Homeostatic Presynaptic Suppression of Neuronal Network Bursts

Dror Cohen and Menahem Segal
Department of Neurobiology, The Weizmann Institute, Rehovot, Israel
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Cohen D, Segal M. Homeostatic presynaptic suppression of neuronal network bursts. J Neurophysiol 101: 2077–2088, 2009. First published January 28, 2009; doi:10.1152/jn.91085.2008. Spontaneous synchronized bursts of activity play an essential role in the maturation and plasticity of neuronal networks. To investigate the cellular properties that enable spontaneous network activity, we used dissociated cultures of hippocampal neurons that express prolonged network activity bursts. Acute exposure to a low concentration of N-methyl-D-aspartate (NMDA) caused an increase in spontaneous firing rates and intracellular calcium concentration ([Ca\textsuperscript{2+}]\text{[i]}). However, in the course of a chronic (>1 day) exposure to NMDA, [Ca\textsuperscript{2+}]\text{[i]} recovered back to normal baseline levels, and only sporadic asynchronous calcium transients were detected. Spontaneous network bursts were still absent 1 h after the removal of NMDA, indicating a persistent downregulation of network activity, which did recover eventually 2 days later. This effect of NMDA was activity-dependent as it was blocked by co-application of tetrodotoxin (TTX). The chronic NMDA-treated neurons expressed normal morphology and active membrane properties as well as spontaneous miniature excitatory postsynaptic currents and postsynaptic reactivity to glutamate. However, in response to trains of afferent stimulation in paired recordings, the treated neurons expressed synaptic depression as opposed to synaptic potentiation seen in control cells. Also, treated neurons did not respond to low-intensity electrical field stimulation as did control cells. Finally, Western blot analysis revealed that chronic exposure to NMDA altered presynaptic but not postsynaptic protein expression patterns, suggesting a presynaptic locus of effect. Thus a long-lasting increase in activity downregulates neurotransmitter release to prevent over-excitation of the network and, consequently, blocks the generation of network bursts.

INTRODUCTION

Prolonged reverberations of neuronal network activity underlie brain development, maturation and plasticity (Mongillo et al. 2008; Wang 2001). Different models suggested different neuronal properties as key players in enabling the occurrence of prolonged activity periods, including recurrent excitation (Wang 2001), intrinsic cell properties (Fransen et al. 2006; Loewenstein and Sompolinsky 2003), and short-term synaptic facilitation (Mongillo et al. 2008). Yet addressing this issue experimentally requires more simplified systems than the intact brain (Lau and Bi 2005). In the later report, the authors have focused on synaptic mechanisms and demonstrated that evoked reverberations in cultured hippocampal neuronal networks could be blocked by either blocking excitatory inputs or buffering the residual presynaptic calcium. Similar observations were made on spontaneously occurring prolonged network bursts (Bacci et al. 1999). Past activity regimes shape neuronal intrinsic and synaptic properties. Yet the effects of persistent alterations in network activity on the ability to generate prolonged network bursts are not fully understood.

Homeostatic plasticity is a concept used to describe the modifications that neurons undergo while adapting to persistent alterations in input strength. It assumes a negative feedback process so as to stabilize the overall network activity and allow the system to oppose a forced change in activity pattern and return to a tolerated dynamic range (Turrigiano 1999). Possible cellular mechanisms underlying this plasticity include postsynaptic scaling up of synaptic currents (O’Brien et al. 1998; Turrigiano et al. 1998), modifications of intrinsic properties (Turrigiano et al. 1994, and presynaptic modifications of release properties (Bacci et al. 2001; Murthy et al. 2001). However, although assumed, adaptations in network activity patterns when these are persistently shifted from their initial set point have only few documented examples. Galvan et al. (2003) demonstrated an increase in network activity following a chronic activity blockade. Kim and Tsien have shown a similar increase in the frequency of spontaneous bursts in hippocampus slice cultures following activity blockade, presumably resulting from increased mossy fiber transmission (Kim and Tsien 2008). Yet an experimental system in which neuronal network activity is dramatically modified, such that spontaneous network bursts are completely abolished in a homeostatic manner, has not been documented. Investigating such system may better our understanding of the mechanisms underlying prolonged network activity states.

One of the major factors leading to neuronal death in brain injuries such as stroke and head trauma is glutamate excitotoxicity (Arundine and Tymianski 2004). Yet the injured brain may demonstrate partial recovery of brain functions, attributed in part to plasticity of the peri-injured tissue (Nudo 2007). Such plasticity may be generated by nontoxic prolonged activation of glutamate receptors in the vicinity of the necrotic tissue (Di Filippo et al. 2008). Thus understanding the plastic behavior of neuronal networks after nontoxic persistent activation of glutamate receptors might extend our understanding of the plastic injured brain.

In the present study, we investigated the effect of a chronic exposure to a low concentration [15–20 μM] of NMDA on network activity in hippocampal cultures. NMDA application increased the level of [Ca\textsuperscript{2+}]\text{[i]} and firing rates of the cells, yet in the course of a chronic exposure to NMDA, intracellular calcium returned back to normal levels. Surprisingly, the network-wide spontaneous bursts disappeared, only to recover within 2 days of removal of the drug. Paired recordings from treated neurons revealed a shift from synaptic facilitation to synaptic depression suggesting a presynaptic locus of effect.

Address for reprint requests and other correspondence: M. Segal, Dept. of Neurobiology, The Weizmann Institute, Rehovot 76100, Israel (E-mail: menahem.segal@weizmann.ac.il).
Treated neurons also demonstrated alterations in expression patterns of presynaptic markers. Thus a long-lasting increase in activity downregulates neurotransmitter release expressed through the elimination of network bursts.

METHODS

Cultures

Animal handling was done in accordance with the guidelines published by the Institutional Animal Care and Use Committee of the Weizmann Institute and with the Israeli national guidelines on animal care. Cultures were prepared as detailed previously (Papa et al. 1995).

Briefly, Wistar rats were decapitated on embryonic day 19, and their brains were removed and placed in a chilled (4°C), oxygenated Leibovitz L15 medium (Gibco, Paisley, UK) enriched with 0.6% glucose and gentamicin (20 μg/ml; Sigma, St Louis, MO). Hippocampal tissue was mechanically dissociated and passed to the plating medium consisting of 5% heat-inactivated horse serum (Gibco), 5% fetal calf serum, and B-27 (1 ml/10 ml; Gibco) prepared in minimum essential medium (MEM) Earl salts (Gibco), enriched with 0.6% glucose, gentamicin (20 μg/ml), and 2 mM glutamax (enriched MEM). About 6–8 × 10⁵ cells in 1 ml medium were plated in each well of a 24-well plate, onto 12-mm poly-L-lysine-coated glass coverslips. For pair recordings, 1–2 × 10⁵ cells in 1 ml medium were plated in each well of a hippocampal glial feeder.
layer that was grown on the glass for 2 wk prior to the plating of the neurons. For Western blots, ~1–1.2 × 10⁶ cells in 2 ml medium were plated directly on poly-l-lysine-coated 35 mm Petri dishes. In all conditions, cells were left to grow in the incubator at 37°C, 5% CO₂ for 3 days at which time the medium was changed to 10% horse serum in enriched MEM, plus a mixture of 5'-fluoro-2-deoxyuridine/uridine (20 µg and 50 µg/ml, respectively, Sigma), to block glial proliferation. Three days later, the medium was replaced by 10% horse serum in enriched MEM. Chronic treatments were added to the growth medium at 6–10 days in culture for 2–7 days. Experiments were conducted at 9–14 days in culture. The cultures were transferred to a recording chamber and bathed in standard recording medium [containing (in mM) 10 HEPES, 4 KCl, 2 CaCl₂, 1 MgCl₂, 139 NaCl, 10 d-glucose, adjusted with sucrose to an osmolarity of 325 mOsm and with HCl to a pH of 7.4).
Transfection and morphology assessments

For morphological assessments, cells were transfected with enhanced green fluorescent protein (eGFP) using lipofectamine 2000TM (Invitrogen, Carlsbad, CA) as detailed previously (Cohen et al. 2008). Transfections were conducted on day 8 in culture. Cells were visualized 5 days after transfection using inverted Zeiss confocal laser scanning microscope (LSM-510). Dendritic protrusions having a diameter of ≥0.5 μm at one point and a maximum length of 2.5 μm were considered as spines, whereas dendritic protrusions thinner than 0.5 μm were considered as filopodia. Measurements were made using the LSM image analysis software.

Calcium Imaging and culture stimulation

Cultures were Incubated for 1 h at room temperature with the standard recording medium containing 2 μM Fluor4-AM (Invitrogen). Cells were imaged thereafter on the stage of an inverted Olympus microscope equipped with a Till Photonics light source and an Andor Technology Ixon CCD camera (Belfast, Northern Ireland). Cells were imaged at 5 Hz with 20-ms exposure time to a 488-nm light. The results are presented as fluorescence units which reflects the concentration of [Ca2+]i. The regions of interest in which fluorescence was measured included the whole soma. Network bursts were considered as calcium surges that induced an increase of ≥50% in dF/dF in more then three cells simultaneously. When imaging somatic responses to exogenous glutamate application, TTX (1 μM) was added to the standard recording medium, and 400 μM glutamate was applied by a 400-ms-long air-pressure application out of a patch pipette. Responses to 200 μM N-methyl-d-aspartate (NMDA) applications were imaged in the standard recording medium containing 0 Mg2+, 100 mM glycine, TTX (1 μM), bicuculline (20 μM), and 6,7-dinitroquinoxaline-2,3-dione (DNQX, 10 μM).

Stimulation of the entire culture was performed after placing the culture in a chamber that contains two parallel platinum wires fixed to the bottom and separated by 15 mm. Neurons were stimulated electrically using an Isosflex current source (AMPI Jerusalem, Israel) by applying a train of 15 4-ms bipolar pulses at 20 Hz through the wires. The amplitude was either 6 mA for weak stimulation or 10 mA for strong stimulation. In the latter case, the volume of the recording medium was reduced from 700 to 500 μl to further increase current density.

Electrophysiology

Hippocampal neurons were recorded with patch pipettes containing (in mM) 136 K-gluconate, 10 KCl, 5 NaCl, 10 HEPES, 0.1 EGTA, 0.3 Na-GTP, 1 Mg-ATP, and 5 phosphocreatine, pH 7.2 (with a resistance of 5–10 MV). For measuring burst reversal potential and Ca2+ conductance, the pipette solution contained CsCl instead of K-gluconate. When recording burst reversal potential lidocaine N-ethylbromide quaternary salt (QX-314 2 mM, Alomone Labs, Jerusalem, Israel) was added to pipette solution for intracellular blockade of Ca2+ current. When recording Ca2+ conductance, Ba2+ replaced Ca2+ in the recording medium. The activation of fast K+ currents was investigated by delivering consecutive 200-ms-long current steps between −60 and 0 mV after a 100-ms-long hyperpolarizing step to −80 mV. The inactivation protocol included delivering 150-ms-long current steps between −90 and −20 mV followed by a 300-ms-step to +20 mV. The slow K+ currents were evoked by delivering consecutive 400 ms long current steps between −75 and +10 mV. Ca2+ channels were activated by delivering a 100-ms hyperpolarizing step to −80 mV followed by consecutive 300-ms-long current steps between −50 and +30 mV. To study synaptic connections, dual whole cell recordings were conducted in a recording medium containing 1.5 mM CaCl2 and 1.5 mM MgCl2. Minimal DC required to evoke an action potential was injected into the presynaptic neuron in current-clamp mode, and an average of four to five postsynaptic responses was recorded in voltage-clamp mode at 10-s intervals. Signals were amplified with a multiclamp700B amplifier and recorded with Clampex 9.2 (Axon Instruments, Union City, CA). The electrophysiological data were subjected to a 600-Hz low-pass filter and analyzed off-line using Clampfit-9. mEPSC recordings were analyzed off-line using MiniAnalysis software (Synaptosoft, Decatur, GA) with a detection threshold of 10 pA.

Western blot analysis

Cells were harvested in a Tris-Triton buffer (20 mM Tris-HCl, pH = 8, 100 mM NaCl, 1 mM EDTA, 0.1% Triton X-100) supplemented with a cocktail of protease inhibitors (Sigma) and mechanically lysed by vortex. Protein concentration in the cleared lysate was determined using the Bradford assay (Bio-Rad, Hercules, CA). Samples containing equal amounts of protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The membrane was blocked in PBS containing 5% skim milk powder. The primary antibodies were rabbit anti synaptotagmin1L, rabbit anti syntaxin1L, rabbit anti VAMP2, rabbit anti SNAP25 (Alomone Labs), rabbit anti synapsin1, rabbit anti GluR1, mouse anti NRI (Millipore, Bedford, MA), and rabbit anti Neuron Specific Enolase (NSE) (Zymed, San Francisco, CA). Horseradish-peroxidase-conjugated secondary antibodies and ECL were added for the visualization of the immunoreactive proteins. Two different experiments, each of them included two repeated measurements of the immunoreactive signals, were averaged after background subtraction from each blot, using Adobe Photoshop software (v 7.0).

RESULTS

Hippocampal neurons generate AMPA-R-dependent network bursts

Spontaneous network bursts in the hippocampus have been recorded in vivo as early as postnatal day 4 (Leinekugel et al. 2002). In agreement with prior reports (Bacci et al. 1999), imaging of calcium variations in hippocampal neurons revealed the occurrence of spontaneously synchronized bursts also in culture (Fig. 1A). These network bursts were correlated with prolonged inward currents recorded with whole cell patch pipettes (Fig. 1B and C). Note that under the current experimental conditions, we did not record individual action potentials but rather prolonged activity bursts. The spontaneous network bursts (Fig. 1, D–F) depended on synaptic inputs as they were diminished by an acute blockade of AMPA-Rs (Fig. 1E) but were not blocked by the NMDA-R antagonist APV (Fig. 1F).

To further evaluate the role of synaptic inputs in the network bursts, we investigated the reversal potential of the spontaneous prolonged bursts while blocking voltage gated Na+ and K+ channels intracellularly using QX-314 and Cs+, respectively (Fig. 1G). The reversal potential of these events was around 0 mV and the length and shape of the spontaneous events did not change across voltages, suggesting that they represent a concerted AMPA-dependent synaptic burst.
Chronic exposure to NMDA abolished spontaneous network bursts

We investigated the effects of a low concentration (15–20 μM) of NMDA on spontaneous activity in the culture. An acute application of NMDA resulted in a typical increase in intracellular [Ca²⁺]i [Fig. 2, A and C, P < 0.001 Tukey post hoc test following 1-way ANOVA F(2,14) = 29.772, P < 0.001] and in firing rate (Fig. 2D). However, when neurons were imaged after chronic exposure to NMDA, and still in the presence of NMDA, their baseline [Ca²⁺]i was not different from that of control cells (Fig. 2, B and C). Moreover, the treated neurons demonstrated asynchronous calcium transients instead of the normal synchronized network bursts (Fig. 2, A and B). Intracellular recordings under these conditions did not detect the typical prolonged activity bursts although individual EPSPs were frequently seen (Fig. 2E). Taken together, the chronically treated neurons exhibited markedly reduced activity compared with the one exhibited by acutely treated neurons.

FIG. 3. Parametric analysis of effects of NMDA. A–E: time course of induction of activity suppression, monitored 1 h after the removal of NMDA. A: 1-h treatment. B: 3-h treatment. C: 6-h treatment. D: 13-h treatment. E: summary: average number of bursts measured after each treatment in a 2-min recording. F–J: dose dependence, network activity dependence and reversibility of the effect. F: network bursts observed after a chronic 5 μM NMDA treatment. G: network bursts observed after a chronic 20 μM NMDA treatment combined with APV. H: network bursts observed after a chronic 20 μM NMDA treatment combined with TTX. I: network bursts observed 2 days after the chronic NMDA treatment was washed out. J: summary: averaged number of bursts measured after each treatment in a 2-min recording period monitored 1 h after NMDA removal.

FIG. 4. Chronically depolarizing neurons with high [K⁺]o also exert a long-lasting effect on network activity. A: calcium imaging of control neurons before, during, and after acute application of 7 mM KCl, indicated by ■. Zoom in box focused on the network activity before the treatment demonstrates the synchronization exhibited by the control neurons during the normal network bursts. B: calcium imaging of chronically treated neurons kept in 7 mM KCl until the imaging started. Excessive KCl was washed out and in again as indicated by ■. Zoom in box focused on the network activity before the KCl wash out demonstrates the lack of network-wide bursts. C: average [Ca²⁺]i baseline levels measured in control neurons before and during acute KCl treatment, and in chronically treated neurons still in the presence of excess KCl. D: 1 h after the wash out of excess KCl network activity is still suppressed in chronically treated neurons.

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under the same recording conditions. When imaged 1 h after washout of NMDA, cultures still did not express spontaneous 
\([Ca^{2+}]_i\) variations (Fig. 2F), and only sporadic EPSPs were recorded intracellularly. This demonstrates that the downregulation of network activity persists for at least an hour after the removal of NMDA.

We investigated the duration of exposure to NMDA needed for the persistent suppression of the spontaneous network bursts monitored 1 h after the NMDA washout. While network bursts were still detected after 1–3 h of exposure to the drug, (Fig. 3, A and B), they were completely abolished when the cultures were exposed to NMDA for ≥6 h [Fig. 3, C, D, and E, \(P < 0.008\) Tukey post hoc test following 1-way ANOVA \(F(4,28) = 5.07, P < 0.003\)]. This suggests that several hours of exposure to NMDA are sufficient to persistently modify network activity. A lower concentration of \(5 \mu M\) NMDA was not effective in silencing spontaneous network bursts (Fig. 3F). The effect was blocked by the NMDA-R antagonist APV (Fig. 3, G and J). Surprisingly, the suppression of network activity depended on action potential discharges during the treatment as it was blocked when TTX was applied together with NMDA (Fig. 3, H and J). The silencing effect of NMDA was reversed 2 days after washout of the drug (Fig. 3, I and J). The dependence of network suppression on spontaneous action potential discharges suggested that other treatments that chronically increase network activity may also generate a similar negative feedback effect.

**Chronically depolarizing neurons with high \([K^+]_o\) also blocks network activity**

To examine the specificity of NMDA, we chronically depolarized the cultured neurons by the addition of 7 mM KCl to the external medium (which depolarized neurons by 22 mV from resting level). This treatment caused an acute increase in intracellular \([Ca^{2+}]_i\) and the appearance of an asynchronous activity pattern [Fig. 4, A and C, \(P < 0.02\), Tukey post hoc test following 1-way ANOVA \(F(2,12) = 6.784, P < 0.011\)]. Chronically treated neurons imaged in the presence of high \([K^+]_o\) returned to baseline \([Ca^{2+}]_i\) levels yet they lacked the normal network bursts. Spontaneous network bursts were still absent 1 h after washout of the excess KCl (Fig. 4D). Thus depolarizing neurons by raising \([K^+]_o\), increases network activity acutely but generates a persistent downregulation of

![A Control gross morphology](image1.jpg)  ![B NMDA treated gross morphology](image2.jpg)

**FIG. 5.** Chronically NMDA-treated neurons demonstrate normal morphology. A: a GFP-transfected control cell. B: a GFP-transfected, NMDA-treated cell. C: Sholl analysis demonstrating that cells in both groups expressed similar gross morphology. D: average number of cells observed in each field in control and NMDA treated cultures. E and F: a representative dendrites of control (E) and NMDA-treated (F) cells. G and H: averaged number of spines (in which spine “head” exceeded the diameter of 0.5 \(\mu m\), G) and filopodia (“headless” protrusions, H) measured in a 30-\(\mu m\) dendritic segment.
network activity when administered chronically. Interestingly, \([K^+]_o\)-mediated depolarization was also shown in another test system (Molder et al. 2006) to reduce transmitter release probability.

**NMDA treated neurons have normal morphology**

A massive depolarization, over an extended period of time, associated with a rise in \([Ca^{2+}]_i\) is expected to cause massive changes in the morphology of the exposed neurons (Papa and Segal 1996). We studied the morphology of the chronically treated cells using Sholl analysis of randomly GFP-transfected neurons. The NMDA-exposed cells demonstrated normal gross morphology \(F(1,13) = 1.046, P < 0.325\), Fig. 5, A–C, and normal neuronal density (Fig. 5D), indicating there was no cell loss. The treated cells also expressed normal spine and filopodia density (Fig. 5, E–H).

**NMDA-treated neurons do not express altered membrane excitability**

After confirming that chronic NMDA treatment did not generate significant cell loss or morphological abnormalities, we investigated the intrinsic membrane properties of the chronically treated neurons. Neurons were not affected in the ability to produce a train of action potentials in response to a 300-ms depolarization step (Fig. 6, A and B). Spike threshold, peak amplitude, and width (Fig. 6, C–E) were unaffected as well, and there was no significant effect on input resistance and resting membrane potential (Fig. 6, F and G). We further focused on voltage-gated potassium conductances of the treated cells. We did not detect any change in the activation and inactivation kinetics of fast \(K^+\) currents while blocking the slow \(K^+\) currents with TEA (Fig. 6H, left). The activation of slow \(K^+\) currents exhibited by the treated cells was also normal (Fig. 6H, right). Voltage-gated \(Ca^{2+}\) conductance recorded in treated cells, when \(K^+\) and \(Na^+\) currents were blocked with \(Cs^+\) and TTX, respectively, and \(Ba^{2+}\) replaced \(Ca^{2+}\) in the recording medium, also demonstrated normal activation curves (Fig. 6I). Taken together, the suppression of network bursts cannot be attributed to changes in intrinsic membrane properties of the affected neurons.

**Treated neurons expressed impaired evoked synaptic responses**

The persistent suppression of network activity can be caused by a reduction in postsynaptic response to afferent activation, a change in threshold for firing action potentials, or a modification of presynaptic release properties. To examine these possibilities, we studied evoked synaptic responses at 1 h after NMDA washout. Control cultures that burst spontaneously (Fig. 7A), responded synchronously to weak electrical field stimulation (15 pulses of 6 mA at 20 Hz, Fig. 7B, 9 fields). This population response was dependent on synaptic transmission as it was blocked by the glutamate receptors antagonists DNQX and APV (Fig. 7C, 6 fields). A nonsynaptic response was detected when a strong stimulation (15 pulses of 10 mA at 20 Hz, Fig. 7D, 6 fields) was applied in the presence of the glutamate receptors antagonists to directly activate action potential discharges. In contrast, the NMDA-treated neurons did not respond to the weak stimulation (Fig. 7F, 10 fields) but did respond to the strong stimulation (Fig. 7G, 8 fields). The response to the strong stimulation was not blocked by glutamate receptor antagonists (Fig. 7H, 6 fields), indicating that this effect resulted from a direct stimulation of the neurons. Thus the NMDA-treated neurons exhibited impaired evoked synaptic responses but were not impaired in the ability to generate action potential-related calcium surges.

**In search for the locus of change I: post synaptic responses**

Given that the NMDA-treated neurons demonstrate normal active membrane properties, including spike threshold and cell excitability, leaves the synapse as the likely locus of change. We recorded miniature synaptic currents in the presence of
TTX and found no differences in the frequency or amplitudes of the mEPSCs (Fig. 8A). The fact that the mEPSCs amplitude of the chronically treated neurons was normal suggested normal postsynaptic receptors efficacy. To corroborate this observation, we imaged somatic rise in intracellular \([\text{Ca}^{2+}]_i\) following exogenous glutamate application in the presence of TTX 1 h after the NMDA washout, a time point in which network activity in the treated cultures is fully suppressed. The treated neurons expressed normal baseline calcium levels and normal responses to the glutamate application (Fig. 8B). This demonstrates that a large-scale activation of glutamate receptors produced a similar somatic response both in control and in treated neurons. To test possible desensitization of NMDA receptors, we imaged somatic rise in \([\text{Ca}^{2+}]_i\) following NMDA application. We found a 26% reduction in the responses to NMDA (\(P < 0.001\) Fig. 8C). This minimal effect on NMDA-receptor (NMDAr) activation is probably masked by other glutamate receptors such that responses to glutamate are unaffected, and by no means can it explain network suppression because a complete blockade of NMDAr with APV did not block spontaneous network bursts (Fig. 1F). Thus we concluded that network suppression is not attributed to reduced postsynaptic responses.

In search for the locus of change II synaptic depression

If indeed the spontaneous release of neurotransmitter as well as the postsynaptic response to the quantum of released transmitter are not different between the two groups, perhaps the recruitment of releasable pools is affected by NMDA. To examine this, we investigated the synaptic properties of the treated neurons using paired recordings, such that action po-

**FIG. 7.** NMDA-treated neurons are impaired in evoked synaptic responses to electrical stimulation. Top: control culture. A: spontaneous activity. B: evoked responses to weak stimulations (15 pulses of 6 mA at 20 Hz; ↓). C: evoked responses to weak stimulations in the presence of APV and DNQX. D: evoked responses to strong stimulations (15 pulses of 10 mA at 20 Hz) in the presence of APV and DNQX. Bottom: NMDA-treated culture. E: spontaneous activity. F: lack of evoked responses to weak stimulation. G: evoked responses to strong stimulations. H: evoked responses to strong stimulation is not blocked by APV and DNQX.

**FIG. 8.** Lack of effect of NMDA on post synaptic responses. A, left: representative traces of control (top) neuron and NMDA-treated (bottom) neuron recorded in the presence of TTX. Right: average miniature excitatory postsynaptic current’s (mEPSC’s) frequency (top) and amplitude (bottom). B: imaging of calcium responses to exogenous glutamate application. C: imaging of responses to NMDA applications.
tentials were evoked in one neuron and EPSC or inhibitory PSC (IPSC) were recorded from a follower cell. In control cells both excitatory and inhibitory synapses exhibited synaptic facilitation between the second and the fifth responses in a train given at 22 Hz (Fig. 9, B and D). In striking contrast, NMDA-treated excitatory as well as inhibitory synapses demonstrated synaptic depression during a train of evoked responses (Fig. 9, C and E). A significant effect was observed in the ratio between consecutive pulses and the first pulse in the train in both excitatory \(F(1,22) = 12.35, P < 0.002\) post hoc t-test: pulse 2/pulse 1 \(P < 0.001\), pulse 3/pulse 1 \(P < 0.006\), pulse 4/pulse 1 \(P < 0.016\), pulse 5/pulse 1 \(P < 0.025\) and inhibitory synapses.

synapses \( F(1,16) = 11.34, P < 0.004 \) post hoc \( t \)-test: pulse 2/pulse 1 \( P < 0.001 \), pulse 3/pulse 1 \( P < 0.003 \), pulse 4/pulse 1 \( P < 0.009 \), pulse 5/pulse 1 \( P < 0.024 \); Fig. 9, F and J). Although the treated evoked responses differed from control ones during a train, the single evoked responses in both groups were not different (Fig. 9, H and I). In addition, the paired-pulse ratio did not correlate with the first response amplitude (slope = \(-0.0023\), \( r^2 = 0.0256 \), Fig. 9J), suggesting that the synaptic depression demonstrated by the treated neurons is not a result of vesicle depletion but rather downregulation of a facilitatory release mechanism and/or potentiation of a depressive release mechanism in the affected neurons.

**Treated neurons express different patterns of presynaptic markers**

We then investigated the expression of synaptic markers in control and treated cells using Western blot analysis. The glutamate receptors subunits NRI and GluRI immunoreactivity levels were not different between control and treated culture extracts (Fig. 10). In contrast, the expression of presynaptic proteins synaptotagmin I and synapsin I was downregulated (55 and 30% reduction, respectively), and the expression of the SNARE complex proteins Vamp2 and SNAP25 was upregulated (30% and 40% increase, respectively) in the treated cells. The fact that the treated neurons exhibited altered expression of presynaptic markers further supports the hypothesis of a presynaptic locus of control.

**DISCUSSION**

In this study, we describe the suppression of prolonged network bursts in the course of chronic exposure to low concentrations of NMDA. These network bursts are spontaneously occurring synaptic driven events that do not depend on NMDAr activation. The NMDA-treated neurons demonstrated normal morphology, culture confluency, and normal intrinsic membrane properties, leaving synaptic properties as the locus of effect of the drug. Indeed culture-wide stimulations revealed impaired evoked synaptic responses to tетanic stimuli. Interestingly, the post synaptic responses of the NMDA-treated neurons were normal as indicated by the mEPSC’s amplitude, responses to exogenous glutamate applications, and single spike evoked responses. In contrast, the NMDA-treated neurons demonstrated synaptic depression instead of synaptic facilitation in response to a train of five afferent stimuli, suggesting a presynaptic locus of change. The NMDA-treated neurons also demonstrated alterations in expression patterns of presynaptic markers, further supporting a presynaptic locus of action. Thus the chronic NMDA treatment induced a presynaptic modification of release properties that blocked the generation of network bursts.

Stimulus-specific prolonged network activity periods are considered as the neural correlate of working memory (Monteiro et al. 2008; Wang 2001). Yet a comprehensive investigation of the cellular mechanisms that enable the generation of such network activity patterns requires simplified experimental systems such as the recurrent network established in primary hippocampal cultures (Lau and Bi 2005). In the later report, the authors demonstrated that evoked reverberations in small networks of hippocampal neurons are not dependent on NMDAr activation but could be blocked by buffering the residual presynaptic calcium with EGTA-AM. In agreement, our present results demonstrate that larger networks which are spontaneously active are also not dependent on NMDAr activation for generation of bursts and are most sensitive to changes in presynaptic release properties. In addition, we demonstrate that such network bursts are sensitive to past activity regimes and could be suppressed in a homeostatic manner.

The concept of homeostatic plasticity assumes a negative feedback process working to stabilize the overall network activity such as to allow the system to oppose a forced change in activity pattern and return to a tolerated dynamic range (Turrigiano 1999). Preliminary steps in demonstrating homeostatic modifications in network activity patterns were made by Turrigiano et al. demonstrating alterations in spike frequency following chronic activity blockade with TTX or chronic increased activity with bicuculline (Turrigiano et al. 1998). Galvan et al. (2003) further demonstrated increased spontaneous network activity following a chronic activity blockade by TTX. Kim and Tsien have shown a similar increase in the frequency of spontaneous bursts in hippocampus slice cultures following activity blockade, presumably resulting from increased mossy fiber transmission (Kim and Tsien 2008). However, disrupting the ability of neuronal networks to generate prolonged network bursts in a homeostatic manner has not been documented.

In the course of the chronic NMDA treatment described here, the intracellular \([\text{Ca}^{2+}]\) has returned to baseline levels, and occasional asynchronous calcium transients replaced the large-scale repetitive network bursts even in the presence of NMDA. This indicates that the persistent downregulation of the network activity pattern took place before the removal of NMDA. The silencing effect was reversed 2 days after the NMDA washout, indicating that the treatment was not too aggressive and allowed neurons to readjust their network activity pattern once more. Taken together with the fact that network suppression depended on ongoing activity during the treatment, it can be concluded that the chronic NMDA treatment has activated homeostatic mechanisms to regulate activity and eventually eliminate network bursts.

While moderate activation of NMDA-Rs promote neuronal survival, excessive activation of NMDA-Rs induce neuronal death (Hardingham and Bading 2003; Lipton and Nakanishi 1999). The chronic NMDAr activation described here is by no means neurotoxic as no cell loss was found in the treated cultures. Thus the suppression of network activity cannot be attributed to reduced culture confluence. This is further cor-
roborated by the reappearance of network bursts 2 days after the removal of NMDA.

In agreement with previous reports demonstrating adaptations in presynaptic release properties in response to chronic changes in activity (Bacci et al. 2001; Molder et al. 2004, 2006; Murphy et al. 2001; Virmani et al. 2006), we present here two lines of evidence indicating that the chronic NMDA treatment caused a persistent modification of presynaptic functions: first, the shift from synaptic facilitation to depression and second, the altered expression pattern of presynaptic markers.

While the rules governing short term plasticity are still being revised, the current dogma suggests that the direction of short-term plasticity is correlated with synaptic efficacy. Stronger synaptic contacts are more likely to exhaust their releasable pool of vesicles and express synaptic depression, while synapses with low release probability are more likely to be potentiated in response to residual calcium build up. However, the distinct short-term plasticity characteristics of control and NMDA-treated neurons described here appear without significant alterations in the initial synaptic efficacy, expressed through the normal responses to single spikes generated in the presynaptic cell and the normal mEPSC frequency. Sippy et al. demonstrated a switch from synaptic depression to synaptic facilitation without altering the basal synaptic transmission or the initial neurotransmitter release probability following the overexpression of the calcium binding protein calcium sensor-I (Sippy et al. 2003). This observation suggested separated mechanisms governing the initial release probability and short-term plasticity, while the latter is sensitive to the expression level of calcium binding proteins that may affect the calcium sensitivity of the release machinery. Such possible modification in short-term plasticity is more likely to be expressed in bursting activity patterns and not in spontaneous release of neurotransmitter.

The robust effect described here is presumably a concerted response of different proteins. However, the shift toward synaptic depression is most likely not attributed to the increased expression of the SNARE complex proteins SNAP25 and VAMP2 as the expression of these proteins is suggested to have a positive effect on Ca\(^{2+}\)-dependent vesicle release (Schoch et al. 2001; Washbourne et al. 2002). The decreased expression of synapsin-I is also not an attractive explanation because synapsin-I knockout mice do not demonstrate reduced paired-pulse ratio but rather increased paired pulse facilitation (Rosahl et al. 1993). Thus the most intriguing altered expression pattern we found is the downregulated expression of synaptotagmin I, known to act as a calcium sensor profoundly affecting synchronous Ca\(^{2+}\)-dependent transmitter release (Geppert et al. 1994; Nishiki and Augustine 2004).

Possible regulation of Ca\(^{2+}\) sensitivity of the release machinery as a presynaptic homeostatic mechanism has been suggested by Pedras-Renteria et al. (2004), demonstrating neurotransmission preservation in hippocampal synapses lacking the pore-forming subunit of the P/Q-type Ca\(^{2+}\) channel, a major Ca\(^{2+}\) entry pathway in presynaptic terminals. In agreement with this model, we propose that the NMDA-triggered increased activity leads to chronically increased levels of intracellular [Ca\(^{2+}\)] in the presynaptic terminals. This is followed by a downregulation of Ca\(^{2+}\) sensitivity of the release machinery, attributed at least in part to the downregulated expression of the Ca\(^{2+}\) sensor synaptotagmin I, acting downstream to the intracellular [Ca\(^{2+}\)] in the presynaptic terminals. This leads to a decreased Ca\(^{2+}\)-dependent transmitter release, which in turn, dampens network bursting activity.

A similar effect may be generated when other stimuli which increase network activity are used. Downregulated release probability after a chronic increased network activity triggered by elevated extracellular [K\(^+\)] was reported by others (Molder et al. 2006). However, as in many other reports in the field of homeostatic plasticity, the scope of this work did not include the consequences of this treatment on network activity patterns. We have demonstrated that such chronic treatment eliminates spontaneous network bursts. Thus two different paradigms that increase neuronal activity downregulate neurotransmitter release to prevent overexcitation of the network, and consequently, block the generation of network bursts.

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