The Dynamic Range for Gain Control of NMDA Receptor-Mediated Synaptic Transmission at a Single Synapse

Lu-Yang Wang

The Program for Brain and Behavioral Research and Division of Neurology, The Hospital for Sick Children and Department of Physiology, University of Toronto, Toronto, Ontario, Canada M5G, 1X8

Although the level at which NMDA receptors (NMDARs) are activated can profoundly influence the direction and extent of long-term changes in synaptic strength, the probabilistic nature of quantal release at individual synapses makes it difficult to determine the dynamic operating range of NMDAR-mediated synaptic transmission. By continually driving glutamate release from a single high-fidelity auditory synapse with bursts of high-frequency stimuli, I show here that NMDAR-mediated EPSCs exhibited incremental summation in their amplitude and did not reach a plateau until six or seven consecutive stimuli into the train. An increase in the initial quantal output, by broadening presynaptic spikes with the potassium channel blocker tetraethylammonium (TEA, 0.2 mM), slightly increased the plateau amplitude at 200/300 Hz but shifted its peak temporally toward the earlier stimuli. These results suggest that the plateau amplitude in TEA reflects the activation of the entire population of synaptic NMDARs and hence the maximal gain of NMDAR-mediated synaptic transmission. This maximum was estimated to be 3.2-fold of the basal synaptic strength, giving a 31% occupancy of synaptic NMDARs by glutamate. Thus, synaptic NMDARs possess a broad dynamic range within which the activity-dependent control of synaptic strength and plasticity can potentially be tuned by the amount of Ca^{2+} influx associated with different levels of NMDAR occupancy within the same synapse.

Key words: calyx of Held–MNTB synapse; Ca^{2+}; glutamate release; synaptic NMDA and AMPA receptors; gain range; synaptic plasticity

NMDA receptors (NMDARs) are known to play indispensable roles in neural development, neurotoxicity, and various forms of synaptic plasticity; however, the dynamic operating range of the NMDAR-dependent synaptic transmission at a single synapse level remains unknown. Recent evidence suggests that synaptic NMDARs are not saturated by a single quanta (Mainen et al., 1999; McAllister and Stevens, 2000), raising the possibility that the synaptic strength of NMDAR-mediated neurotransmission can be regulated in an activity-dependent manner at individual synapses. Given that activation of NMDARs with different input patterns can induce distinct forms of synaptic plasticity, such as long-term potentiation (LTP) and depression (LTD) (Bear and Abraham, 1996; Chittajallu et al., 1998; Feldman et al., 1999), I have explored the dynamic range of NMDAR-mediated synaptic transmission using the calyx of Held–principal neuron synapse in the medial nucleus of the trapezoid body (MNTB) of the auditory brainstem. Because each postsynaptic neuron at this synapse is innervated at the soma by a single terminal (Morest, 1968; Kuwabara et al., 1991), direct and accurate measurement of EPSCs can be reliably made using electrophysiological means (Forsythe, 1994; Borst et al., 1996). Because the calyx of Held–MNTB synapse is highly specialized for high-fidelity synaptic transmission at high frequencies (Wu and Kelly, 1993; Wang and Kaczmarek, 1998), the full gain range of NMDAR-mediated synaptic transmission can be readily tested by delivering short trains of stimuli to activate the total population of NMDARs within the same synapse. The results presented in this study demonstrate that the maximal gain of NMDAR-mediated synaptic transmission is approximately three-fold of the basal synaptic strength.

MATERIALS AND METHODS

Preparation of brainstem slices. Slices were prepared as described previously (Forsythe, 1994). Briefly, postnatal mice (CD1xCD57 or CD1x129SV/EMS) of age 12–14 d were decapitated, and the brains were removed rapidly and submerged in an ice-cold bicarbonate-buffered artificial CSF (ACSF) solution gassed with 95% O_2 and 5% CO_2. The brainstem was glued to a vibratome stage (Leica VT1000S), and the segment containing MNTB nuclei was cut into four to six transverse slices (250 μm in thickness). The slices were incubated at 37°C for 1 hr and thereafter kept at room temperature (20–22°C) for recording.

Electrophysiological recordings. The slice was transferred to a recording chamber mounted on a Zeiss microscope fitted with Normarski optics and a 40× water immersion objective. The chamber was continuously

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Correspondence should be addressed to Dr. L.-Y. Wang, Division of Neurology, The Hospital for Sick Children, 555 University Avenue, Toronto, Ontario, Canada M5G, 1X8. E-mail: luyang.wang@utoronto.ca.

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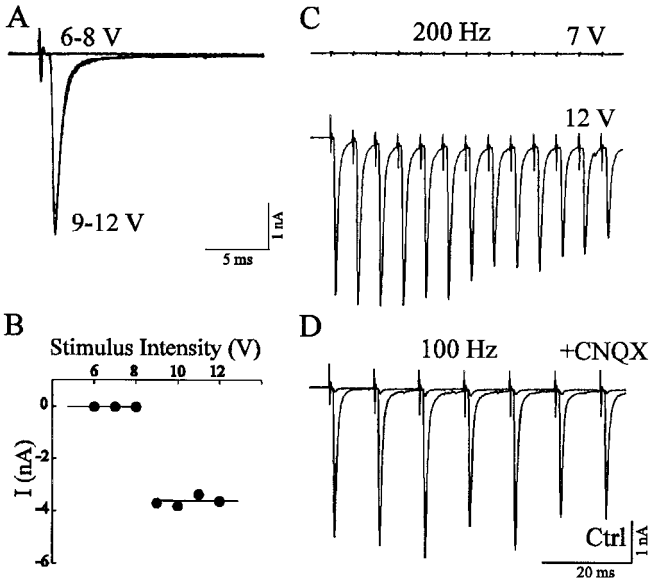

Figure 1. All-or-none synaptic transmission at single calyx of Held–MNTB synapses. *A*, A typical example of AMPAR EPSCs evoked by stimulating the presynaptic axon with increasing intensity (6–12 V, 1 V per increment). *B*, The peak amplitude of postsynaptic responses measured from *A* is plotted against the stimulus intensity, giving a threshold of 9 V at this synapse. Note that suprathreshold stimulation did not increase the amplitude of EPSCs. *C*, Postsynaptic responses to a train of subthreshold (7 V, *top*) and suprathreshold stimuli (12 V, *bottom*) at 200 Hz. *D*, EPSCs were effectively blocked by CNQX (5 μM), an antagonist for AMPARs. The holding potential for experiments included in this figure was −60 mV.

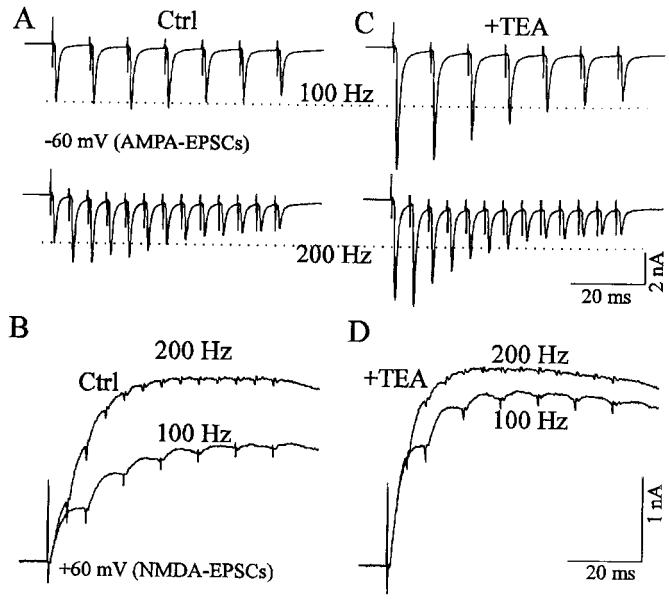

Figure 2. Multiple release events are required to saturate synaptic NMDA receptors. *A*, *C,* Glutamate release profiles in the absence and presence of TEA (0.2 mM), at a negative holding potential of −60 mV, were assessed by recording AMPAR EPSCs in response to a 60 msec train of stimuli at 100 Hz (*top panel*) and 200 Hz (*bottom panel*). *B*, *D,* After blocking AMPARs with CNQX (5 μM), NMDAR EPSCs recorded from the same synapse at a positive holding potential of +60 mV using stimulation protocols identical to *A* are shown. Note the shift of the plateau peak in the presence of TEA. The *dashed lines* in *A* and *C* indicate the level of initial quantal output. Stimulation artifacts in *B* and *D* during trains of NMDAR EPSCs are largely removed for clarity in this and subsequent figures.

perfused (1 ml/min) with oxygenated ACSF containing (in mM): NaCl 125, KCl 2.5, $NaHCO_3$ 26, NaH_2PO_4 1.25, Na pyruvate 2, myo-inositol 3, glucose 10, $CaCl_2$ 1.5, $MgCl_2$ 1, pH 7.4. Whole-cell voltage-clamp recordings were made from visually identified MNTB neurons using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The patch electrodes had a resistance of 2–4 MΩ and were filled with an intracellular solution containing (in mM): K-gluconate 97.5, CsCl 32.5, EGTA 5, HEPES 10, $MgCl_2$ 1, ATP 2, GTP 0.2, lidocaine *N*-ethyl bromide (QX314) 3 (an intracellular blocker for Na^+ currents), and TEA 30, pH 7.2. No apparent difference was observed when EGTA was replaced with BAPTA in some cases because the latter buffers Ca^{2+} more effectively. EPSCs were evoked by stimulating the presynaptic axon fiber bundle (20–30% above threshold) with a platinum bipolar electrode placed near the midline of the slices. Bicuculline (10 μM) and strychnine (10 μM) were added to the ACSF to block inhibitory inputs. The series resistance was 4–8 MΩ and compensated by 90% with a lag of 10–15 μsec. Data were filtered at 2 kHz, digitized at 10 kHz, acquired on-line, and analyzed with pClamp7 software (Axon Instruments). Averaged data were expressed as mean ± SE. TEA, kynurenic acid, strychnine, and bicuculline were obtained from Sigma (St. Louis, MO). QX-314, 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), and L(+)-2-amino-5-phosphonopentanoic acid (L-AP5) were purchased from Tocris Cookson.

RESULTS

High-fidelity synaptic transmission at the calyx of Held–MNTB synapse

Synaptic fidelity of the calyx of Held–MNTB synapse was first confirmed by whole-cell voltage-clamp recording of EPSCs while presynaptic axons were stimulated with a bipolar electrode (Forsythe, 1994; Borst et al., 1996; Wang and Kaczmarek, 1998). These EPSCs could be evoked in an all-or-none fashion (Fig. 1*A*,*B*). Suprathreshold stimulation consistently evoked a train of EPSCs without failures (Fig. 1*C*), confirming that these large responses originate from a single input. At a negative holding potential of −60 mV, the NMDAR EPSC was absent in the presence of Mg^{2+}, whereas the fast EPSCs mediated by α-amino-3-hydroxy-5-methyl-4-isoxazole propionic receptors (AMPAR EPSCs) were sensitive to CNQX (5 μM), a potent antagonist for AMPARs (Fig. 1*D*).

Saturation of synaptic NMDARs during high-frequency stimulation

To establish a correlation between the activity of synaptic NMDARs and the amount of glutamate release, it is important to measure the quantal output during a short burst of synaptic activity. Because AMPARs have rapid kinetic properties and a much lower affinity for glutamate than NMDARs (Patneau and Mayer, 1990; Clements, 1996), AMPAR EPSCs were first recorded at −60 mV to reflect the release profile of glutamate during high-frequency stimulation. In the physiological concentration of extracellular Ca^{2+} ($[Ca^{2+}]_e$ = 1.5 mM/$[Mg^{2+}]_e$ = 1 mM), the amplitude of AMPAR EPSCs typically showed facilitation within the first few pulses and then depressed slightly when stimulated at high frequencies (100 or 200 Hz) (Fig. 2*A*). This depression may be attributed to a reduction in the size of the readily releasable pool of synaptic vesicles and/or the release probability (Wang and Kaczmarek, 1998; Schneggenburger et al., 1999; Wu and Borst, 1999). Desensitization of postsynaptic AMPARs has been shown previously to make minor contribution to synaptic depression at this synapse (Wang and Kaczmarek, 1998).

Having established the release profile of each synapse in response to repetitive stimuli, I examined the behavior of synaptic NMDARs by clamping the postsynaptic neuron at +60 mV after blocking AMPARs with CNQX (5 μM). These experimental conditions were formulated to minimize the Ca^{2+}-dependent

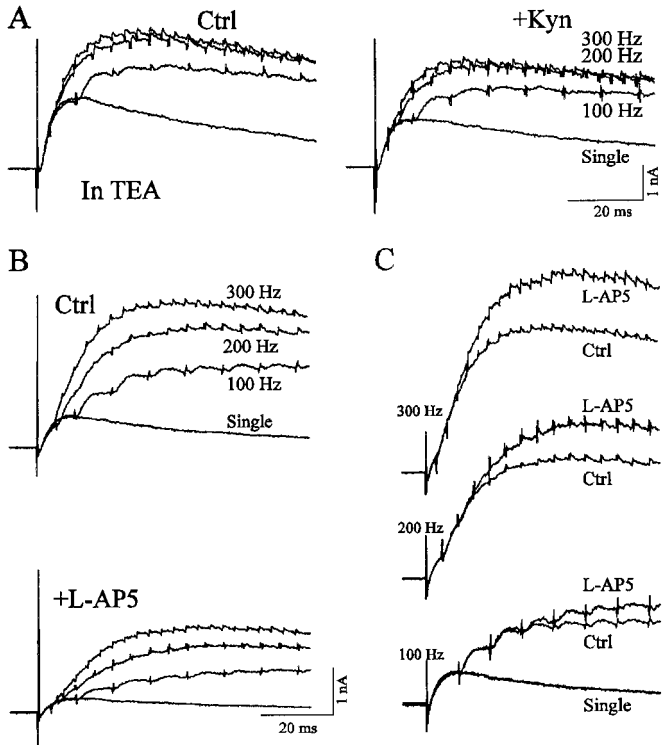

Figure 3. NMDARs are unsaturated at individual release sites. *A*, NMDAR EPSCs in response to a single stimulus or trains of stimuli (100–300 Hz) in the absence (*left panel*) and presence (*right panel*) of kynurenic acid (50 μM) are shown. The plateau levels at 200 and 300 Hz are about the same with or without kynurenic acid. Note that TEA (0.2 mM) was included throughout the experiment. *B*, Same experiment as in *A* with the exception that no TEA was added. Single and trains of NMDAR EPSCs are compared before (*top panel*) and after (*bottom panel*) addition of L-AP5 (50 μM). *C*, NMDAR EPSCs in the presence of L-AP5 are normalized to that of control traces by scaling the single EPSC and superimposed at corresponding frequencies. Similar observations were made in three other synapses.

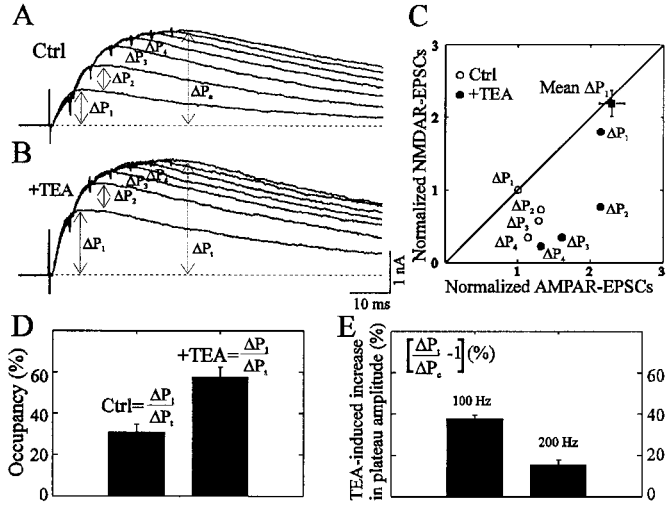

Figure 4. Low occupancy of synaptic NMDARs and large gain range of NMDAR-mediated synaptic transmission. *A*, *B*, Superimposed traces of NMDAR EPSCs in response to an incremental number of stimuli (*1-7*) before (*A*) and after TEA application (*B*) (0.2 mM). *C*, The amplitude of each NMDAR EPSC (ΔP_x) was measured individually, normalized to the first EPSC, and plotted against that of the corresponding AMPAR EPSC (also normalized from Fig. 2*A*,*C*). Note that *x* denotes the number of EPSCs, and the *square symbol* near the unity line reflects the pooled result from seven synapses in the presence of TEA. *D*, The amplitude ratio ($\Delta P_1/\Delta P_t$) between the first NMDAR EPSC and plateau in the presence of TEA are estimated to obtain the occupancy of synaptic NMDARs during basal transmission. The occupancy before and after TEA application is 30.8 ± 3.8% and 57.7 ± 4.7%, respectively ($n = 7$). *E*, The relative increase in the plateau amplitude induced by TEA is plotted to show a greater potentiation at 100 Hz (37.6 ± 1.9%) than at 200 Hz (15.3 ± 2.5%) ($n = 7$).

inactivation of NMDARs (Jahr and Stevens, 1993; Legendre et al., 1993; Wang and MacDonald, 1995) and the interaction between AMPARs and NMDARs (D'Angelo and Rossi, 1998; Yu and Salter, 1999), because NMDA EPSCs are carried by outward currents. When the same stimulation patterns were applied, NMDAR ESPCs summated incrementally to the maximal plateau after six to seven stimuli (Fig. 2*B*). Notably, this plateau has a much higher amplitude at 200 Hz than that at 100 Hz. Because the amount of glutamate release at 200 Hz was ~80% of the initial quantal output in response to the sixth or seventh stimulus (i.e., AMPAR EPSCs), this maximum indicates that the entire population of synaptic NMDARs is approaching saturation. However, the plateau at 100 Hz may instead be a result of an equilibrium between binding and unbinding of glutamate to synaptic NMDARs.

If the entire population of synaptic NMDARs were nearly saturated during the train stimulation at 200 Hz, one may predict that an increase in the quantal output would produce little increase in the plateau amplitude but may shift the saturation point temporally. The quantal output can be increased by either raising $[Ca^{2+}]_e$ or broadening the width of presynaptic spikes. Because the former is known to directly reduce the NMDAR conductance and induce Ca^{2+}-dependent inactivation (Jahr and Stevens, 1993; Legendre et al., 1993; Wang and MacDonald, 1995), the latter approach was chosen. Application of a low concentration of TEA (0.2 mM), a potassium channel blocker previously shown to induce presynaptic spike broadening at this synapse (Wang and Kaczmarek, 1998), dramatically increased the initial quantal output without changing the amplitude of AMPA EPSCs near the end of stimulation trains (Fig. 2*C*). Under such a condition, the plateau was reached within the first three or four stimuli (Fig. 2*D*). The plateau amplitude at 100 Hz showed a robust increase, whereas only a marginal increase was seen at 200 Hz. This observation suggests that synaptic NMDARs are fully saturated at the latter frequency in the presence of TEA (Fig. 2*D*). This interpretation is further supported by two additional experiments. Stimulation at higher frequency (i.e., 300 Hz) failed to further increase the plateau amplitude in the presence of TEA alone (Fig. 3*A, left panel*) or in combination with kynurenic acid (50 μM), a noncompetitive NMDAR antagonist that partially blocked NMDAR EPSCs (Fig. 3*A, right panel*). Hence, the plateau level at 200 Hz in the presence of TEA is regarded as the maximal gain of NMDAR-mediated synaptic transmission, which normally operates well below its gain ceiling at this synapse.

Previous studies have shown that NMDARs are not saturated by single quanta at typical hippocampal synapses with single release sites (Mainen et al., 1999; McAllister and Stevens, 2000). However, the anatomic structure of the calyx of Held–MNTB synapse, which contains many release sites, clearly differs from that of the hippocampal synapse. It is thus possible that NMDARs opposing individual release sites in the calyx are in fact saturated, and the summation of NMDA EPSCs represents a progressive activation of different release sites. To address this possibility, I made use of a low-affinity NMDAR competitive antagonist, L-AP5, which can be rapidly displaced by a rise in endogenously released glutamate and thus produces a degree of

block inversely proportional to the extent of NMDAR saturation (Clements, 1996; Rusakov and Kullmann, 1998; Bergles et al., 1999). Figure 3*B* shows a typical recording of single and train responses (100–300 Hz) before and after addition of L-AP5 (50 μM). When the single EPSC in the presence of L-AP5 was scaled to that of control, train responses showed relatively higher plateau levels (Fig. 3*C*), suggesting that synaptic NMDARs at individual release sites are unsaturated.

Gain range of NMDAR-mediated synaptic transmission

To directly measure the amplitude of individual EPSCs in the context of dynamic changes of glutamate binding and unbinding during each stimulation paradigm, single or multiple NMDAR EPSCs were evoked in a sequential manner in the absence and presence of TEA (Fig. 4*A*,*B*). The peak amplitude of each NMDAR EPSC in response to any given stimulus within a train was resolved by subtracting that of the preceding event(s). Figure 4*C* depicts the relationship between the glutamate release (i.e., AMPAR EPSCs) and NMDAR EPSCs at 200 Hz. The amplitude of the first AMPAR EPSC (Fig. 2*A*,*C*) and the first NMDAR EPSC (Fig. 4*A*) was used to normalize subsequent EPSCs. The relative increase in the first EPSC by TEA is well correlated (Fig. 4*C*, ■), but the ratio between subsequent NMDA EPSCs and AMPA EPSCs falls away from the unity line as the number of stimuli is increased. This is in sharp contrast to the parallel depression of both AMPAR EPSCs and NMDAR EPSCs at low frequency (i.e., 1 Hz) (von Gersdorff et al., 1997). These observations suggest that the fraction of available synaptic NMDARs after the first stimulus at high frequencies decreases dramatically. The results of these experiments, as summarized in Figure 4, *D* and *E*, demonstrate that synaptic NMDARs are not saturated in response to a single stimulus during which only 31% of total synaptic NMDARs are occupied. Thus, such a low occupancy provides a remarkably large dynamic range for the gain control of NMDAR-mediated synaptic transmission.

DISCUSSION

The observations made using a single synapse in this study provide direct evidence that synaptic NMDARs are unsaturated and that their activity can summate to different levels depending on the input patterns. Because the calyx of Held–MNTB synapse is known to contain many release sites (Morest, 1968; Kuwabara et al., 1991), and a single action potential triggers fusion events from a fraction of synaptic vesicles in the readily releasable pool (Schneggenburger et al., 1999), it is entirely possible that only those synaptic NMDARs facing the active release sites are exposed to glutamate. Therefore, the graded increase in NMDAR EPSCs during a train of stimuli may represent the summated activity of the NMDAR clusters near each release site. Assuming that NMDARs around each release site are indeed clustered and operate independently from each other, and that an action potential induces the release of 20% synaptic vesicles in the readily releasable pool (Schneggenburger et al., 1999), one can predict that the maximal gain would be 5 if each cluster is fully saturated. However, the maximal gain should be even higher than 5 when a single quanta is not sufficient to saturate each cluster. Nevertheless, what was actually measured in this study (i.e., 3.2) is clearly lower than the predicted value. This implies that NMDAR clusters are unlikely to operate independently within the same synapse. Because the structural constraints for diffusion within the large calyx synapse would likely promote spillover and rebinding of glutamate to the neighboring NMDAR clusters, particularly those located in the central area of large terminals (Trussell et al., 1993; Silver et al., 1996), it is reasonable to postulate that the basal NMDAR EPSC in the calyx–synapse may represent a summated current of immediate and peripheral clusters near each release site. This may lead to a reduction in the estimated value of the maximal gain.

Using the low-affinity NMDAR competitive antagonist L-AP5, I have also shown that there is an activity-dependent reduction in the block of synaptic NMDARs, consistent with the notion that synaptic NMDARs at individual sites are not saturated by single quanta, as suggested for synapses with a single release site (Mainen et al., 1999; McAllister and Stevens, 2000) (but see Holmes, 1995; Clements, 1996; Frerking and Wilson, 1996; Rusakov and Kullmann, 1998; Bergles et al., 1999). It should be noted, however, that the release and clearance of glutamate at the calyx-type synapses that contain multiple release sites may be quite different from other central synapses with single release sites. The most straightforward interpretation for the incremental changes in NMDAR EPSCs described here is that synaptic NMDARs are not saturated by single quanta (Fig. 3*B*,*C*) and that progressive activation of these receptors summates as more release sites are activated during a train of stimuli. Regardless of the anatomic difference between these synapses, the results presented in this study provide compelling evidence that synaptic NMDARs are unsaturated in synapses with multiple release sites.

It is well known that calyx-type auditory synapses are specialized for preserving the fidelity of synaptic transmission at high frequencies (up to several hundred Hertz) (Trussell, 1999). Frequency-dependent summation of NMDAR EPSCs may be a physiologically significant mechanism for synaptic fidelity, because it likely leads to a small and sustained depolarization of the membrane potential during trains of activity and may potentially facilitate spike generation at this giant synapse. Because central synapses have various shapes and sizes, many of which contain multiple release sites (Walmsley et al., 1998; Conti and Weinberg, 1999), it is likely that the dynamic range for NMDAR-mediated transmission may vary in different synapses. This range can be influenced by many factors, including the release probability from each release site, the peak concentration of glutamate and its fate in the synaptic cleft, and the subsynaptic arrangement of NMDARs and AMPARs (Holmes, 1995; Clements, 1996; Frerking and Wilson, 1996; Rusakov and Kullmann, 1998; Walmsley et al., 1998; Bergles et al., 1999; Conti and Weinberg, 1999). Furthermore, because activity-dependent changes in the number of release sites (i.e., perforated synapse) have been demonstrated (Bolshakov et al., 1997; Neuhoff et al., 1999), I suggest that such changes are particularly important for determining the dynamic range of NMDAR-mediated synaptic transmission. In conclusion, glutamate molecules released during a single action potential typically occupy 31% of synaptic NMDARs, and the dynamic range, which can vary by a factor of ~3, is primarily determined by the occupancy of binding sites for glutamate. The large gain range may allow activity-dependent fine tuning of Ca^{2+} influx, leading to various forms of synaptic plasticity and metaplasticity in central synapses (Bear and Abraham, 1996; Abraham and Tate, 1997; Chittajallu et al., 1998; Feldman et al., 1999). An understanding of the operating range of NMDAR-mediated synaptic transmission may also be important for the strategic design of effective therapeutics targeting glutamate binding sites for the treatment of neurological disorders.

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