in sham-treated animals are different from those measured in neurons from untreated tecta (P < 0.05, 2-tailed t-test). The increase in sham versus untreated was significant at P < 0.01 level with the use of a one-tailed test. Thus it appears that there is an NMDA-receptor-mediated current contributing to the later portion of the sEPSCs in untreated and in sham-treated tecta. Unexpectedly, this current is larger in sham-treated animals. However, a similar NMDA-receptor-mediated current is undetectable or absent in NMDA-treated animals.

Most normal tectal cells show EPSCs mediated by both NMDA and non-NMDA glutamate receptors in response to optic tract stimulation (Hickmott and Constantine-Paton 1993a). Therefore it seems unlikely that the sample of 10 neurons contributing to the sEPSC analysis from NMDA-treated tecta could, by chance, all be members of a minority tectal population that normally do not show NMDA receptor function. Alternatively, the apparent reduction in the NMDA receptor contribution to the sEPSCs could be an artifact resulting from changes in the morphology and therefore in the electrotonic filtering of tectal dendrites. For example, we could be sampling a different population of synapses in each of the three treatment groups. To examine this possibility, we analyzed the raw sEPSC data for evidence of treatment-associated changes in the cable properties of the cells. As stated previously, there was no significant change in the mean resting potential, input resistance, or series resistance with either treatment. Figure 4A shows graphs of the rise time versus the peak amplitude for each individual sEPSC for the three treatment groups. The line in each graph is the standard linear regression line for the data. For each treatment group there is a significant correlation between sEPSC rise time and peak amplitude (P < 0.0001, F test), suggesting that there is electrotonic filtering of the sEPSCs across the dendritic tree of tectal neurons. However, neither
FIG. 4. Chronic treatment does not change cable properties of tectal neurons. A: rise-time-vs.-amplitude plots for all sEPSCs from each neuron in the 3 treatment groups. Lines: standard linear regression line fit to points. \( R \) values for lines: 0.607, 0.608, and 0.639; slopes: 0.46, 0.49, and 0.41 for untreated, NMDA-treated, and sham-treated animals, respectively. B: cumulative frequency histogram of peak amplitude of sEPSCs for untreated (black bars), NMDA-treated (gray bars), and sham-treated (hatched bars) neurons. Binwidth: 2 pA.

Analysis of agonist-evoked currents

Another possible explanation for the effects of NMDA treatment on sEPSCs is that NMDA treatment causes a redistribution of NMDA receptors such that there are fewer NMDA receptors at synapses. Because there is no reduction in the number of NMDA binding sites in the tectal neuropil with chronic NMDA treatment (Debski et al. 1991), such a redistribution would be unlikely to affect agonist-evoked current, which can activate NMDA receptors over a large portion of a tectal neuron. Consequently we examined the combined responses of synaptic and nonsynaptic glutamate-receptor-mediated currents in voltage-clamped neurons due to iontophoresis of AMPA or NMDA. For these experiments, the slices were bathed in 0-Mg\(^{2+}\)/Ca\(^{2+}\) buffer supplemented with 1 \( \mu \)M tetrodotoxin to remove the Mg\(^{2+}\) block on the NMDA channel and isolate the responses of a single neuron, respectively. The maximal responses of each neuron to AMPA and NMDA were analyzed at a variety of holding potentials (Fig. 5A). The NMDA-evoked currents were unaffected by bath application of 10 \( \mu \)M CNQX but were blocked by 100 \( \mu \)M APV, whereas AMPA-evoked currents were unaffected by 100 \( \mu \)M APV but blocked by 10 \( \mu \)M CNQX (\( n = 4 \) neurons, data not shown). Thus this iontophoresis protocol activated AMPA and NMDA receptors specifically, with little cross activation. Each neuron’s response to the particular agonist could be described by the slope of the \( I-V \) relation, which was linear for both NMDA and AMPA because of the absence of external Mg\(^{2+}\) (Fig. 5B). To control for differences in the relative position of the iontophoresis electrode and the ejection characteristics of the electrode, the NMDA response was normalized with respect to the AMPA response for each neuron. Thus the overall measure of the relative contribution of the NMDA current (referred to as the N/A ratio) was the slope of the linear regression line of the NMDA \( I-V \) plot divided by the slope of the linear regression line of the AMPA \( I-V \) plot. This showed that no significant differences were observed in the N/A ratio for this cell was 1.0.
line of the AMPA I-V plot (AMPA slope). For the neuron shown in Fig. 5, the N/A ratio is 1.0, reflecting nearly equal contributions, at least for the peak currents, of NMDA and AMPA receptor channels.

Quantification of the agonist iontophoresis data is shown in Fig. 6. Chronic NMDA treatment (gray bars) resulted in a significant decrease in the mean N/A ratio (Fig. 6, right) of tectal neurons when compared with the mean ratio from neurons in untreated (black bars; $P < 0.01$, 2-tailed $t$-test) or sham-treated (hatched bars; $P < 0.005$, 2-tailed $t$-test) tecta. This decrease in N/A ratio resulted from a decrease in NMDA slope (Fig. 6, middle) rather than an increase in AMPA slope (Fig. 6, left). A significant increase in the N/A ratio and NMDA slope in sham-treated neurons compared with untreated neurons ($P < 0.05$, hatched bars, Fig. 6, middle and right) was also observed in these data. Thus, in agreement with the data obtained from analysis of sEPSCs, chronic NMDA treatment caused a decrease in NMDA current evoked by agonist iontophoresis, whereas the plastic alone appeared to actually increase the responsiveness of tectal neurons to NMDA. These results suggested that the changes in NMDA receptors produced by chronic exposure to the plastic or to the plastic plus NMDA were not simple relocations of NMDA receptor complexes on tectal dendrites and prompted an analysis of individual NMDA receptor channels.

**Analysis of single channels**

NMDA receptors on neurons from the three treatment groups were examined directly by recording currents resulting from single NMDA channel openings in outside-out patches from tectal neuron somata. Figure 7A shows examples of such channels, evoked by iontophoresis of NMDA, from patches from untreated (top trace) and NMDA-treated (bottom trace) tecta. Qualitatively, it appeared that the primary effect of NMDA treatment was a reduction in the amplitude of the current passed by the receptor channel. For quantification, mean channel amplitude and open time were determined for each patch. Figure 7B shows summary plots indicating how these two parameters were measured. Each of these plots presents, for each treatment group, the pooled data from each patch. The distributions in the top row graph the amplitude of the current for each channel opening against the frequency with which currents of that amplitude were observed. Each distribution is fit with the best-fit Gaussian distribution ($\cdot \cdot \cdot$) to determine the overall mean amplitude for the entire data set. The mean current amplitudes were $-3.8$, $-3.1$, and $-3.7$ pA for currents from untreated (left), NMDA-treated (middle) and sham-treated (right) tecta, respectively. The value for NMDA-treated animals (middle) is significantly smaller ($P < 0.01$, 1-way ANOVA, followed by 2-tailed $t$-tests) than the values for untreated (left) and sham-treated (right) animals. The bottom row contains the frequency distributions of the open time for each channel opening for all patches from each treatment group. Each plot is fit with the least-squares minimized single-exponential fit ($\cdot \cdot \cdot$). These plots were also fit with the use of double exponentials; however, these fits were no better than the single-exponential fits, so the single-exponential fits were used in this analysis. The mean values for the populations are 2.2, 2.8, and 3.8 ms, respectively. The open time for sham-treated animals (right) is significantly larger than those for untreated (left) or NMDA-treated (middle) animals ($P < 0.05$, Kolmogorov-Smirnov test).

The mean values for NMDA channel amplitude and open time were also averaged for each treatment group from the channel measurements of individual neurons, and these are plotted as histograms in Fig. 8. The mean current amplitudes were $-3.78 \pm 0.22$, $-3.16 \pm 0.08$, and $-3.77 \pm 0.05$ pA and the mean open times were $1.73 \pm 0.31$, $1.79 \pm 0.13$, and $3.08 \pm 0.19$ ms for untreated, NMDA-treated, and sham-treated animals, respectively. As in the distribution analysis (Fig. 7B), the mean channel amplitude was significantly decreased by NMDA treatment ($P < 0.01$, unpaired 2-tailed $t$-tests) and the mean open time was increased by sham treatment ($P < 0.025$, unpaired 2-tailed $t$-tests). Thus it appears that chronic NMDA treatment has the direct effect of changing the NMDA receptor/channel complex so that it passes less current when a ligand is bound. Agonist treatment may also be capable of decreasing NMDA channel open time, but it would be impossible to detect such an effect when NMDA is applied with the use of the slow-release plastic because the plastic alone appears to increase open time.

**DISCUSSION**

In this study we used three different techniques to examine the efficacy of NMDA receptors after chronic exposure to agonist. The results from these three approaches are internally consistent, but they are also unexpected in some respects. In addition, frog tectal responses have never been studied quantitatively with these techniques. Consequently, we first briefly discuss the methods and compare the properties of tectal glutamatergic transmission they reveal with those observed in other glutamatergic pathways, and then we summarize and consider the broader significance of the findings in experimentally manipulated tissue.
sEPSC analysis

sEPSCs were used in this study to assay the relative contribution of the NMDA receptor to synaptic events. This technique does not accurately sample all NMDA receptor function, because a cell’s maximal NMDA current is only observed when large and prolonged dendritic depolarization is produced by both monosynaptic and polysynaptic activity (Hickmott and Constantine-Paton 1993). However, because the presence of polysynaptic activity precludes a consistent assessment of NMDA current, the tectal sEPSCs remain the most accurate and reliably obtainable indexes of relative receptor contributions to active synapses. The properties of sEPSCs can vary widely. Typically, purely NMDA-mediated sEPSCs or composite non-NMDA plus NMDA mediated EPSCs have falling phases that are best fit by a double exponential (e.g., Carmignoto and Vicini 1992; D’Angelo et al. 1990, 1994; Hestrin 1992b). However, the falling phases of the tectal sEPSCs we have recorded were well fit by a single exponential (Fig. 1B). Our sEPSC kinetics closely resembles that of non-NMDA sEPSCs recorded in mammals (e.g., Hestrin 1992a, 1993). However, sEPSCs recorded from turtle cortex (Blanton and Kriegstein 1991) have both an NMDA component and kinetics that are best fit by a single exponential. Furthermore, NMDA currents typically peak later than our sEPSCs (~30 ms, as opposed to ~10 ms for our data). However, fast-rising (~3 ms) and fast-decaying (~40 ms) NMDA sEPSCs have been recorded in mammalian cerebellar granule cells (D’Angelo et al. 1990, 1994). The decay times of these sEPSCs were also shown to decrease with hyperpolarization (D’Angelo et al. 1994). Thus the fast kinetics of sEPSCs in our tectal preparation probably reflects an unusually small contribution of NMDA current to the sEPSCs (~1 pA, Fig. 3) at our hyperpolarized holding potential of ~100 mV, and only the early, fast decay component of the NMDA current would be resolvable above the noise. Our hyperpolarized holding potential could further decrease the fall time of tectal NMDA-mediated sEPSCs.

Agonist-evoked currents

Agonist iontophoresis was used to activate non-NMDA and NMDA receptors across large regions of pharmacologically isolated tectal neurons. Iontophoretic data can be difficult to compare across different neurons because of inconsistencies in iontophoretic electrode position and in the amount of drug ejected from trial to trial. For these reasons we used a multibarreled iontophoresis electrode and interspersed AMPA and NMDA responses in the same pattern for each
cell. We analyzed maximal currents in an attempt to saturate the cells’ response to the agonist and thus control for differences in the distance of the cell from the electrode and variability in the electrodes’ ejection characteristics. Finally, by normalizing NMDA current to AMPA current for each neuron, we attempted to factor out more variability in distance between cell and electrode. This protocol appeared to activate specifically AMPA and NMDA currents in a reproducible manner. The slow kinetics of the iontophoretically induced currents probably reflects primarily the diffusion kinetics of the synthetic agonists used under the conditions of our assay. Thus we made no attempt to compare the kinetics of the responses among different cells or treatment groups. We only compared the peak amplitude of the maximal current. This measure reflects the binding of agonist at the receptor as well as the characteristics of the underlying ion channel.

Single-channel currents

Most outside-out patches pulled from these somata showed no channel activity in response to NMDA. To assure the accumulation of sufficient records for a quantitative analysis across treatment groups it was necessary to record prolonged intervals of channel activity at one holding potential whenever active patches were obtained, rather than attempting to hold the patch at a variety of different potentials. Thus we did not determine the reversal potential of the NMDA channel currents directly, and we can only estimate the channel conductance. If, as suggested by our present and previous whole cell recordings from tectal neurons (Hickmott and Constantine-Paton 1993a), NMDA-receptor-mediated currents reverse at ~0 mV, then the estimated conductance for untreated tadpole NMDA receptor channels would be 32 pS. This value is within the range of conductance values for the NMDA channel isolated from cerebellar neurons (30–50 pS) (Cull-Candy and Usowicz 1987) and is somewhat smaller than the values obtained from hippocampal CA1 neurons (35–60 pS) (Jahr and Stevens 1987) or from striatal/mesencephalic neurons (~40–60 pS) (Nowak et al. 1984). The open time value of 1.73 ± 0.31 ms for untreated tectal NMDA channels is comparable with the open times of some populations of the NMDA-evoked events in cerebellar (2.7 ms) or hippocampal (1.9 ms) cells. Our value is considerably shorter than the open times determined for striatal/mesencephalic neurons (4.7 ms). These data, combined with the sEPSC data, imply that tectal NMDA channels may be more closely related to cerebellar NMDA channels than to those from hippocampal CA1 or striatum. Whether this similarity means that the tectal NMDA receptor expresses an NR2C-like subunit, as is observed in the mammalian cerebellum (Kutsuwada et al. 1992), remains to be determined.

It is clear from the data in Figs. 7 and 8 that both treatments also caused a decrease in the variability of the NMDA channel amplitude. This decrease might merely reflect the smaller number of animals used to obtain the single-channel data for the treated animals (6 for untreated, 4 for NMDA-treated, and 3 for sham-treated animals). Alternatively, chronic treatment might have caused the loss of a relatively uncommon, higher conductance state of the channel. Loss of such a conductance state could explain the decrease in mean amplitude observed with NMDA treatment. However, the sham treatment results argue against this interpretation. In the sham data, mean amplitude is not significantly different from the untreated mean amplitude, yet the decrease in variability of the current measurements is comparable with that seen in the NMDA-treated currents.

Changes in NMDA receptors after chronic treatments

The major finding in this study is that the tectal NMDA receptors present after chronic exposure to NMDA pass smaller currents than do the receptors on untreated tectal neurons or tectal neurons exposed for the same period to the plastic without NMDA. It is likely that this change in the properties of single NMDA channels is the basis of the observed decrease in whole cell current evident in response to NMDA iontophoresis and the decreased contribution of NMDA-receptor-mediated currents to sEPSCs. However, the effect on the NMDA receptor is not generalized to all ionotropic glutamate receptors (See Figs. 2 and 6).

In the developing amphibian visual system, the retina (Hollyfield 1968) and tectum (Currie and Cowan 1973; Reh and Constantine-Paton 1983) grow continuously throughout development. Nevertheless, the retinotectal map is maintained throughout. Therefore retinal synapses are constantly broken and reformed at new sites, presumably with the use of correlations among RGC activity to maintain a high-resolution retinotectal projection (Fraser 1983; Reh and Constantine-Paton 1984). If the NMDA receptor functions as the correlation detector during this period, receptor down-regulation should have the effect of retaining correlation detection but reducing its sensitivity. Thus a high-fidelity map should be maintained in NMDA-treated tecta, but inputs that would normally be stabilized in regions of neuropil where correlations among neighboring RGCs are low would fail to reach the more stringent stabilization threshold and would be lost. These anatomic results have been observed in NMDA-treated tecta in previous studies in which a variety of techniques was used (Cline and Constantine-Paton 1990; Cline et al. 1987; Yen et al. 1995).

It has been argued that some decrease in excitatory transmission, rather than a specific block of the NMDA receptor, is responsible for the failure to stabilize synapses of neighboring RGCs with chronic APV treatment. Similar criticisms have made it difficult to attribute the effects of chronic APV application in mammals to a specific blockade of a correlation detector function (e.g., see Fields and Nelson 1992; Scheetz et al. 1996). Transmission of visual information through the tectum is not prevented during chronic APV treatment (Udin et al. 1992), but not all tectal activity can be effectively assayed. Thus the possibility that APV application is having its effects on synaptic refinement and competition merely by decreasing excitatory transmission is difficult to exclude.

The criticism applied to the interpretation of APV treatment cannot be applied to chronic NMDA treatment. Although some excitatory drive is probably removed by receptor down-regulation, the anatomic responses to this treatment suggest an increased threshold of the postsynaptic correlation detection/stabilization system rather than decreased
synaptic transmission. This increase in stringency is evident in the loss of synapses only in the region of the doubly innervated tectum where correlations among RGC activity are lowest, not throughout the tectum. In fact the synapses that remain in these tecta show morphological signs of increased transmission efficacy (Yen et al. 1993) and synapse distribution is not altered in the proximal regions of the same terminals that show distal pruning (Yen et al. 1995). With APV or tetrodotoxin treatment all regions of RGC terminals are similarly affected and the positioning of terminals is disorganized. Thus the previous anatomic data and these physiological data suggest a link between the functional efficacy of the NMDA receptor and the mechanism that registers the degree of correlation among RGC inputs.

The current results also provide insight into other factors that might potentially have contributed to the anatomic changes in doubly innervated tecta after chronic NMDA treatment. For example, AMPA channel function is not altered in treated tecta, suggesting that normal non-NMDA glutamate receptor current is not sufficient to ‘rescue’ poorly correlated inputs in the face of decreased NMDA-mediated currents. In addition, we have been unable to detect any physiological evidence that the size of the dendritic arbors of tectal cells is significantly reduced by the chronic treatment: neither the input impedance of the whole cells nor the cable properties of the dendrites in the NMDA-treated group differ significantly from those of sham-treated or untreated neurons. It is therefore unlikely that the synaptic loss produced by chronic NMDA treatment (Yen et al. 1995) occurs because of an NMDA-induced reduction of dendritic surface area. Finally, one mechanism for competitive synapse elimination derived from work on the neuromuscular junction (Balice-Gordon and Lichtman 1994) is the physical withdrawal of receptors from the postsynaptic membrane. We cannot rule out the possibility that NMDA receptors are physically absent from subsynaptic sites, because our sEPSC analyses failed to find any evidence of an NMDA-mediated component of these events in NMDA-treated neurons. However, given the observed decreased NMDA responsiveness across the entire neuron, a simple relocation of synaptic receptors is clearly not the only basis of reduced NMDA receptor effectiveness in these cells.

Effects of sham treatment

Surprisingly, chronic treatment of tecta with Elvax lacking NMDA (sham treatment) caused an apparent increase in NMDA receptor efficacy as assessed by all three techniques (Figs. 3, 6, and 8). Possible reasons for these increases following sham treatment are currently under investigation. One likely explanation is that the plastic, by mechanical pressure, may decrease retinotectal transmission and initiate a compensatory up-regulation of NMDA receptor function. This compensation would be consistent with the observation that blockade of activity in rat visual cortex eliminates a normal developmental decrease in NMDA receptor channel open times (Carmignoto and Vicini 1992). Alternatively, removal of the pia before placement of the Elvax plastic on the tectum almost certainly causes some damage to the superficial tectal neuropil. Reactive synaptogenesis might occur after this damage in a manner similar to that described in hippocampus, where it has been shown that the relative amount of NMDA current at such synapses is increased (Turner and Wheal 1991). Regardless of its cause, the sham effect in tecta cannot account for any of the effects of NMDA treatment, because the sham effect is in the opposite direction to that observed for NMDA treatment (i.e., sham treatment causes an increase in NMDA efficacy, whereas NMDA treatment causes a decrease). Thus the response to the sham rather than invalidating the NMDA effect probably mitigates some of the effect that could be produced by NMDA alone.

Given the large increase in NMDA receptor efficacy with sham treatment, it might be expected that there would be an increase in the amount of overlap among RGC terminals in the tectum with sham treatment. However, previous anatomic studies have documented no apparent effect of sham treatment (Cline and Constantine-Paton 1990; Cline et al. 1987). Two possible explanations for this lack of effect are as follows. 1) The anatomic assays may not have been sensitive enough to detect the increases in overlap. 2) RGCs in normal tissue may already overlap as much as is possible, perhaps due to other factors that may control RGC terminal extent. Further research on sham effects is necessary to reconcile the data.

Significance of NMDA receptor regulation during development

In several regions of the mammalian CNS, including superior colliculus (Hestrin 1992b), visual cortex (Carmignoto and Vicini 1992), frontal cortex (Burgard and Hablitz 1993), cerebellum (Garthwaite et al. 1987), and hippocampus (Kleckner and Dingledine 1991), the efficacy of the NMDA receptor has been shown to decrease during normal development. It is likely that the normal developmental decreases, like the decreases we experimentally induce, reflect regulation of the NMDA receptor due to enhanced NMDA receptor activation.

These developmental changes in NMDA receptor function could be extremely important to the successful establishment of effective synapses for the following reason: early in development, glutamatergic inputs are scarce and not very effective at driving putative target neurons (Blanton and Kriegstein 1991). If current flow through the NMDA receptor is important for stabilizing these young contacts, they must be capable of triggering an effective NMDA-receptor-mediated response. Thus in early neuropil the NMDA receptor channel would have to be highly responsive to any transmitter release. As development proceeds, the presence of more and better correlated inputs will increase the activation of NMDA receptors and receptor down-regulation would function effectively in this normal context to buffer the numerous intracellular events regulated by cytoplasmic Ca2+ levels (for review see Fields and Nelson 1992; Kennedy 1989) and to prevent excitotoxic cell death (for review see Choi and Rothman 1990). An intriguing possibility is that the decreases in functional synaptic plasticity associated with the down-regulation of NMDA receptor function in mammals (for review see Fox and Daw 1993) could be due to the inability of NMDA receptors, once down-regulated, to stabilize new synaptic contacts that are likely to be poorly correlated with inputs already established on the maturing target cells.
The authors thank Drs. Dean Buonomano, Nigel Daw, and Patricia Steen for comments on various versions of this manuscript. We also thank Dr. Peter Sargent for use of Microcal Origin software.

This work was supported by National Eye Institute Grant EY-06039 to M. Constantine-Paton.

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Received 28 January 1997; accepted in final form 6 May 1997.

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