

NMDA Receptor-Dependent Periodic Oscillations in Cultured Spinal Cord Networks

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Keefer, Edward W., Alexandra Gramowski, and Guenter W. Gross. NMDA receptor-dependent periodic oscillations in cultured spinal cord networks. *J Neurophysiol* 86: 3030–3042, 2001. Cultured spinal cord networks grown on microelectrode arrays display complex patterns of spontaneous burst and spike activity. During disinhibition with bicuculline and strychnine, synchronized burst patterns routinely emerge. However, the variability of both intra- and interculture burst periods and durations are typically large under these conditions. As a further step in simplification of synaptic interactions, we blocked excitatory AMPA synapses with 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoxaline-7-sulphonamide (NBQX), resulting in network activity mediated through the *N*-methyl-D-aspartate (NMDA) receptor (NMDA_{ONLY}). This activity was APV sensitive. The oscillation under NMDA_{ONLY} conditions at 37°C was characterized by a period of 2.9 ± 0.3 s (16 separate cultures). More than 98% of all neurons recorded participated in this highly rhythmic activity. The temporal coefficients of variation, reflecting the rhythmic nature of the oscillation, were 3.7, 4.7, and 4.9% for burst rate, burst duration, and interburst interval, respectively [mean coefficients of variation (CVs) for 16 cultures]. The oscillation persisted for at least 12 h without change (maximum observation time). Once established, it was not perturbed by agents that block mGlu receptors, GABA_B receptors, cholinergic receptors, purinergic receptors, tachykinin receptors, serotonin (5-HT) receptors, dopamine receptors, electrical synapses, burst afterhyperpolarization, NMDA receptor desensitization, or the hyperpolarization-activated current. However, the oscillation was destroyed by bath application of NMDA (20–50 μ M). These results suggest a presynaptic mechanism underlying this periodic rhythm that is solely dependent on the NMDA synapse. When the AMPA/kainate synapse was the sole driving force ($n = 6$), the resulting burst patterns showed much higher variability and did not develop the highly periodic, synchronized nature of the NMDA_{ONLY} activity. Network size or age did not appear to influence the reliability of expression of the NMDA_{ONLY} activity pattern. For this reason, we suggest that the NMDA_{ONLY} condition unmasks a fundamental rhythmogenic mechanism of possible functional importance during periods of NMDA receptor-dominated activity, such as embryonic and early postnatal development.

INTRODUCTION

The mechanisms driving the spontaneous activity of neuronal ensembles and their generation of spatiotemporal spike and burst patterns are difficult to study quantitatively in vivo be-

cause the simultaneous monitoring of neurons in small ensembles is technically extremely difficult (Bragin et al. 2000; McNaughton et al. 1983). However, neuronal networks grown in culture provide simpler systems in which a quasi monolayer architecture can be coupled to planar microelectrode arrays that allow ensemble recording over long periods of time. We used murine spinal cord networks growing on such microelectrode arrays (Droge et al. 1986; Gross 1994) to obtain long-term, multisite recording of spontaneous and pharmacologically modified activity (Fig. 1). The chemical environment can be controlled with precision, and specific activity states can be maintained and investigated for many hours, a time period usually not achievable in vivo. Like in vivo networks, cultured networks exhibit multiple synaptic driving forces resulting in complex spike and burst patterns. Therefore, this in vitro system offers the opportunity to investigate basic cellular and ensemble mechanisms underlying spontaneous activity.

In mammalian development, there is a short period during which neuronal networks are connected by purely excitatory synapses (Habets et al. 1987; Jackson et al. 1982). Throughout this developmental window, or when inhibitory synapses are blocked in more mature tissue, networks exhibit bursts of action potentials which are roughly synchronized across the population. These synchronous population bursts are proposed to be important in pruning and sculpting neuronal circuits during development (Tosney and Landmesser 1985). However, in the absence of inhibitory transmission, the mechanisms that serve to terminate bursts are unclear.

Application of inhibitory neurotransmitter antagonists, resulting in disinhibition of cultured networks, reliably induces oscillatory behavior consisting of bursts of action potentials concomitant with intracellular Ca^{2+} oscillations (Lawrie et al. 1993; Rhoades and Gross 1994; Robinson et al. 1993; Wallen and Grillner 1987; Wang and Gruenstein 1997). Similar results have also been obtained by increasing excitability with ionic manipulations such as low Mg^{2+} or increased extracellular K^{+} . Fictive locomotion can be elicited in intact spinal cord preparations with burst patterns alternating between the right and left sides of the cord by application of serotonin (5-HT), *N*-methyl-D-aspartate (NMDA), 5-HT and NMDA in combination, or elevated extracellular K^{+} (Bracci et al. 1998; MacLean et al.

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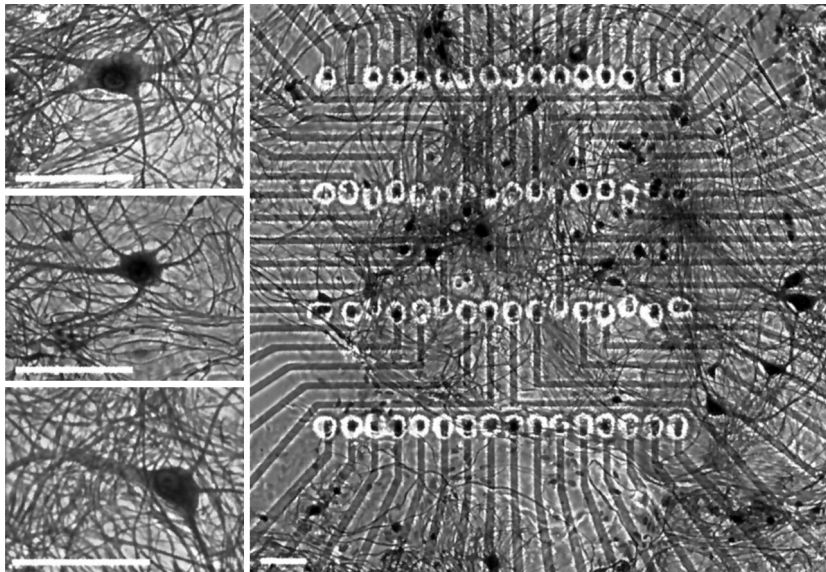


FIG. 1. Spinal cord network grown on 64-electrode array 95 days after seeding. The indium tin oxide conductors are $10\ \mu\text{m}$ wide. Electrode spacing is $40\ \mu\text{m}$, vertical spacing between rows is $200\ \mu\text{m}$. *Insets*: close-ups of neurons within the network. Bars: $80\ \mu\text{m}$. Loois-modified Bodian stain. Primary cultures derived from dissociated embryonic tissue form strong adhesion and stable cell-electrode coupling.

1998; Wallen and Grillner 1987). These studies have shown that inhibitory conductances are not essential for rhythmogenesis, but it remains unclear as to the relative contributions of cellular versus network properties in the production of rhythmic motor output.

To study burst patterns in excitatory-only coupled networks, we pharmacologically reduced the inhibitory and excitatory neurotransmitter systems that drive the spontaneous activity in cultured murine spinal cord networks to one dominated by NMDA receptor activation, a situation reminiscent of that found during the embryonic/early postnatal period (Scheetz and Constantine-Paton 1994). The resulting activity (termed “NMDA_{ONLY}”) is characterized by a highly regular burst oscillation, as evidenced by the low coefficient of variation [$(\pm \text{SD}/\text{mean}) \times 100$] values for population burst variables, burst synchronization among units in the network, and a stationary burst rate of 21.0 ± 2.0 bursts per minute (bpm; mean \pm SD, $n = 16$ cultures) that is independent of the initial activity state. Blocking NMDA receptors in the presence of bicuculline (BCC) and strychnine (STR), leaving the AMPA/kainate glutamate receptor subtypes as the sole driving force, did not produce network oscillations with the same level of precision as the NMDA_{ONLY} activity ($n = 6$). It appears that the NMDA receptor is uniquely suited for the production of the most stable burst oscillations.

We explored burst termination mechanisms with potential contribution to the NMDA_{ONLY} activity. These include after-hyperpolarization (AHP), receptor desensitization, and depression of neurotransmitter release (O'Donovan 1999). Blocking both AHP and NMDA receptor desensitization did not affect established NMDA_{ONLY} oscillations. Synaptic depression, possibly resulting from exhaustion of the readily releasable pool (RRP) of excitatory neurotransmitter vesicles, has been observed in disinhibited whole spinal cord and spinal cord slices (Bracci et al. 1996a; Streit 1993). CA3 population activity can be dominated by vesicle depletion and the time to refill the RRP (Staley et al. 1998). Our results indicate that strong synaptic depression caused by the depletion of the RRP could serve as burst termination mechanism during

NMDA_{ONLY} activity, with the time required to refill the pool setting the period of the oscillation.

METHODS

Cell culture

The techniques used to fabricate and prepare microelectrode arrays (MEAs) have been described elsewhere (Gross 1979; Gross and Kowalski 1991; Gross et al. 1985). The electrode conductor pattern consisted of a central 0.8-mm^2 recording matrix of 64 microelectrodes (Gross 1994). Dissociated tissue cultures were prepared according to the basic method established by Ransom et al. (1977). Spinal cord tissues were harvested from embryonic day 14–15 Hsd:ICR mice. The tissue was dissociated enzymatically and mechanically, then seeded at a density of $0.2\text{--}0.5 \times 10^6$ cells/ cm^2 onto MEA surfaces (Gross 1994; Gross and Kowalski 1991). Cultures were incubated at 37°C in a 10% CO_2 atmosphere until ready for use, generally 3 wk to 3 mo after seeding. Cells media was replenished twice a week with minimal essential medium (MEM) containing 10% horse serum. Spontaneous activity started at approximately one week in the form of random spiking and stabilized in terms of coordinated spike and burst pattern by 15 days in vitro. The age of cultures used in this series of experiments ranged from 18 to 113 days in vitro. Such networks can remain spontaneously active and pharmacologically responsive for more than 6 mo (Gross 1994).

Drugs and solutions

All drug applications were made to a constant volume (1,000–1,500 μl) experimental bath in a consecutive manner. Thus, all drugs for a particular pharmacological treatment (BCC/STR/NBQX for example) were in the experimental bath simultaneously (see Fig. 6). All stock solutions were made on the day of use. Concentrations were calculated to minimize volume additions to the experimental bath. Apamin, atropine, BCC, CaCl_2 , charybdotoxin, curare, Evan's blue, glycine, KCl, MgCl_2 , norepinephrine, and STR were purchased from Sigma Chemical (St. Louis, MO). APV, haloperidol, L-733,060, MCPG, NBQX, and SCH 50911 were purchased from Tocris Cookson (Ballwin, MD). Carbenoxolone was purchased from ICN (Costa Mesa, CA). Methysergide maleate was obtained from RBI (Natick, MA).

Extracellular recording procedures and data analysis

MEAs were placed in constant-bath recording chambers (Gross 1994; Gross and Schwalm 1994) and maintained at 37°C on a microscope stage. The pH was maintained at 7.4 with a continuous stream of filtered, humidified, 10% CO₂. Stainless steel chamber components were sterilized via autoclaving before each experiment. The normal bath solution consisted of a 50/50 mixture of fresh MEM and conditioned MEM with 10% horse serum supplement. The basal ionic concentrations for all experiments were (in mM) 0.8 Mg²⁺, 1.3 Ca²⁺, and 5.4 K⁺. Neuronal activity was recorded with a two-stage, 64-channel amplifier system (Plexon, Dallas, TX), and digitized simultaneously via a Dell 410 workstation (spike analysis) and a Masscomp 5700 computer (burst analysis). Total system gain used was normally 12 K. Spike identification and separation was accomplished with a template-matching algorithm (Plexon) in real time to provide single-unit spike rate data. In addition, whole channel (multiple-units/channel) data were analyzed off-line using custom programs for burst recognition and analysis. Burst patterns derived from spike integration ($\tau = 200$ ms) provided a high signal-to-noise feature extraction that has been shown to reveal modes of neuronal network behavior (Gross et al. 1994). From this approach, burst rate, duration, and interburst interval were readily quantified from individual recording sites. All data presented are based on quantification of whole-channel burst parameters, except for Figs. 5C and 10, B and D. The power spectra in these figures were calculated from spike data, and reveal prominent peaks at 0.37 Hz, corresponding to the period of 2.9 ± 0.3 s obtained from the whole-channel burst data. This confirms that the burst events are an integrated phenomenon, reflecting the ensemble dynamics of a neuronal population. Detailed analysis of the firing of individual neuronal units during their participation in the network burst events may yield insights into the mechanisms underlying the precise oscillation we characterize. However, the precision and culture-to-culture reproducibility of the phenomenon is captured by the burst analysis performed here.

Statistics

Statistical significance was determined using two-sample Student's *t*-test with $P < 0.05$ considered as significant. To quantify the periodicity of bursting, as well as the synchronization of the neuronal population participating in each burst event, we calculated the coefficients of variation over time and across the network after 15- to

30-min stabilization periods to allow the networks to reach a steady state in their activity after pharmacological manipulation. Figure 2 summarizes the calculation of CVs. CV_{time} reflects the periodic behavior of the activity pattern (Bracci et al. 1996a). $CV_{network}$ reflects the synchronization between different neurons. CV_{time} s were obtained from minute means of each channel by averaging over the experimental episode. The episode CVs per channel then were averaged to yield temporal network means. $CV_{network}$ s were obtained by averaging values in 60-s bins, followed by another average across channels that yielded a $CV_{network}$ for each minute. These CVs were averaged for each experimental episode. The CVs were calculated for different burst variables: burst rate (BR), burst duration (BD), and interburst interval (IBI) during different experimental episodes, (spontaneous, partial disinhibition with BCC, and NMDA_{ONLY}). Large CVs imply a wide range of variability in the activity of the networks across both time and the neurons recorded. Hence, if a population was synchronized, but fluctuated together, low $CV_{network}$ and high CV_{time} were obtained. Conversely, a nonsynchronized network with several regular (periodic) patterns yielded high $CV_{network}$ with low CV_{time} .

RESULTS

In their original medium (native state), spinal cord networks typically exhibited multiple patterns of activity (Fig. 3A). Disinhibition with the GABA_A receptor antagonist BCC (40 μ M), elicited robust bursting activity that was synchronized across the network (Fig. 3B). However, under BCC, the temporal variation in the burst period was still high. Further disinhibition by blocking glycine receptors with 1 μ M STR did not reduce the variability of the burst period. When AMPA/kainate receptors were subsequently blocked with 10 μ M 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoquinoline-7-sulphonamide (NBQX), the networks were entrained to one common, highly periodic burst pattern activity (Fig. 3C). This unique activity state persisted for up to 12 h without change in regularity (Fig. 3D).

Data presented in this report were obtained from 16 separate cultures. In a subset of eight experiments, the oscillations began almost immediately after addition of BCC/STR/NBQX with a period of 2.8 ± 0.2 (SD) s, suggesting some common underlying control mechanism. This oscillation period was

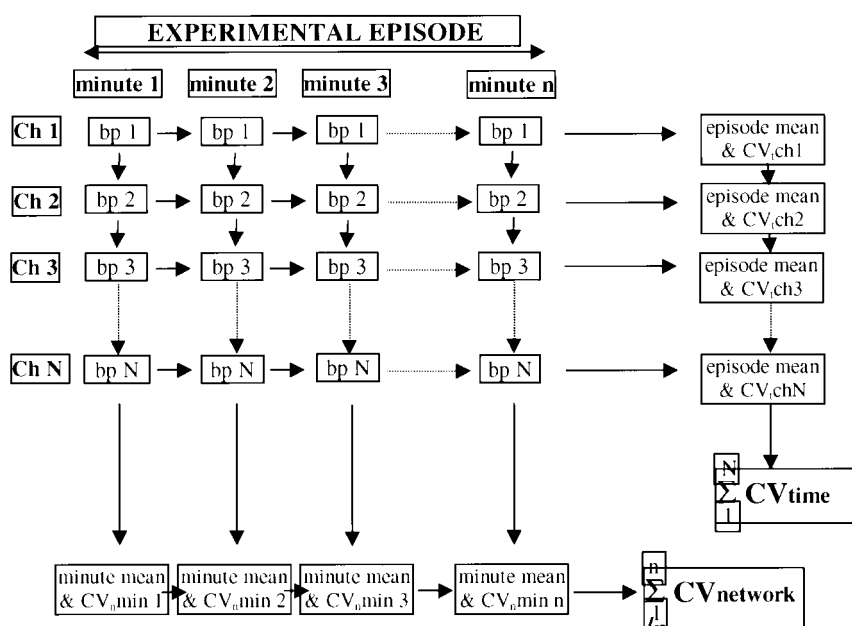


FIG. 2. Determination of temporal regularity and network synchronization using coefficients of variation (CV). All calculations are based on one minute bins where a specific burst variable (bv) is either logged as a number for BR or averaged for BD and IBI. These values are used to obtain episode means with CVs for experimental episodes (left/right) or minute means for each minute of the experimental episode (top to bottom). The episode CVs (CV_{time}) for each channel represent a measure of temporal pattern fluctuation for that channel. Averaged across the network, CV_{time} reflect pattern regularity even if several patterns exist and even if they are not synchronized. Conversely, the minute CVs ($CV_{network}$) represent channel coordination. Averaged across the experimental episode, $CV_{network}$ reflect the degree of network synchronization even if the pattern fluctuates in time.

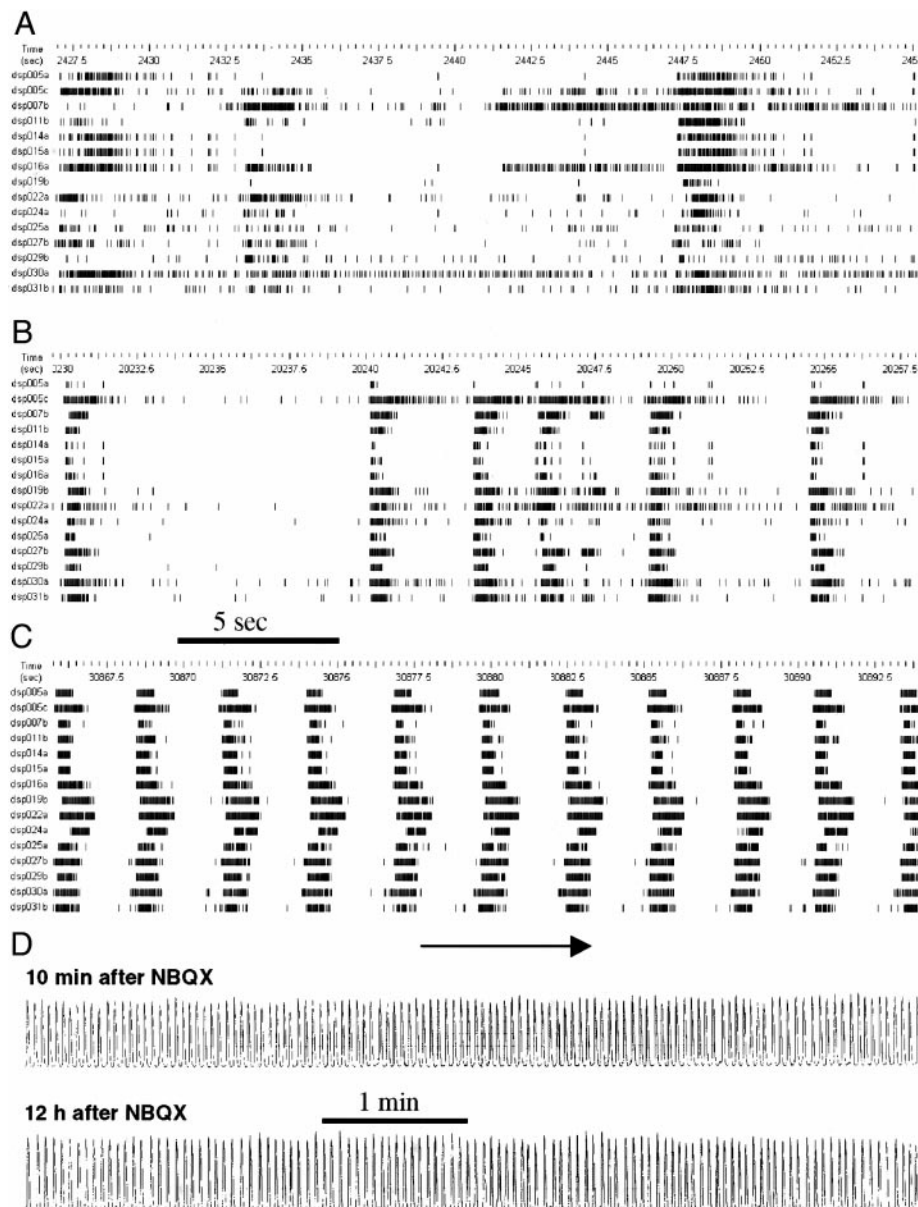


FIG. 3. A–C: simultaneous spike trains from 15 discriminated neurons during different experimental episodes (25-s samples). A: spontaneous native activity displaying tonic and phasic firing with occasional coordinated bursting. B: 40 μ M bicuculline transforms the activity into a bursting pattern that is coordinated across neurons but with a high degree of variability in the interburst intervals (IBI) and burst durations (BD). C: N-methyl-D-aspartate only (NMDA_{ONLY}) activity consists of a synchronized periodic burst pattern. In many cases, the phase relationships between neurons remained stable for hours. D: the long-term stability of the NMDA_{ONLY} burst oscillation is demonstrated with chart recorder trace of integrated activity ($\tau = 0.7$ s) from a single channel at 5 min and 12 h after NBQX application.

associated with high spatial and temporal burst pattern regularity. The eight remaining networks established longer burst periods under NBQX (4.3–3.8 s), with less regularity of the bursts, suggesting that additional inhibitory mechanisms such as GABA_B receptor inhibition of vesicle release (Isaacson and Hille 1997) might have prevented the establishment of the unique 2.8-s oscillation. Indeed, the addition of 50 μ M SCH 50911 (a soluble GABA_B antagonist) decreased the burst period in five of eight networks to 3.0 ± 0.3 s. In the three remaining networks, the application of 40 μ M norepinephrine (NE) in addition to the preceding four compounds, promptly led to the adjustment of the burst oscillation to a regular, periodic burst period of 2.8 ± 0.2 s. NE is known to affect both NMDA receptor desensitization and the slow burst AHP, two other potentially inhibitory mechanisms (Legendre et al. 1993; Madison and Nicoll 1986; Tong and Jahr 1994; Tong et al. 1995). It is important to emphasize that SCH 50911 and/or NE had no effect when they were added after the NMDA_{ONLY} oscillation was established. These results indicate that GABA_B

receptor-mediated inhibition of neurotransmitter release or mechanisms sensitive to NE were sometimes sufficient to prevent the characteristic NMDA_{ONLY} pattern from developing, but were not necessary for its maintenance. It is also important to note that the low CVs characterizing the NMDA_{ONLY} activity pattern were not obtained if the network burst rate following BCC/STR/NBQX was lower than 17 bpm. Thus, the eight cultures that required SCH or SCH/NE to lower their oscillation period into the 2.8 ± 0.2 -s range had significantly higher CVs for each of the burst parameters before the additional pharmacological treatment.

The mean periods with associated SDs of all three pharmacological manipulations (BCC/STR/NBQX, BCC/STR/NBQX/SCH, and BCC/STR/NBQX/SCH/NE) were not significantly different ($P > 0.05$, *t*-test for 2 samples), and thus data from all 16 experiments were pooled for subsequent analysis. To quantify the periodicity of the bursting, as well as the synchronization of the neuronal population participating in each burst event, we calculated the coefficients of variation for

several burst variables over time and across the network. All calculations were done with binned data (60 s means). The results of the CV calculations for three different burst variables, burst rate (BR), burst duration (BD), and interburst interval (IBI), under three different conditions (spontaneous, disinhibited with 40 μ M BCC, and under NMDA_{ONLY}), are summarized in Fig. 4. During native conditions, the CVs are large, implying a wide range of variability in the activity of the networks across time and between the neurons recorded. BCC (40 μ M) reliably reduced the CVs of all three burst parameters, showing that disinhibition has a stabilizing effect on network oscillations. However, reducing the synaptic driving forces to one mediated through the NMDA receptor only, produced CV_{time} of 2.6–5% (Fig. 4A) and entrained virtually all recorded neurons ($n = 678$, 16 experiments), as evidenced by the global CV_{network} for BR of 2.8%. The dependency of the oscillation on the NMDA receptor was demonstrated with 40–100 μ M APV, which abolished activity in all cases ($n = 7$).

Comparing the CVs from the experimental episodes of the eight experiments in which the precise NMDA_{ONLY} oscillation was not established immediately after BCC/STR/NBQX, but before addition of either the GABA_B antagonist SCH50911 or SCH50911 and NE to the CVs of the six experiments under AMPA_{ONLY} conditions, we found that there was no significant difference. In all eight of these NMDA_{ONLY} experiments, the BR was below 17 bpm after

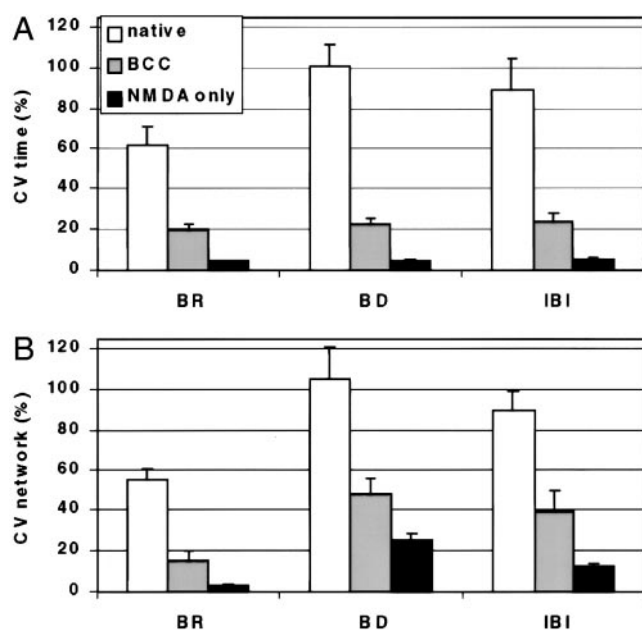


FIG. 4. Mean coefficients of variation (16 experiments) for 3 experimental episodes: native, under 40 μ M bicuculline, and NMDA_{ONLY} (number of channels 185, 185, 87 respectively). A: the CV_{time} for 3 different burst variables: burst rate (BR), BD, and IBI. CV_{time} reflects periodic behavior of the burst variable during a particular experimental episode. Large CV_{time} during the native episodes are due to the complex interaction of multiple neurotransmitter systems. B: the CV_{network} for the same three burst variables. CV_{network} reflects the inter-neuron synchronization across the network. When the GABA_A antagonist bicuculline is applied, both CV_{network} and CV_{time} decrease for all variables to a range of 15–45%. When the activity is mediated solely by NMDA receptors, a further decrease in all CVs occurs (range 2.6–23%), with a concomitant reduction of the standard errors (error bars) obtained from global CVs of 16 different networks. All CVs in the 2 categories (time and network) are significantly different between experimental episodes for all burst variables ($P < 0.05$).

addition of the BCC/STR/NBQX cocktail. Manipulations to maximize neurotransmitter release and prevent NMDA receptor desensitization always increased the BR, indicating that certain inhibitory constraints may be emphasized to a greater extent in some networks. This is an interesting observation in its own right, and may speak to the different homeostatic mechanisms employed by developing neuronal networks to ensure that spontaneous activity falls within an appropriate dynamic range. Burst rates under AMPA_{ONLY} conditions ranged from 13 to 28. None of the pharmacological manipulations we attempted under AMPA_{ONLY} conditions produced the low CVs distinguishing the NMDA_{ONLY} activity (Fig. 5, A and B). Supplementing the BCC/STR/APV treatment with SCH50911 and NE had some minor effects on BR and structure, but did not significantly enhance the precision of the oscillation. In two experiments, we blocked AMPA receptor desensitization with cyclothiazide after adding BCC/STR/APV. In both cases, a rapid and large increase in tonic spiking ensued followed by complete cessation of network activity. This activity inhibition could possibly have been caused by a tonic depolarization of membrane potential produced by the longer open time of the AMPA channel. There was no combination of pharmacology we tried that reproduced the extreme precision of the NMDA_{ONLY} oscillation under AMPA_{ONLY} conditions.

Figure 5C compares the power spectra calculated from the spike trains of 106 neurons from 14 different NMDA_{ONLY} experiments to those of 81 neurons from six different AMPA_{ONLY} experiments. The dotted lines represent 95% confidence intervals, and clearly show that the two pharmacological conditions are distinct in their effects on network oscillations. These results indicate that the NMDA synapse is uniquely suited to provide extremely regular population burst oscillations.

Figure 6 illustrates the evolution of activity with sequential addition of BCC, STR, and NBQX to the culture bath, culminating in the NMDA_{ONLY} activity pattern. The period of spontaneous activity has low rates of bursting and spiking. Addition of 40 μ M BCC produces an increase in both activity variables within 5 min of application. However, the minute-to-minute variation in both variables is still quite large. Addition of 1 μ M STR to remove the second set of inhibitory circuits elicits a further increase in burst and spike rates accompanied by an increase in the temporal variability. The subsequent application of 10 μ M NBQX resulted in a decrease in both burst and spike rates from the totally disinhibited activity. Both measures stabilized at near constant levels after about 20 min. The 30 min following establishment of stable NMDA_{ONLY} activity had CV_{time} for BR, BD, and IBI of 2.1, 4.1, and 2.9%, respectively. The dependence of the stationary activity on NMDA receptor mediated events is shown by the application of 100 μ M APV at 412 min, which resulted in a loss of all bursting within 10 min.

During NMDA_{ONLY} activity, spinal cord networks consistently oscillated within a very narrow range of BRs. This activity was apparently independent of network age or neuronal density, as the 16 cultures from which the data were gathered ranged in age from 18 to 113 days in vitro and were seeded at concentrations of 0.2–0.5 $\times 10^6$ cells/ml. Averaging BRs from all 178 channels resulted in a mean global BR of 21.0 ± 2.0 (SD) bpm. For each channel, the NMDA_{ONLY} BR

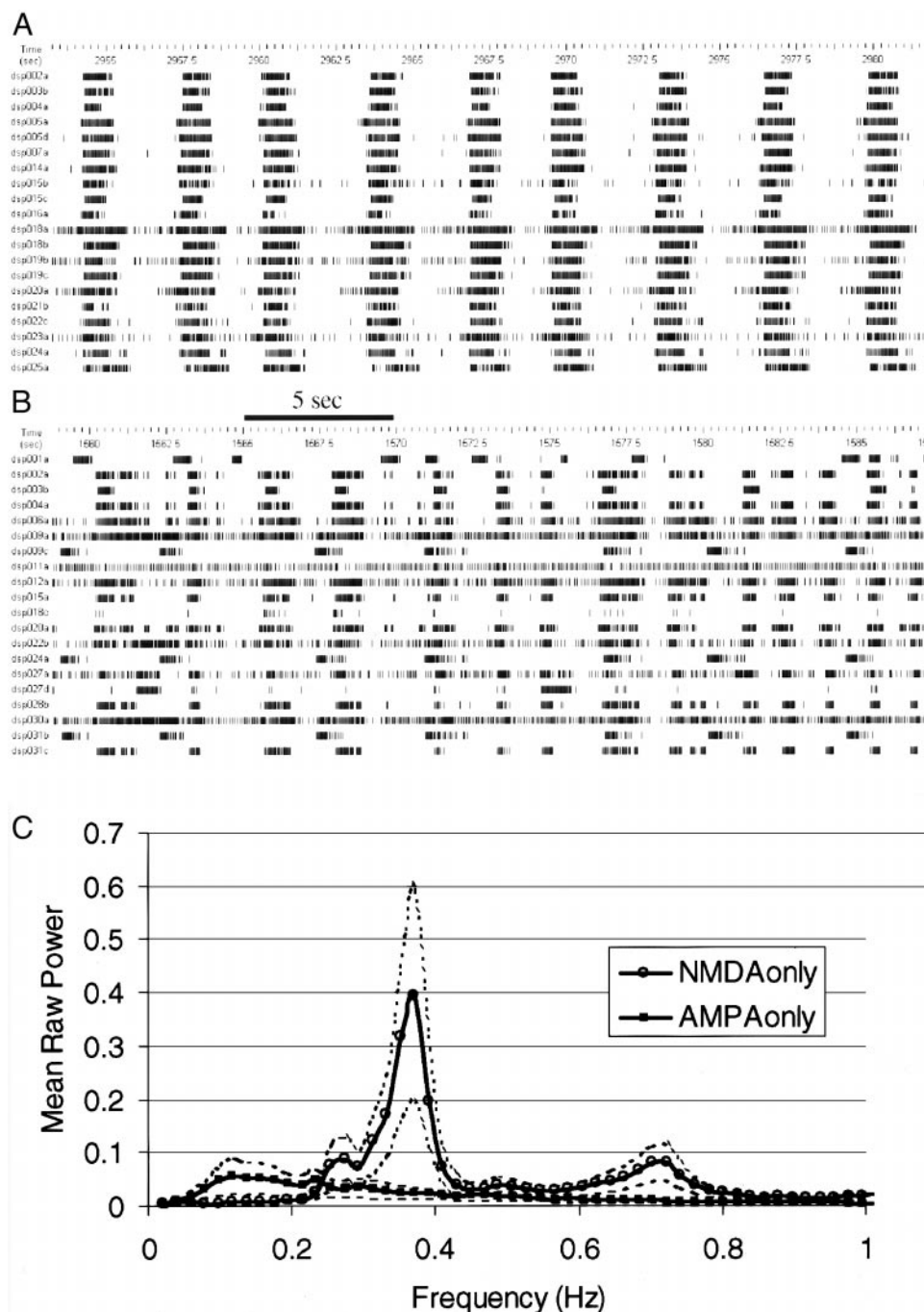


FIG. 5. The highly periodic nature of the NMDA_{ONLY} activity (A) is not approached when synaptic interactions are limited to those mediated by AMPA receptors (B). C: power spectra calculated from the spike trains of 106 neurons/14 separate experiments (NMDA_{ONLY}) compared with spectra calculated from 81 neurons/6 separate experiments (AMPA_{ONLY}). Raw power was calculated for each spike train (0–5 Hz, 256 bins). The resulting histograms were averaged to yield curves for each pharmacological condition. Dotted lines show 95% confidence intervals.

was essentially independent of the initial (native) BR. The distribution of BRs for all 16 cultures (178 channels) under three different conditions is shown in Fig. 7. Native activity (Fig. 7A), prior to any pharmacological additions, ranged from 3 to 27 bpm. When BCC (40 μ M) was added to block GABA_A-mediated inhibition, the BR distribution was broadened, ranging from 3 to 36 bpm (Fig. 7B). A peak in the distribution at 12–15 bpm indicates that the disinhibited network state has some preferential activity modes, though the typical BCC response varies between cultures. Figure 7C, which shows the NMDA_{ONLY} activity distributed from 18 to 24 bpm, demonstrates the reliability of expression of the oscillation in all cultures ($n = 16$).

Mechanisms controlling burst oscillations

The possible mechanisms that contribute to the unique characteristics of the NMDA_{ONLY} activity were explored with a battery of pharmacological agents summarized in Table 1. These agents may be roughly divided into compounds that affect neurotransmitter release, compounds that affect receptor desensitization, compounds that affect burst and spike AHP, and antagonists of neurotransmitter receptors.

COMPOUNDS AFFECTING NEUROTRANSMITTER RELEASE. As previously described, when the main synaptic driving force had been reduced to NMDA_{ONLY} and the network activity had already stabilized to a highly periodic BR of 21.0 ± 2.0 bpm,

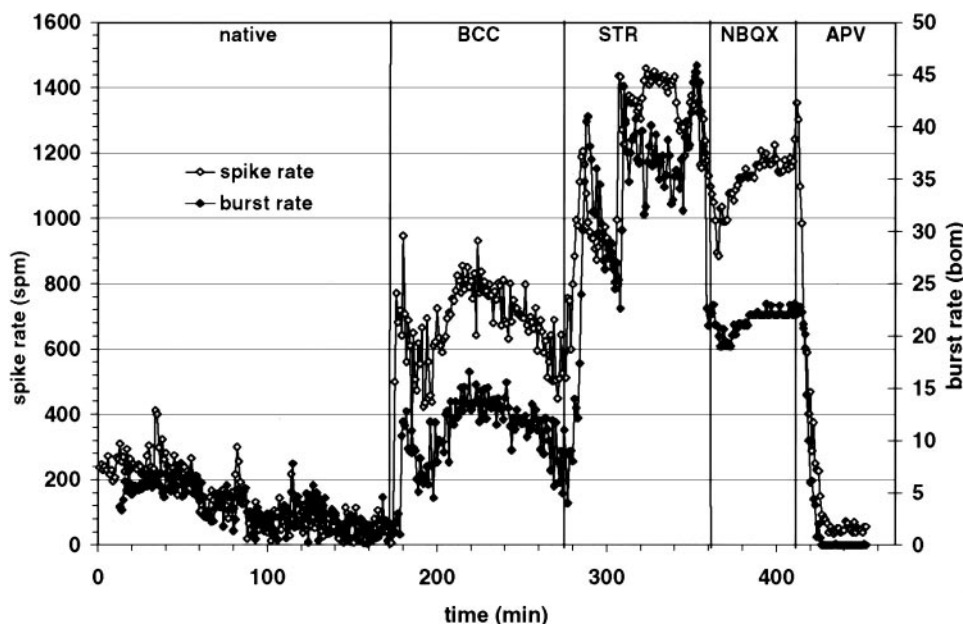


FIG. 6. Changes in spontaneous spike rate (\diamond) and burst rate (\blacklozenge) induced by sequential addition of $40 \mu\text{M}$ bicuculline (BCC), $1 \mu\text{M}$ strychnine (STR), $10 \mu\text{M}$ NBQX, and $100 \mu\text{M}$ APV. Each symbol represents the mean of 27 neurons (spike rate) and 14 channels (burst rate). \downarrow , times of drug application. Spontaneous activity increases within 3 min of bicuculline application at 170 min. Strychnine application at 270 min induces a further increase in both bursting and spiking, accompanied by an increase in temporal variability. Blocking AMPA receptors with NBQX at 360 min reduces both bursting and spiking to levels intermediate to BCC and STR. Note the greatly reduced activity fluctuation within 20 min after NBQX application. APV abolishes all bursting and most spiking within 10 min of application, demonstrating the dependence of the activity on the NMDA receptor.

the GABA_B receptor antagonist SCH 50911 ($50 \mu\text{M}$) had no effect. However, in eight experiments, the combination of BCC/STR/NBQX resulted in a BR of less than 17 bpm. The variability in the burst parameters were also significantly higher than in the eight experiments that responded with precise burst oscillations immediately after application of NBQX. In 5 of 8 of these cultures, the effect of SCH50911 was to increase the BR to 18–24 bpm and to establish a highly periodic pattern of activity. Thus it appears in our preparation, inhibition of neurotransmitter release through activation of GABA_B receptors by endogenous GABA is sometimes sufficient to prevent the characteristic NMDA_{ONLY} pattern of activity from developing. These network specific effects may reflect fluctuations in network structure that are revealed by the pharmacological manipulations.

In three experiments, after NMDA_{ONLY} activity had been established, we titrated the Ca^{2+} concentration from 1.3 to 8.0 mM (Fig. 8). The initial Ca^{2+} increase to 3 mM caused a consistent decrease in both the BR and BD. Further increases in Ca^{2+} suppressed mean BDs from 600 ms at 3 mM Ca^{2+} to 300 ms at 8 mM Ca^{2+} , but did not affect the BR significantly. At all elevated Ca^{2+} concentrations, the precision of the pre-existing NMDA_{ONLY} oscillation was lessened. In two of these experiments, we subsequently raised K^{+} concentrations from the basal level of 5.4 mM to 10 mM. K^{+} increases both the level of neuronal excitability and the level of Ca^{2+} influx at the presynaptic terminal. The effect of elevated K^{+} under these conditions was to increase the BR, consistent with an increase in neuronal excitability (Fig. 8). After the extensive pharmacological manipulations, it is of significance that the network responded to elevated K^{+} concentrations in a predictable manner (Rhoades and Gross 1994).

The contribution of metabotropic glutamate receptors (mGluR) to neurotransmitter release was examined with the nonselective group I/II antagonist MCPG ($200 \mu\text{M}$). The compound was without measurable effect in five experiments, indicating that mGluR activation was not a significant factor in the control of glutamate release in our cultures. This finding is in accord with those of von Gersdorff et al. (1997), who

showed that mGluR activation apparently contributes to only about 10% of the EPSC depression induced by afferent stimulation at the calyx of Held in the rat brain stem.

Titration of the extracellular Mg^{2+} from the normal 0.8 mM to a maximum of 6 mM after establishment of NMDA_{ONLY} activity resulted in a dose-dependent reduction in the BR without affecting the BD except at the higher concentrations immediately prior to network failure (Fig. 9). Like Ca^{2+} , Mg^{2+} has multiple sites of action. It inhibits neurotransmitter release and blocks the NMDA channel in a voltage-dependent fashion. Increasing the concentration of Mg^{2+} raises the threshold for relief of the voltage-dependent blockade. Mg^{2+} is also implicated in suppression of spontaneous activity by charge screening of voltage-sensitive Na^{+} channels (Guth and Drescher 1990).

COMPOUNDS AFFECTING RECEPTOR DESENSITIZATION. NMDA receptors undergo different forms of desensitization that could potentially contribute to the activity pattern. Glycine-dependent desensitization of NMDA receptor-mediated currents has been reported in cultured hippocampal neurons (Mayer et al. 1989; Vyklíček et al. 1990). Both groups found that $3 \mu\text{M}$ glycine was sufficient to alleviate a strong depression of such currents. In addition, the NMDA receptor undergoes a Ca^{2+} -dependent form of desensitization that is mediated by the phosphatase calcineurin (Legendre et al. 1993; Tong and Jahr 1994; Tong et al. 1995). To test the hypothesis that glycine-dependent NMDA receptor desensitization influenced the neuronal network activity under NMDA_{ONLY} conditions, we added $10 \mu\text{M}$ glycine to counteract desensitization ($n = 3$). The glycine had no measurable effect, indicating that glycine-dependent desensitization did not play a role in generating NMDA_{ONLY} oscillations.

Raman et al. (1996) showed that NE increases PKA activity through a β -adrenergic dependent process, which is sufficient to counteract the phosphatase actions of calcineurin and prevent NMDA receptor desensitization. In three experiments, application of $40 \mu\text{M}$ NE increased BRs and stabilized IBI at NMDA_{ONLY} levels. When NE was applied after establishment

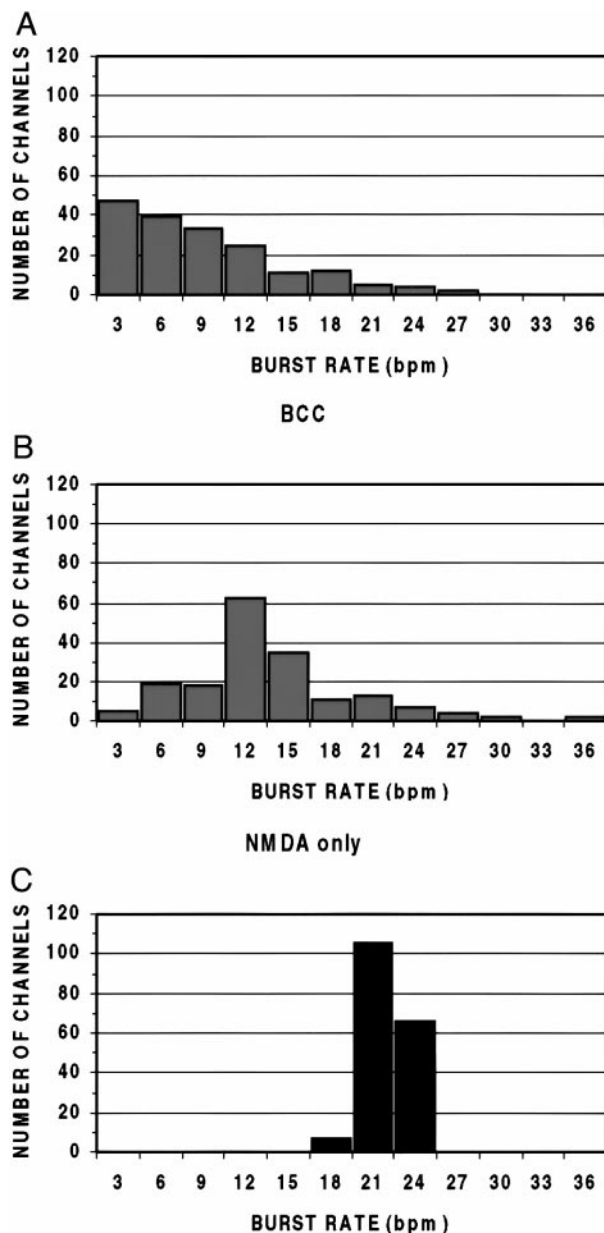


FIG. 7. Burst rate distributions under 3 different conditions. *A*: native activity of 16 cultures ranges from 3 to 27 bursts per minute (bpm). *B*: disinhibition with 40 μ M bicuculline results in a wider range (3–36 bpm), with a small peak at 12–15 bpm, indicating a preference for certain activity states but with relatively low reliability of expression. *C*: NMDA_{ONLY} activity ranges from 18 to 24 bpm. The narrow distribution shows the interculture reproducibility of the oscillation. Mean burst rates for 60 min intervals under each condition were calculated on a per channel basis ($n = 178$ channels).

of a stable NMDA_{ONLY} pattern, it was without effect. However, NE also inhibits the slow AHP (sAHP) that may serve to terminate bursting (Madison and Nicoll 1986). Therefore it is unclear whether the effects produced by NE were a result of blocking receptor desensitization or the sAHP. In either case, the NMDA_{ONLY} pattern of activity, once established, was not dependent on a mechanism sensitive to NE.

COMPOUNDS AFFECTING SHP. Action potentials in many excitable cells are followed by a prolonged AHP that modulates repetitive firing. Apamin has been shown to inhibit the Ca^{2+} -dependent S(K) current that is responsible for this AHP (Sah

and McLachlan 1992). In our experiments, 1 μ M apamin ($n = 4$) increased BRs if it was applied prior to blocking AMPA/kainate receptors (Fig. 8). When apamin was applied after the NMDA_{ONLY} activity was established ($n = 3$), it was without effect. This indicates that the apamin-sensitive AHP does not affect NMDA_{ONLY} oscillations.

A transient AHP was identified in hippocampal slices (Alger and Williamson 1988) that was sensitive to charybdotoxin (ChTX), indicating its mediation by B(K) K^{+} channels. We performed experiments with 20 nM ChTX before ($n = 3$) and after ($n = 3$) network activity was reduced to NMDA_{ONLY}. ChTX increased BRs when it was applied before blockade of AMPA/kainate receptors (Fig. 8), but was without effect when applied after NBQX. These results indicate that the Ca^{2+} -activated B(K) channel does not contribute substantially to NMDA_{ONLY} oscillations.

NEUROTRANSMITTER RECEPTOR ANTAGONISTS. After the NMDA_{ONLY} activity was established, we used atropine (muscarinic receptor antagonist, 1–30 μ M, $n = 3$), curare (nicotinic receptor antagonist, 10–20 μ M, $n = 3$), haloperidol (dopamine receptor antagonist, 2 nM, $n = 2$), methysergide (5-HT_{1/2} receptor antagonist, 20 μ M, $n = 2$), Evan's blue (P2X receptor antagonist, 50 μ M, $n = 2$), and L-733,060 (tachykinin receptor antagonist, 2 nM, $n = 2$). None of these compounds had measurable effects on previously established NMDA_{ONLY} oscillations.

INVOLVEMENT OF ELECTRICAL SYNAPSES AND I_{H} CURRENTS. The functional contribution of gap junctions was investigated with

TABLE 1. List of pharmacological agents tested

| Active Agent, μ M | Mode of Action |
|------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------------------------------------|
| Apamin (1) | Antagonist, small conductance (SK) Ca^{2+} activated K^{+} channels |
| APV (5–100) | NMDA receptor antagonist |
| Atropine (10–30) | Cholinergic muscarinic antagonist |
| Bicuculline (40) | GABA _A antagonist |
| Ca^{2+} (1200–5000) | Facilitation of neurotransmitter release |
| Carbenoxolone (50–100) | Gap junction blocker |
| Charybdotoxin (0.015) | Antagonist, large conductance (BK) Ca^{2+} activated K^{+} channels |
| Curare (10–20) | Cholinergic nicotinic antagonist |
| Evan's Blue (50) | P2X receptor antagonist (adenosine) |
| Glycine (10) | Prevents glycine-dependent NMDA receptor desensitization (strychnine insensitive glycine site) |
| Haloperidol (0.002) | Dopamine receptor antagonist |
| K^{+} (5400–10000) | Enhancer of neuronal excitability |
| L-733,060 (0.002) | NK ₁ receptor antagonist (sub. P) |
| MCPG (100–200) | Metabotropic glutamate receptor antagonist |
| Methysergide (20) | Serotonin receptor antagonist |
| Mg^{2+} (0–6000) | Inhibitor of neurotransmitter release, NMDA receptor blocker |
| NBQX (10–20) | AMPA/kainate antagonist |
| NMDA (50) | Selective agonist |
| Norepinephrine (40–50) | PKA activator (β -adrenergic receptor mediated), blocks burst after hyperpolarization, also counteracts Ca^{2+} -dependent NMDA receptor desensitization |
| SCH 50911 (50–100) | GABA _B antagonist |
| Strychnine (1) | Glycine receptor antagonist |
| ZD7288 (50) | Hyperpolarization activated cationic conductance blocker (I_{h} current) |

NMDA, *N*-methyl-D-aspartate; MCPG, α -methyl-4-carboxyphenylglycine; NBQX, 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzoxazine-7-sulphonamide.

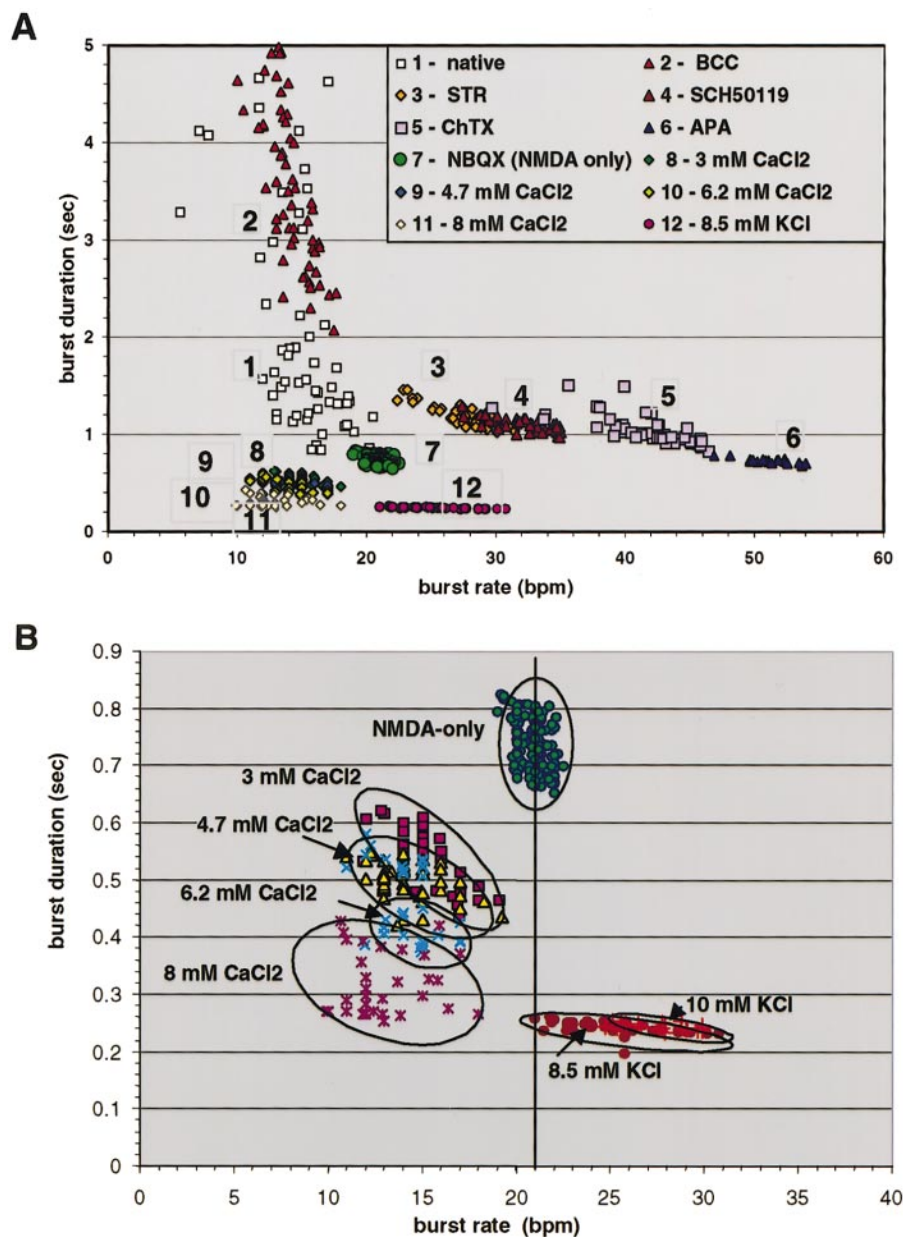


FIG. 8. Changes in burst duration and burst rate elicited by pharmacological manipulation of synaptic driving forces and burst ignition and termination mechanisms (minute means from 14 channels, single experiment). The extremely low variability of the NMDA_{ONLY} activity is indicated by the tight cluster after addition of 10 μ M NBQX (green circles, 7). Further manipulation by titration of Ca²⁺ decreases both duration and rate, while titrating K⁺ to 10 mM increases the burst rate (B).

carbenoxolone (50–100 μ M, $n = 3$). There was no measurable effect on the NMDA_{ONLY} activity, indicating that gap junction coupling played no significant role in these oscillations. ZD7288 (50 μ M, $n = 3$) was used to test for the contribution of the hyperpolarization-activated cationic conductance (I_h) to NMDA_{ONLY} activity. This agent was also ineffective.

PRE- VERSUS POSTSYNAPTIC CONTROL MECHANISMS. The addition of 20–50 μ M NMDA to the experimental culture medium after the NMDA_{ONLY} activity was established resulted in both increased spike production and a disruption of the periodic nature of the activity (Fig. 10, $n = 2$). CV_{time} for BR was 58% in one network and 71% in the other, indicating that the regular oscillations were no longer present. The disruption of the pattern can be demonstrated in the spike frequency domain with Fourier analysis of spike intervals from the same identified units. Figure 9, B and D, reveals a drastic alteration in the spike interval structure. Whereas the NMDA_{ONLY} activity

peaks at 0.34 Hz (B), the addition of NMDA to the medium (D) obliterates the interval organization.

The NMDA receptor channel possesses a unique voltage-dependent Mg²⁺ block that has been shown to induce oscillations in several preparations (Hu and Bourque 1999; Rioult-Pedotti 1997; Wallen and Grillner 1987). This oscillation is elicited by NMDA receptor activation in the presence of physiological levels of Mg²⁺ and is apparently due to the negative slope in the current-voltage relation of the NMDA synapse. Wallen and Grillner (1987) showed that NMDA application produced TTX-resistant oscillations in membrane potential, indicating an intrinsic cellular property. Because bath application of NMDA directly activates the receptors, independent of vesicle release, and essentially uncouples the NMDA synapses from presynaptic influences, the loss of regular oscillations strongly suggests that the periodicity under NMDA_{ONLY} conditions is not induced at the receptor level only.

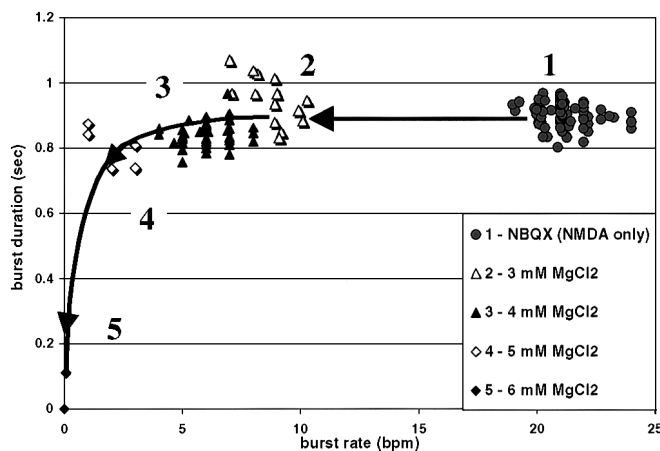


FIG. 9. Effect of increasing Mg^{2+} concentration on BR and BD. Raising Mg^{2+} from the basal 0.8 mM under NMDA_{ONLY} conditions caused a progressive decrease in burst rates without affecting burst durations until 4–5 mM Mg^{2+} . A catastrophic loss of all activity occurred at 6 mM Mg^{2+} . Points represent the minute means of 14 channels under each pharmacological condition.

DISCUSSION

Independent of spontaneous activity or age, 16 spinal cord cultures were entrained to a remarkably stable, highly periodic

burst pattern of 21.0 ± 2.0 bpm under NMDA_{ONLY} conditions. This pattern of activity persisted for up to 12 h (maximum observation time, Fig. 3D), and more than 98% of all recorded neurons participated (40–85 neurons per experiment). NMDA receptor activation was necessary for generation of this unique, highly periodic activity pattern as it was abolished by 40–100 μ M APV. However, tonic NMDA receptor activation was not sufficient to maintain the periodicity, implying that control of the period may originate at the presynaptic level. Additionally, disinhibition with BCC and STR and blockade of NMDA receptors with APV (leaving only the AMPA/kainate synapse as the sole driving force) did not produce the characteristic oscillation.

Mechanisms underlying intrinsic burst generation have been explored in several different cell types (Azouz et al. 1996; Ballerini et al. 1999; Bracci et al. 1996b; Llinas 1988; Streit 1993; Traub et al. 1994). Generally, these investigations have analyzed burst phenomena one cell at a time, although population studies using Ca^{2+} imaging (Lawrie et al. 1993; Robinson et al. 1993; Wang and Gruenstein 1997) or extracellular field potentials (Staley et al. 1998) have provided insights into network bursting. Acutely isolated intact spinal cords, acute spinal cord slices, and organotypic slice preparations have been

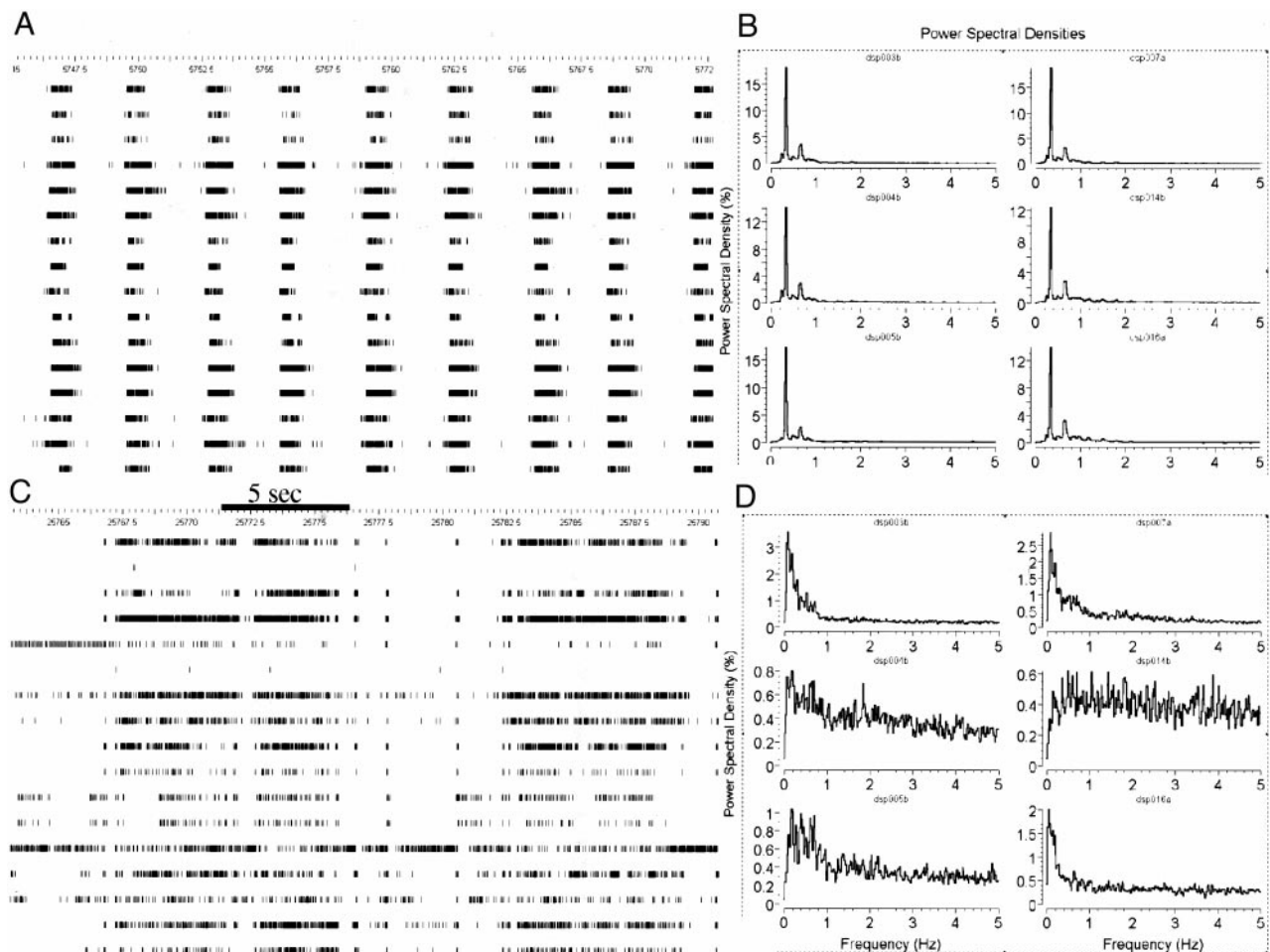


FIG. 10. Uncoupling of presynaptic influences by bath application of 50 μ M NMDA destroys the periodic nature of NMDA_{ONLY} oscillations. *A*: 25 s of activity of 16 neurons under NMDA_{ONLY} conditions. *B*: the period of the NMDA_{ONLY} burst oscillation is reflected in the large peak in the power spectra at 0.34 Hz (2.94 s period) calculated for a sample of 6 of the neurons shown in *A*. *C*: the same 16 neurons 5 min after 50 μ M NMDA was added to the experimental bath. The periodic and synchronized nature of the NMDA_{ONLY} activity is destroyed. *D*: power spectra calculated for the same neurons as in *B* after NMDA addition.

used to study the mechanisms of fictive locomotion and putative central pattern generators. For example, elevated extracellular K^+ resulted in increased neuronal excitability, leading to rhythmic, synchronized oscillations in organotypic rat spinal cord slices (Ballerini et al. 1999). These oscillations were network dependent as they were blocked by TTX and low- Ca^{2+} /high- Mg^{2+} solutions. Comparable oscillations were also seen in isolated spinal cord preparations (Bracci et al. 1998).

Ballerini et al. (1999) reported in one experiment a persistent, network-dependent, rhythmic burst oscillation in ventral horn interneurons with a period of 2.8 ± 1.5 s under elevated (6–7 mM) extracellular K^+ (a period close to the 2.9 ± 0.3 s that we observed during NMDA_{ONLY} activity). Thus, the oscillation rate of the NMDA_{ONLY} activity is not unique to the dissociated cell culture condition. However, this is the first report of an oscillation characterized not only by remarkable temporal and spatial stability of all burst parameters within individual experiments but also by high interexperiment reproducibility with networks that varied widely in age and cell density. This suggests the NMDA_{ONLY} network state may be unmasking a fundamental cellular mechanism rather than a circuit specific network property.

CA3 network activity can be dominated by strong synaptic depression presumably induced by depletion of a finite supply of releasable glutamate (Staley et al. 1998). They suggested that the period of the population bursts was determined by the rate of refilling of this pool. Recent investigations have provided insights into the dynamics of neurotransmitter vesicle pools (Goda and Stevens 1998; Ryan et al. 1993, 1996). The RRP in central neurons has been shown to contain from 5 to 20 vesicles (Dobrunz and Stevens 1997; Stevens and Tsujimoto 1995). This pool refills with a time constant of 5–12 s (Rosenmund and Stevens 1996). High-frequency stimulation of presynaptic terminals with trains of 100–300 Hz significantly enhances the rate of refilling when compared with square wave depolarizing input (Wang and Kaczmarek 1998). Stevens and Wesseling (1998) reported that the synaptic recovery period was reduced from 6 to 3 s when focal hyperosmotic conditions were paired with presynaptic 10-Hz stimulation. The recovery time for CA1 synapses depleted by 10-Hz stimulation in hippocampal slices was 2.8 ± 2.0 s (Dobrunz and Stevens 1997), a time remarkably similar to our NMDA_{ONLY} period. By comparison, the NMDA_{ONLY} bursts in our studies produced long plateaus (0.2–0.4 s) at spike frequencies of 200–250 Hz. The close agreement between the NMDA_{ONLY} oscillation period of 2.9 ± 0.3 s and the time constants reported for refilling of the RRP during electrical stimulation, in conjunction with the results of our pharmacological manipulations implying a presynaptic control of the NMDA_{ONLY} activity, suggest the vesicle depletion/refilling cycle is a good candidate for controlling the period of the unique burst oscillations observed.

Although presynaptic mechanisms may underlie the unique NMDA_{ONLY} oscillations, it is also necessary to survey postsynaptic responses that can influence spike frequencies in bursts as well as BDs. NMDA receptor activation is associated with Ca^{2+} conductances supporting plateau potentials, with effects on burst mechanisms that are not seen with AMPA receptor activation (MacLean et al. 1997). Calton et al. (2000) showed that single stimulation pulses were sufficient to elicit Ca^{2+} spikes through activation of voltage-dependent calcium channels (VDCCs) in pyramidal cells of rat amygdala slices.

AMPA receptor-mediated depolarization was unable to elicit these spikes, despite providing a greater depolarizing current. Schiller et al. (1998) showed that pairing of glutamate application with postsynaptic action potential firing resulted in a threefold increase of Ca^{2+} entry through the NMDA receptor compared with glutamate alone. In cultured cortical neurons, Robinson et al. (1993) found that Ca^{2+} oscillations concomitant with action potential burst firing was dependent on NMDA receptor activation in low- Mg^{2+} medium. Bacci et al. (1999) established that NMDA receptors along with VDCCs provide a long-lasting depolarization that is necessary to support action potential burst firing in cultured hippocampal neurons. These results all highlight the unique relationship of the NMDA receptor to intracellular Ca^{2+} dynamics. Hence, NMDA receptor-dependent Ca^{2+} influx is likely to influence the character of the burst generated, which, in turn, influences the degree of vesicle depletion at the presynaptic terminal.

Preliminary results of Ca^{2+} imaging experiments performed under three different conditions (NMDA_{ONLY}, partial disinhibition with BCC, and AMPA_{ONLY}) indicate that intracellular Ca^{2+} oscillations occur with the same frequency and precision under NMDA_{ONLY} conditions as the extracellularly recorded burst oscillations. The baseline level of intracellular Ca^{2+} during NMDA_{ONLY} appears to be significantly elevated when compared with the other two conditions. This is possible evidence for the NMDA_{ONLY} condition being particularly efficacious in affecting intracellular Ca^{2+} dynamics. This heightened Ca^{2+} concentration could in turn promote maximal vesicle release during the active phase of each oscillation.

In the lamprey, NMDA application induces a robust fictive swimming rhythm which is dependent on NMDA-induced Ca^{2+} currents and Ca^{2+} -dependent K^+ currents in pacemaker cells (Brodin et al. 1991). Rat spinal neurons exhibit membrane potential oscillations in response to NMDA application which are TTX resistant, but dependent on serotonin (Hochman et al. 1994a,b; MacLean et al. 1998). These membrane potential oscillations have been hypothesized to contribute to the phasic population activity occurring during fictive locomotion, allowing the expression of a precise oscillation period by filtering temporally dispersed synaptic activity (Hochman et al. 1994b). While our experiments certainly do not preclude this mechanism from contributing to the oscillatory nature of the NMDA_{ONLY} activity, disruption of the periodicity of preexisting oscillations by application of NMDA indicates that the receptor alone is not sufficient to drive the bursting with great precision.

Staley et al. (1998) speculated that depletion of the excitatory neurotransmitter vesicle pool might be necessary for burst termination, with other mechanisms such as inhibitory conductances and AHP acting to modulate the postsynaptic effect of glutamate. It is of interest that early in postnatal development, GABA application depolarizes neurons (Ben-Ari et al. 1997; Menedez de la Prida and Sanchez-Andres 1999). During this period, bursting patterns of coordinated activity occur (Habets et al. 1987; Jackson et al. 1982). This coordinated activity selectively activates subsets of synapses and functions as an important mechanism for sculpting circuitry that is preserved in the adult animal (Landmesser and Pilar 1978; Tosney and Landmesser 1985). During the period when GABA is depolarizing, mechanisms other than fast synaptic inhibition must serve to terminate bursting. Strong synaptic depression induced

by vesicle depletion would allow the coordination of phasic activity between recurrently connected neurons, permitting the expression of fundamental motor patterns such as the respiratory rhythm prior to establishment of mature synaptic circuitry. The dependence of the NMDA_{ONLY} oscillation on such a mechanism might be explored with compounds that affect the rate of vesicular recycling, such as bafilomycin (Cousin and Nicholls 1997). Quantification of miniature excitatory postsynaptic potentials occurring immediately prior to and after a burst event could give a direct measure of the amount of vesicle depletion caused by a burst and provide information on the refilling rate of the releasable vesicle pool. Initial results from Ca²⁺ imaging studies indicate that intracellular Ca²⁺ concentrations are tonically elevated during the NMDA_{ONLY} condition and that Ca²⁺ oscillations occur with the same frequency and precision as the burst events.

Few neuronal systems can be forced into a specific dynamic state with high reliability and remain entrained for many hours. The availability of such a stable dynamic system provides a tool for the study of structure/function relationships controlling network behavior, excitotoxicity, and quantitative assessment of functional synaptic deficits, as well as therapeutic measures designed to restore synaptic efficacy. In any case, NMDA_{ONLY} activity represents a simplification of synaptic driving forces, which will allow the study of excitatory-coupled network dynamics and basic burst mechanisms, providing insight into the complex interactions of cellular and network properties.

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