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Controlling bursting in cortical cultures with closed-loop multi-electrode stimulation

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One of the major modes of activity of high-density cultures of dissociated neurons is globally synchronized bursting. Unlike *in vivo*, neuronal ensembles in culture maintain activity patterns dominated by global bursts for the lifetime of the culture (up to two years). We hypothesize that persistence of bursting is due to a lack of input from other brain areas. To study this hypothesis, we grew small but dense monolayer cultures of cortical neurons and glia from rat embryos on multi-electrode arrays (MEAs), and used electrical stimulation to substitute for afferents. We quantified the burstiness of the cultures' firing in spontaneous activity and during several stimulation protocols. While slow stimulation through individual electrodes increased burstiness due to burst entrainment, rapid stimulation reduced burstiness. Distributing stimuli across several electrodes, as well as continuously fine-tuning stimulus strength with closed-loop feedback, greatly enhanced burst control. We conclude that externally applied electrical stimulation can substitute for natural inputs to cortical neuronal ensembles in transforming burst-dominated activity to dispersed spiking, more reminiscent of the awake cortex *in vivo*. This non-pharmacological method of controlling bursts will be a critical tool for exploring the information processing capacities of neuronal ensembles *in vitro*, and has potential applications for the treatment of epilepsy.

Introduction

The mammalian cortex has been studied *in vitro* in the form of dissociated monolayer cultures for several decades. Such cultures retain many morphological, pharmacological and electrical properties of cortical networks *in vivo* (Dichter, 1978) and allow much more detailed observation and manipulation than intact brains, at the molecular, cellular, and network levels (Droge et al., 1986; Emery et al., 1991; Curtis et al., 1992; Wilkinson, 1993; Bove et al., 1994; Rhoades et al., 1996; Bove et al., 1997; Canepari et al., 1997; Gross et al., 1997; Liu et al., 1997; Harsch et al., 1998; Honma et al., 1998; Jimbo et al., 1998, 1999;

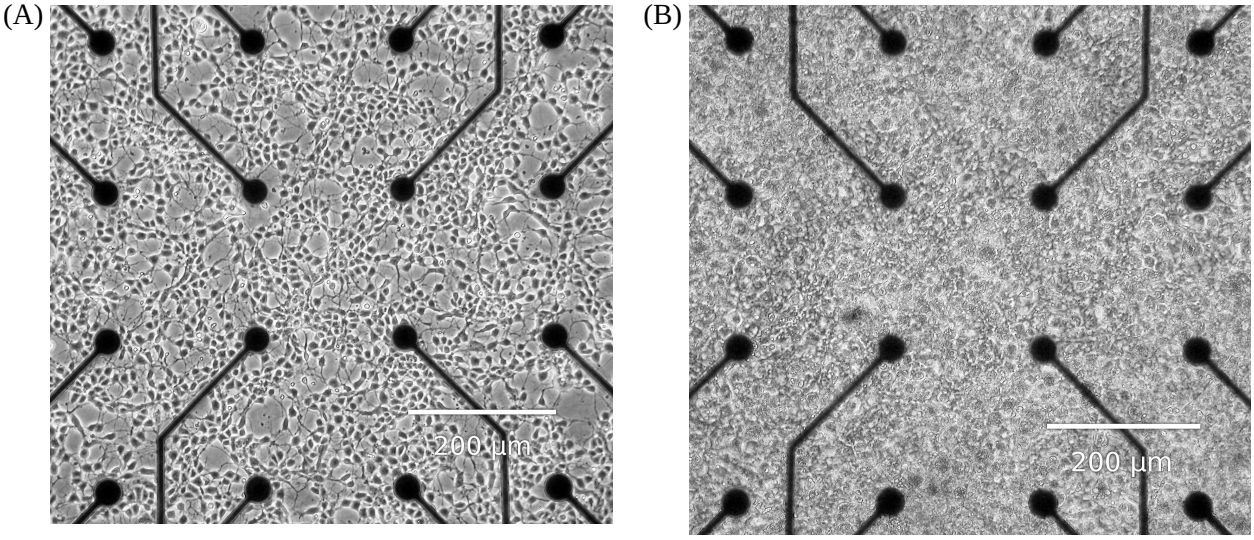


Figure S1: A typical culture growing on an MEA. (A) At 2 div. (B) At 34 div. At this age, glia have formed a carpet covering the culture. Both photographs show the same central part of the MEA. The electrodes are clearly visible. [NB: This figure is 'supplementary' in the published paper.]

Turrigiano, 1999; Harsch and Robinson, 2000; Zhu et al., 2000; Keefer et al., 2001; Streit et al., 2001; Shahaf and Marom, 2001; Corner et al., 2002).

The most prominent feature of the electrical activity of high-density dissociated cortical cultures is their propensity for synchronized bursting (Murphy et al., 1992; Gross et al., 1993; Wong et al., 1993; Kamioka et al., 1996; Canepari et al., 1997; Voigt et al., 1997; Gross and Kowalski, 1999). The cells in these cultures (Figure S1) begin to start firing after about 4 days *in vitro*, and soon after synchronize their activity globally across the culture. This synchronization takes the form of intense bursts of activity, 0.5–2 s in duration, that recur several times per minute. During global bursts, a large fraction of cells in the culture rapidly increase their firing rates by a factor of 10 or more. Bursting persists for the lifetime of the culture, although the fully synchronized bursts of young cultures are gradually replaced by more spatially localized bursts in maturity (Maeda et al., 1995; Corner et al., 2002). Globally synchronized bursting is an extremely robust phenomenon. Suppressing it using pharmacological agents like glutamate receptor blockers (Furshpan and Potter, 1989; Gross et al., 1993; Kamioka et al., 1996) also abolishes most or all other spontaneous electrical activity.

In vivo, bursting occurs during development and plays a role in establishing appropriate connections (Meister et al., 1991; Ben-Ari, 2001; Zhang and Poo, 2001; Leinekugel et al., 2002). However, this phase only lasts for days or at most weeks. The persistence into maturity of bursting in culture may then be interpreted as a sign that cultures are arrested in their development (Corner et al., 2002). Bursting in cultures has also been likened to spindles observed in the EEG of sleeping brains (Krahe and Gabbiani, 2004), as well as to epileptic activity (Furshpan and Potter, 1989; Litt and Echauz, 2002). Techniques that reduce bursting in culture are therefore of potential importance for the treatment of epileptic patients.

We hypothesize that the persistence of global bursts in dissociated cortical cultures is a result of deafferentation. Deafferentation has two effects. Firstly, the lack of (thalamocortical) input might lead to increased strength of connections within the network. Indeed, Turrigiano (1999) showed that blocking the inputs to cortical neurons using TTX during development significantly increased the strength of excitatory connections. Secondly, the lack of structured input and presence of strong excitatory connections puts the network in a highly unstable state in which positive feedback between excitatory cells can easily lead to synchronized bursts of activity (Corner and Ramakers, 1992). Latham et al. (2000) found that bursting

results when too few cells in the network are tonically active. This auto-regulation may be due to slow after-hyperpolarization or regulation of intracellular Ca^{2+} (Darbon et al., 2002). We propose that substituting multi-electrode stimulation for sensory input (Heck, 1995) has the same effect as an elevated tonic firing rate, and should therefore reduce the predominance of global bursts, favoring more locally differentiated neuronal activity.

Methods

Cell culture

Neocortical cells were dissociated from the brains of E18 rats and plated on multi-electrode arrays (MEAs). Timed-pregnant Wistar rats were euthanized using CO_2, according to NIH-approved protocols. Embryos were removed and euthanized by chilling and decapitation. The entire neocortex, excluding the hippocampus, was dissected under sterile conditions. Cortices were cut into 1-mm^3 cubes in Segal's medium (Segal et al., 1998). (In mM: $MgCl_2$: 5.8; $CaCl_2$: 0.25; HEPES: 1.6; Na_2SO_4: 90; K_2SO_4: 30; Kynurenic acid: 1; DL-2-Amino-5-phosphonovaleric acid (APV): 0.05. pH-ed to 7.3 using NaOH and 0.001% Phenol Red.) After enzymatic digestion for 30 minutes by 2.5 U/mL Papain (Roche 108014) in Segal's medium, cells were separated by 6 or 9 trituration passes using a 1 mL pipette tip, in Neurobasal medium with B27 (Gibco; Brewer et al., 1993), 0.5 mM GlutaMax (Gibco) and 10% equine serum (Hyclone). After every 3 passes, the cells already in suspension were transferred to a separate tube to reduce stress on them. Cells were centrifuged at $160 \times g$, onto 5% bovine serum albumin (BSA) in phosphate buffered saline (PBS), resuspended by very gentle trituration and passed through a 40 μm cell strainer (Falcon) to remove large debris. 50,000 cells were plated in a 20 μL drop of Neurobasal, on MEAs pre-coated with poly-ethylene-imine (PEI) and laminin as previously described (Potter and DeMarse, 2001). This led to a plating density of 2500 cells/mm^2 in a monolayer. After 1h of incubation, 1 mL of Neurobasal was added to each culture dish. After 24h, the plating medium was replaced by a medium adapted from Jimbo et al. (1999): Dulbecco's modified Eagle's medium (DMEM, Irvine Scientific 9024) with 0.5 mM Glutamax and 10% equine serum, but no antibiotics or antimycotics. Cultures were maintained in an incubator with 5% CO_2 and 9% O_2 (Brewer and Cotman, 1989). We replaced half the medium every 5–7 days. Glial growth was not suppressed, since glia are essential to long-term culture health. As a result, glia gradually formed a carpet over the neurons. Our use of Teflon-sealed dishes (Potter and DeMarse, 2001) allowed us to maintain the incubator at 65% relative humidity, making it an electronics-friendly environment. Thus we could perform all experiments inside the incubator, ensuring long-term stability of recording conditions. Experiments took place at 25–45 days *in vitro* (div). At this age, over 90% of electrodes recorded spikes. Only cultures that fired at least three bursts in 10 minutes of pre-experimental screening were used.

To determine the fraction of inhibitory cells, we stained two cultures at 16 div for microtubule-associated protein-2 (MAP-2) and γ-amino butyric acid (GABA), as described under *Immunostaining* below. In the two cultures, 10 randomly selected images showed 29 out of 499 neurons (5.8%) and 16 out of 440 (3.6%) neurons with anti-GABA immunoreactivity (Figure 1).

Recording system

Electrical activity was recorded with a square array of 60 substrate-embedded titanium-nitride electrodes, 30 μm in diameter, with 200 μm spacing (MultiChannel Systems, Reutlingen, Germany; www.multichannel-systems.com). After 1200x amplification, signals were sampled at 25 kHz using a MultiChannel Systems data acquisition card, controlled through our MeaBench software[1]. MeaBench's digital filtering system for reducing stimulus artifacts (Wagenaar and Potter, 2002) allowed us to detect action potentials as early as

[1]Software available for free public download: http://www.its.caltech.edu/~wagenaar/meabench.html.

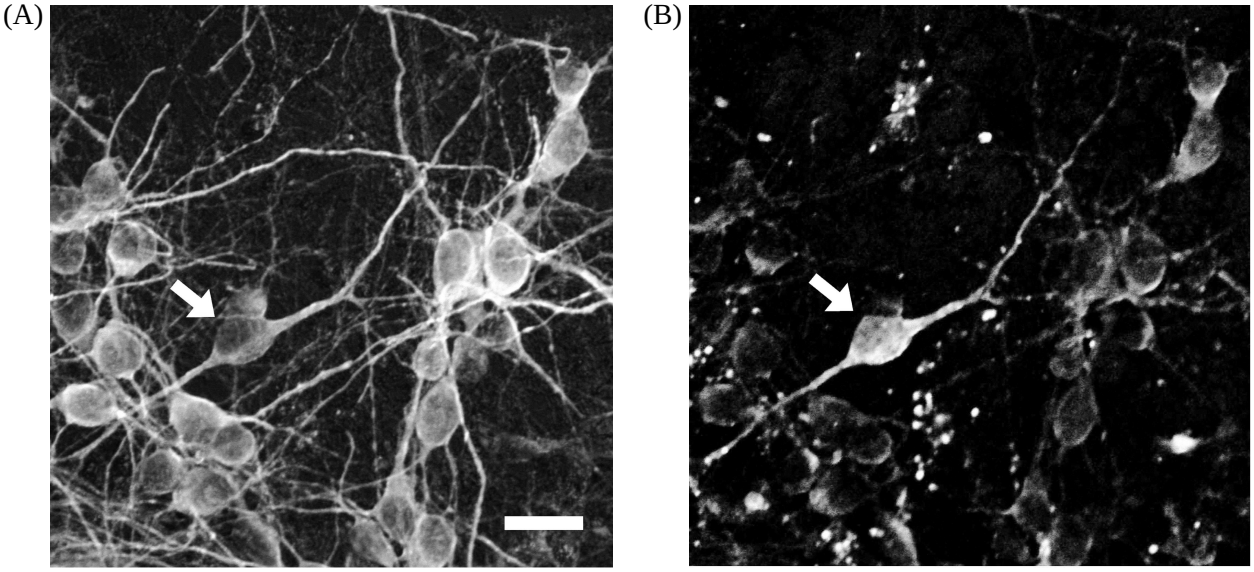


Figure 1: Two-photon images of immunocytochemically stained neurons. (A) MAP2. (B) GABA. The two images show the same field of view. Arrow indicates a GABA-positive cell. Negative controls showed no visible signals. Images were taken with a Carl Zeiss LSM510 multiphoton microscope. Scale bar: 20 μm.

2 ms after stimulation[2]. Spikes were detected online by thresholding at 5x RMS noise, and later validated based on the shapes of their waveforms (P. P. Mitra, personal communication).

Stimulation system

Stimuli were generated using our custom-made 60-channel stimulator (Wagenaar and Potter, 2004). We used biphasic rectangular voltage pulses, positive phase first, since these were found to be the most effective stimulus at any given voltage (Wagenaar et al., 2004). We used stimulus pulse widths of 400 μs per phase and voltages between 100 and 900 mV. Higher voltages were not used, to prevent possible electrochemical damage to electrodes and nearby cells. The stimulator was switched to high impedance output 100 μs after each pulse using the built-in switches of our stimulator.

Experimental protocols

Before experimenting on any MEA, we probed each electrode in the array with voltage pulses between 100 and 900 mV, in random order. For each electrode, we determined the voltage V^* at which the response was five times the spontaneous firing rate. Typically, 40–50 electrodes per dish were in sufficiently close contact with the culture to attain that level of response by voltages in the range tested. For each experimental series, we selected either individual electrodes or groups of 2 to 25 electrodes randomly from this pool.

We used three stimulation protocols:

'S' — Single electrode stimulation: One electrode was stimulated repeatedly at its voltage V^*. We used this protocol at ten different frequencies between 0.05 and 50 stimuli-per-second (stim/s).

'M' — Multi-electrode stimulation: A group of 2 to 20 electrodes was stimulated cyclically at 2 to 20 stim/s, such that each electrode received stimuli once per second, or 25 electrodes were stimulated cyclically at 50 stim/s, each receiving two stimuli per second.

[2]Except on the electrode used for stimulation, which remained saturated by stimulation artifacts for 50–150 ms.

Each electrode was stimulated at its own previously determined V^*. Five different group sizes with corresponding stimulation rates were tested with this protocol.

'FB' — Closed-loop feedback stimulation: Ten electrodes were stimulated cyclically at 10 stim/s (so that again each electrode was stimulated once per second), with voltages continuously tuned to maintain a constant tonic firing rate, as decribed under *Tuning the feedback*, below. With this protocol we could stably maintain firing rates between spontaneous levels and 500 spikes per second array-wide (spsa) using voltages not exceeding 900 mV (Figure 2).

Experimental runs lasted 5 minutes each and were randomly interleaved with each other and with control runs in which we recorded spontaneous (unstimulated) activity. Protocols 'M' and 'S' were performed on N=11 cultures from 3 platings; protocol 'FB' was performed on N=10 cultures from 2 platings. In all cases, each condition was tested 10 times on each culture, with a new random selection of electrodes each time.

Quantifying the level of bursting

Bursts come in different forms, so simply tallying up the number of bursts is not sufficient to describe the burstiness of a culture: it is essential to account for the size of bursts, measured in terms of number of participating neurons, aggregate number of spikes, or duration. Fortunately, we found that it is not necessary to identify individual bursts in order to quantify the level of burstiness of a recording. Instead, we used the following method: divide a 5-minute recording into 300 one-second time bins, and count the number of spikes (total across all electrodes) in each bin. Compute the fraction of the total number of spikes accounted for by the 15% of bins with largest counts. If the firing rate is tonic, this number, f_{15}, will be close to 0.15. Conversely, if a recording is so bursty that the majority of spikes are contained in bursts, f_{15} will be close to one, since even at the highest burst rates observed during these experiments, bursts did not occupy more than 45 one-second bins (15%) in a 5-minute recording. We then defined a *burstiness index*, normalized between 0 (no bursts) and 1 (burst dominated) as $BI = (f_{15} - 0.15)/0.85$. (Statistical fluctuations make BI deviate slightly from zero even in complete absence of bursts.)

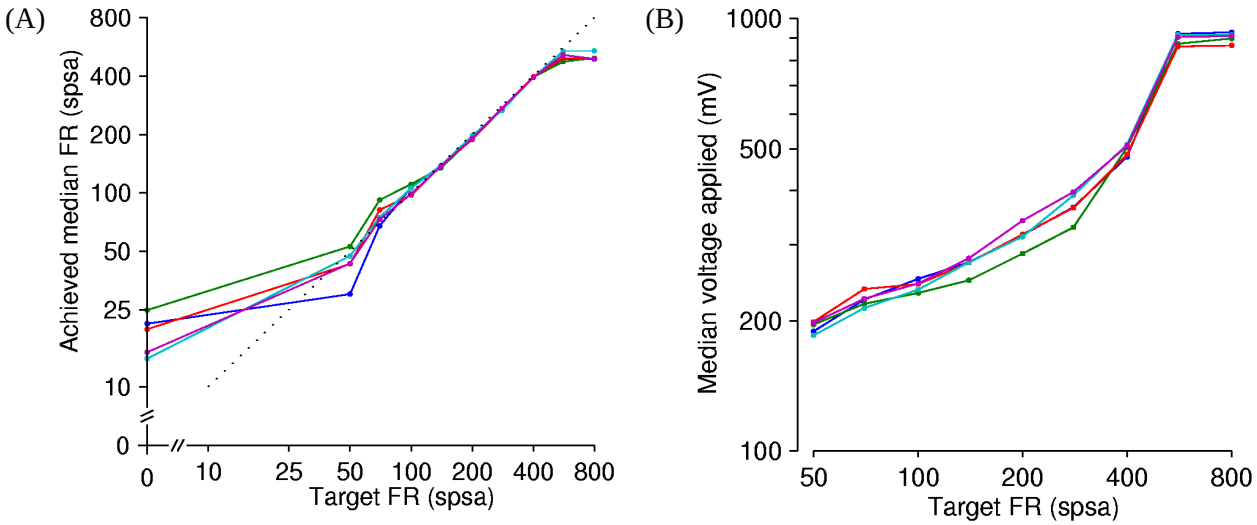


Figure 2: Performance of feedback protocol. (A) Median firing rate (dishwide) achieved vs target. Any firing rate between the spontaneous rate and 500 spsa could be stably maintained. Higher rates were not achievable in this culture without exceeding the safe voltage limit of 900 mV. Dotted line marks equality of achieved and target rates. Data from 5 series on different sets of electrodes; 45 div. (B) Stimulus voltage used to control firing rate at different levels.

Tuning the feedback

For the closed-loop stimulation protocol, we continuously monitored a culture's actual firing rate, and adjusted the stimulation voltages for each electrode to maintain the target firing rate, f_0, as follows. Initially, we used a base voltage, $\bar{V} = 200$ mV, applied to all electrodes. We then measured the (culture-wide) firing rate, $\bar{f}$, in 2-s sliding windows, and used this to update $\bar{V}$ every 100 ms according to:

$$\bar{V} \leftarrow \bar{V}\left(1 - \varepsilon\frac{\bar{f}}{f_0}\right),$$

where ε is a gain factor which determines how fast $\bar{V}$ reacts to changes in $\bar{f}$. We set $\varepsilon = 0.02$, corresponding to a time constant of 5 s. This ensured rapid feedback, while preventing oscillations due to overcompensation.

To account for variations in stimulation efficacy between electrodes, we measured the firing rates in the first 100 ms after each stimulus individually. For each electrode *k*, we used these measurements to maintain a running average, f_k, of the firing rates after the most recent 20 stimuli to that electrode. Every 100 ms, we recalculated fine-tuning factors, α_k:

$$\alpha_k \leftarrow \mathcal{N}\, f_k^{-1},$$

where $\mathcal{N}$ is a normalization factor to make the average of all α_k's be 1. We then set the voltage for the next stimulus on electrode *k* to

$$V_k = \alpha_k \bar{V}.$$

Thanks to MeaBench and our custom-made stimulator (Wagenaar and Potter, 2004), these adjustments could be made in real-time without interrupting the stimulation process.

Since we wanted to control the tonic firing rate, updating $\bar{V}$ and α_k was suspended during putative bursts, detected using a simple heuristic: any 100 ms windows that had a spike count higher than $5\times$ the target were considered potential bursts, and thus excluded for the estimate of the tonic firing rate.

Immunostaining

The fraction of GABAergic neurons was determined as follows. Cultures were fixed with 4% paraformaldehyde at room temperature for 30 minutes. After treatment with 0.1% Triton-X-100 in PBS for 20 minutes, they were incubated in 2% goat serum for 1.5 hours and then in the primary antibodies anti-MAP2 (mouse, 1:200; MAB378 from Chemicon, CA) and anti-GABA (rabbit, 1:100; AB131 from Chemicon, CA) overnight at 4 °C. After washes, cells were incubated with secondary antibodies (Alexa Flour 488 goat anti-mouse, 1:200; Alexa flour 594 goat anti-rabbit, 1:1000; and Hoescht, 1:1000; all from Molecular Probes, CA) for 1 hour at room temperature. Fluorescence images were obtained from a Sony digital camera on a Nikon TE300 fluorescence scope and a Carl Zeiss LSM510 multiphoton microscope.

Results

Spontaneous bursting

Before developing a method to control bursting, we needed to characterize the different kinds of bursting encountered in the spontaneous activity of cultured cortical networks. The frequencies of bursts as well as their sizes were highly variable between cultures from different platings, and even between cultures within platings. Additionally individual cultures showed large variations from day to day. Cultures spontaneously exhibited a wide range of bursting behaviors, from short single cell bursts, to small local bursts involving

2–5 electrodes, to long global bursts (Figure 3). Some cultures exhibited 'superbursts': stereotyped sequences of global bursts, separated by several minutes devoid of bursts (Wagenaar, Z. Nadasdy, and Potter, *in preparation*). Global bursts were typically first observed at around 7 div, after which burstiness steadily increased until they dominated the activity at around 20–25 div. After that, burstiness fluctuated somewhat, but remained high for as long as we looked (up to 45 div in these experiments).

Response to stimulation

The immediate response to stimulation at any electrode consisted of three phases (Wagenaar et al., 2004): (1) Direct, non-synaptically-propagated responses, with very precise timing (typical jitter: 100 μs), and latencies of 3 to 10 ms; (2) Post-synaptic responses, mostly with latencies between 5 and 50 ms; (3) Bursts, often evoked by strong or low-frequency stimuli. Such bursts were time-locked to the stimulus pulse with latencies characteristic of the local network around the electrode stimulated—usually in the range of 50–200 ms—but were otherwise similar to spontaneous bursts. Examples of early responses are shown in Figure 4.

During slow single electrode stimulation (0.05 stim/s), most or all stimuli entrained bursts as previously reported by Gross et al. (1993) and Maeda et al. (1995). At slightly higher frequencies (0.1–0.5 stim/s), bursts were elicited less consistently, depending on stimulation electrode. At still higher frequencies (1–5 stim/s), most stimuli did not elicit bursts, and in fact the burstiness began to drop below spontaneous levels. Increasing the stimulation rate further (10–50 stim/s) did not reduce burstiness more (Figure 5A). The best burst control on average was achieved at 10 stim/s: $BI = 0.19 \pm 0.02$ (mean $\pm$ standard error of the mean (SEM), N=105 runs using different electrodes in 11 cultures; range of per-culture means: 0.04–0.55). This level of burstiness was significantly below the average spontaneous level $BI = 0.48 \pm 0.02$ (N=199 runs, same 11 cultures; range: 0.19–0.86).

When stimuli were applied through a single electrode at high rates, the immediate response to stimulation (spikes recorded 2–20 ms post-stimulus) dramatically decreased with increasing stimulation frequencies (Figure 6). This was likely responsible for the lack of improvement of burst control at those high frequencies. However, the responses to infrequent stimulation through one electrode were not affected by rapid stimulation through another electrode, so this reduction of efficacy was due to a mechanism local to the stimulated electrode, and not to a network-level fatiguing effect.

Burst control by distributed stimulation

Based on the observation that rapidly stimulating single electrodes reduced the efficacy of those stimuli but not of stimuli to other electrodes, we proceeded to test whether better burst control could be achieved by distributing the stimulus load across several electrodes, using protocol 'M' (see Methods). At intermediate frequencies (2–10 stim/s), this protocol resulted in somewhat higher burstiness than single-electrode stimulation, but at frequencies above 10 stim/s, multi-electrode stimulation resulted in greatly improved burst reduction (Figure 5B–C). At the highest stimulation rate tested, 50 stim/s distributed across 25 electrodes, bursts were completely suppressed in all cultures tested. In all cases, a change of stimulation protocol rapidly affected burstiness, and bursting resumed as soon as stimulation was stopped (Figure 7).

Burst control by closed-loop stimulation

Perfect burst control was achieved using protocol 'M', but only at very high stimulation rates and using a large number of electrodes. If good burst control could be attained using fewer electrodes or lower stimulation rates, this would have practical advantages. We noted that the bursts that occurred in protocol 'M' at intermediate stimulation rates were mostly entrained by only one of the electrodes used in a given run, indicating that the calibration of stimulus efficacy performed before the experiment (see Methods) was not

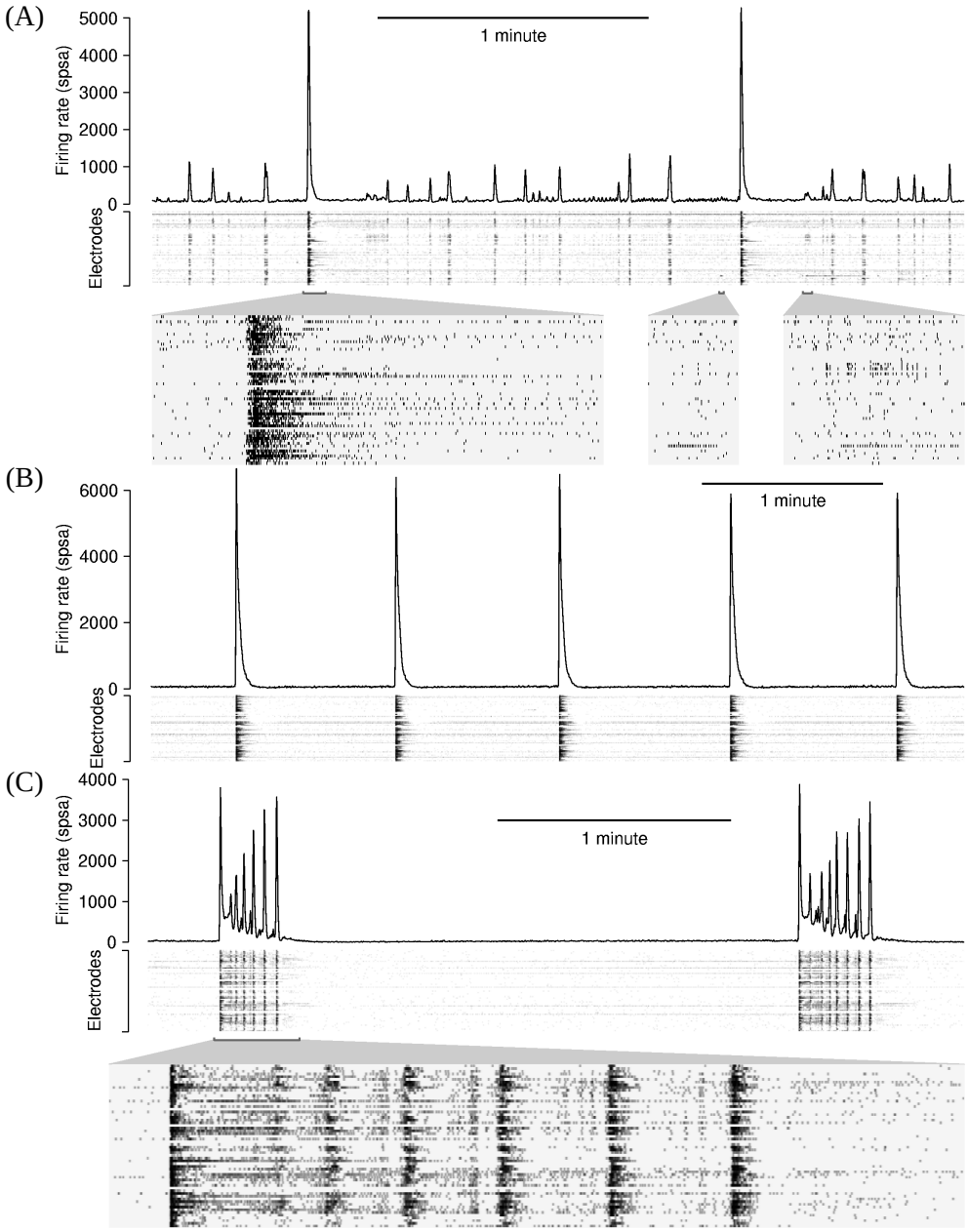


Figure 3: Examples of different spontaneous bursting patterns, with array-wide firing rate (line graphs) as well as per-electrode firing rates (greyscale plots). (A) Chaotic bursting. Insets below show spike raster plots for a large global burst, a single channel burst and a small local burst, at 20x magnification. Recorded at 25 div. (B) Spontaneously regular bursting. Recorded at 39 div. (C) Superbursts. Inset shows spikes at 10x magnification. Recorded at 34 div.

Figure 4: Array-wide responses to stimulation. Each graph shows the responses on one electrode, represented according to the geometry of the array. The stimuli were delivered to the marked electrode. Vertical line indicates time of stimulation. Spikes were detected after artifact suppression (Wagenaar and Potter, 2002); TTX control confirmed the biological origin of all detected spikes.

(A)

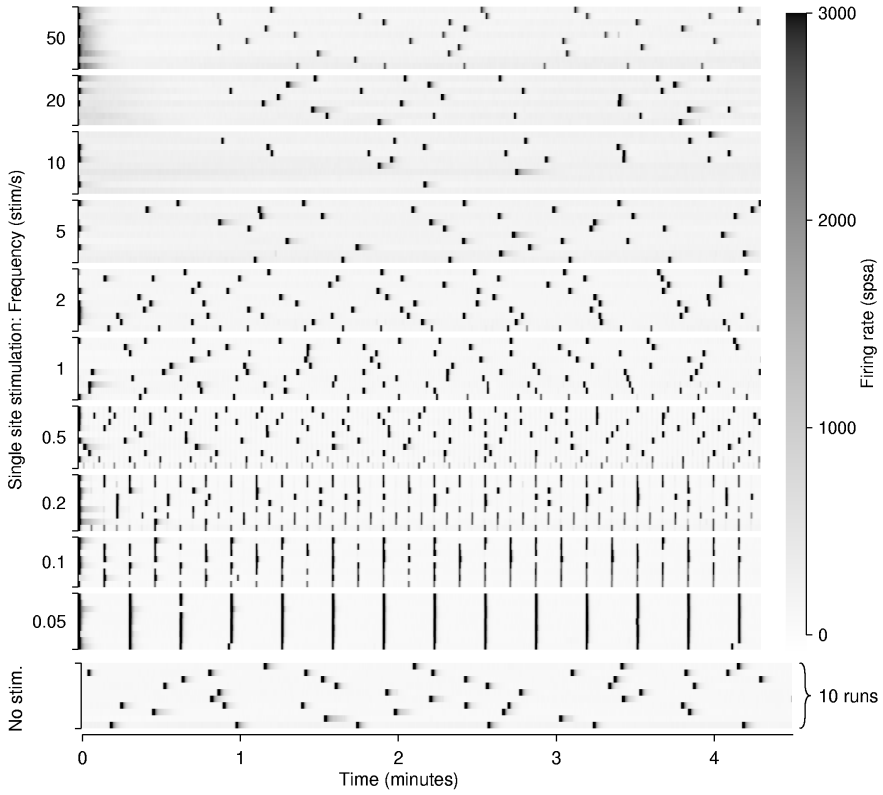
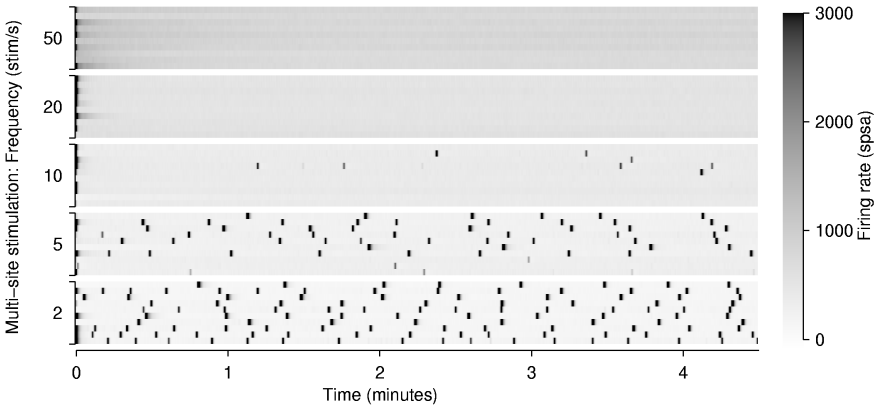


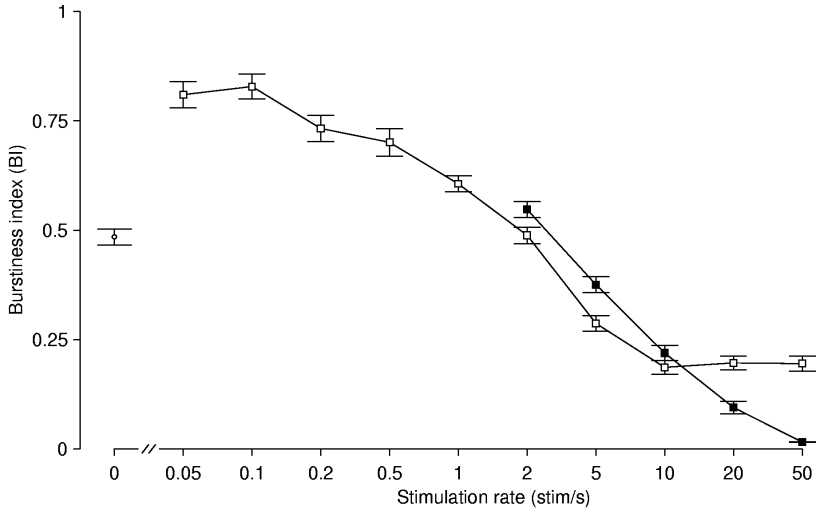
Figure 5: (A) Burstiness during single electrode stimulation (protocol 'S') and spontaneous activity (no stimulation). Each row shows the arraywide firing rate (coded by the grey scale at right) as a function of time during one 5-minute experimental run. In the 10 examples of spontaneous activity shown (bottom), bursts occurred irregularly about once per minute. In the 10 examples of stimulation at 0.05 stim/s, bursts were perfectly aligned with stimuli, except in a few cases where a spontaneous burst just preceded the stimulus. (The stimulating electrode was different in each of the 10 rows.) At 0.1–0.2 stim/s, bursts underwent period doubling. Bursts during stimulation at 1–5 stim/s were less frequent, but still mostly stimulus-locked. In the 10–50 stim/s runs, burst control was perfect for the first 45 s, after which a spontaneous-like pattern returned. Data from a culture at 39 div. Note that experimental runs were executed in random order. (B) Bursting during multi-electrode stimulation (protocol 'M'), same culture. Perfect and sustained burst control is attained at the higher stimulation frequencies. Note the increase in tonic firing rate (background shading) as the stimulation frequency is increased. (C) Burstiness index as a function of stimulation frequency, for single-electrode stimulation (open squares) and multi-electrode stimulation (filled squares). Slow single-electrode stimulation elevates the burstiness over spontaneous (unstimulated) levels (open circle), while rapid stimulation reduces it. Values are mean $\pm$ SEM from N=100 runs on 10 cultures. The most effective protocol tested, 50 stim/s distributed across 25 electrodes, suppressed bursts completely (N= 60 runs, 6 cultures).

— Please see next page for (B) and (C) —

(B)



(C)



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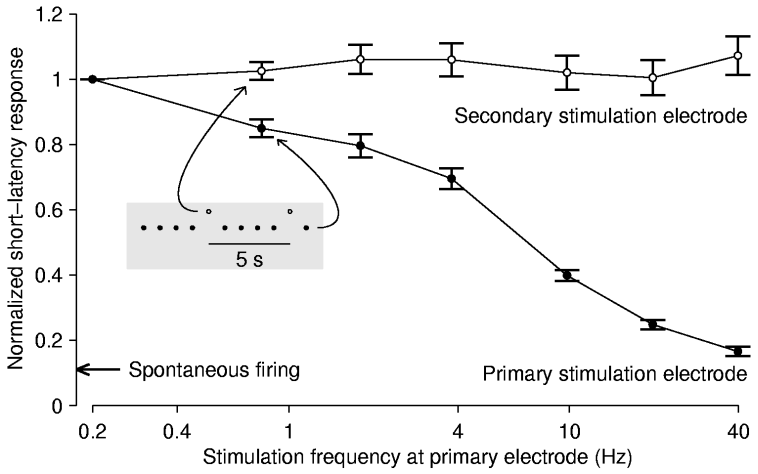


Figure 6: Stimuli presented to a single electrode ('primary stimulation electrode', filled circles) yielded much reduced responses in the first 20 ms post-stimulus when the stimulation rate was increased. (We focused on short-latency responses, because the majority of response spikes occurred at short latencies, and because responses cannot be unambiguously defined beyond one inter-pulse-interval, i.e. 25 ms for the highest stimulation frequency.) In fact, at a stimulation frequency of 40 stim/s, the response was not much higher than the spontaneous firing rate (arrow at left). Each stimulation series lasted five minutes, and we discarded the responses recorded during the first 30 seconds so as to measure the sustained response rate. Results are mean $\pm$ SEM from 53 electrode pairs in 4 cultures. During these experiments, we presented stimulus pulses to a second electrode every five seconds. The responses in the first 20 ms after these latter stimuli (open circles) were not affected by the rate at which the first electrode was stimulated. Response strength in all cases was normalized to the results obtained from single-electrode stimulation at 0.2 stim/s. The response strengths are plotted as a function of the frequency at which the primary electrode was stimulated. Inset and associated arrows: Explanation of stimulation protocol. Irrespective of the frequency of the primary stimulation electrode, the secondary electrode was stimulated once every 5 seconds.

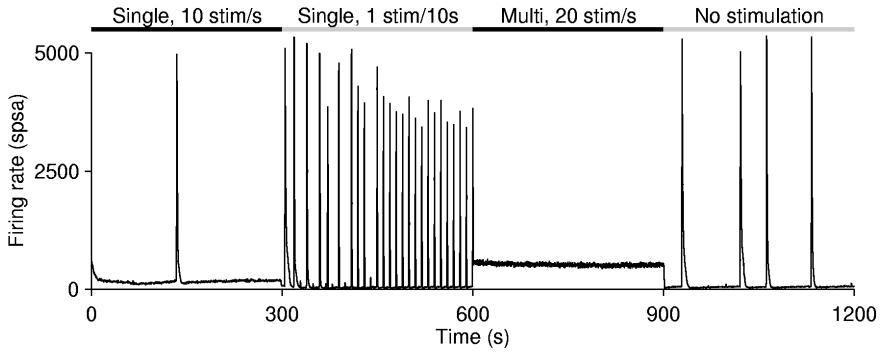


Figure 7: When switching between stimulation protocols, a culture's activity pattern rapidly changed to match the new stimulation context. Here we show switches from rapid single electrode stimulation to slow single electrode stimulation, to rapid multi-electrode stimulation, to no stimulation.

a very good predictor of efficacy in the context of much more intense multi-electrode stimulation (data not shown). Thus we hypothesized that the level of burst control attained by pre-defined voltage pulses could be further improved by tuning the stimulation voltages in real time to obtain a constant level of response. We used feedback control (protocol 'FB'; see Methods) to regulate the median firing rate at 9 fixed levels between 50 and 800 spikes per second array-wide (spsa). Increasing the median firing rate over spontaneous levels reduced burstiness monotonically (Figure 8). At the highest target rate of 800 spsa, this protocol was significantly more effective than either single-electrode or multi-electrode stimulation compared at the same stimation rate (10 stim/s). This held despite the fact that the spontaneous *BI* was 50% higher on average for those cultures on which we tested feedback stimulation compared to those tested with single or multi-electrode stimulation. (This difference in spontaneous behavior was due to variability between cultures, not to our intervention.)

A final comparison of the various protocols tested was made by counting in what percentage of cultures each protocol suppressed bursts completely during a 5-minute run (Figure 9). Any run with $BI<0.05$ was considered burst-free for this assessment. The most intense protocol 'M' stimulation (50 stim/s distributed across 25 electrodes) suppressed bursts in all cultures, independent of the selection of stimulation electrodes. Although a set of electrodes could be found to suppress bursts at 10 stim/s with fixed voltages in over 50% of cultures (white bars), a random selection of electrodes suppressed bursting in only 1 in 5 cultures (grey bars). Closed-loop feedback did much better: a random selection of electrodes suppressed bursting in over 50% of cultures (grey bar), and in 30% of cultures, all 10 random selections of electrodes tested suppressed bursts (black bar).

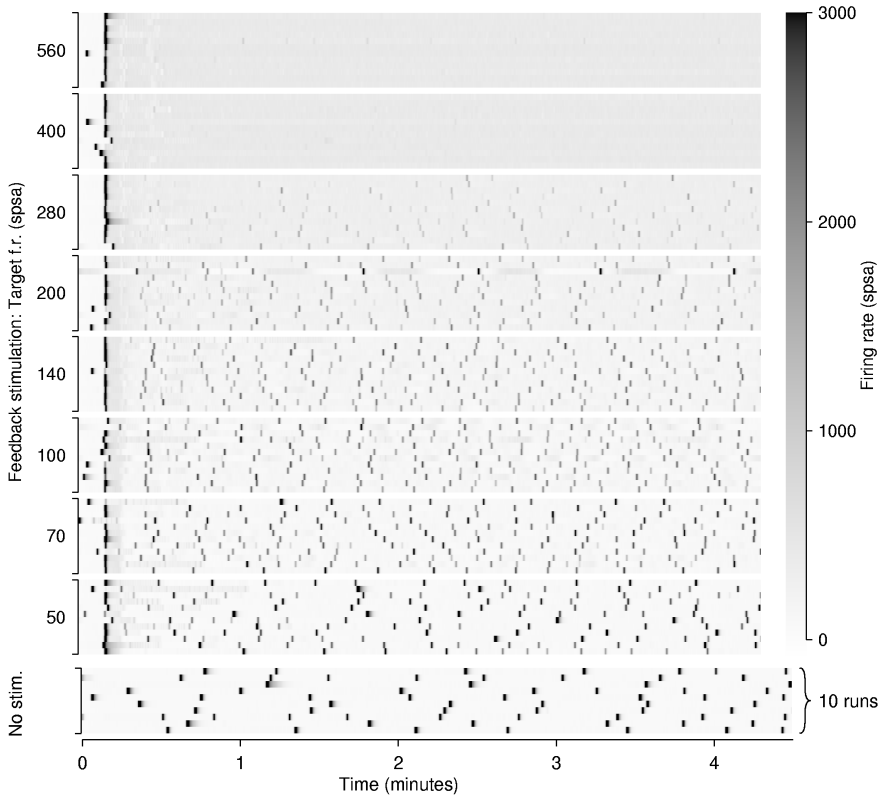
Discussion

Several years ago, Latham et al. (2000) showed that networks with a large fraction of intrinsically active neurons have a reduced tendency to burst. We extend this finding by demonstrating that increasing the tonic activity above spontaneous levels by high-frequency multi-site electrical stimulation also reduces or suppresses bursting. Strikingly, complete suppression of bursts was achieved by a combination of stimuli that entrained bursts when applied singly. Rapid stimulation through single electrodes yielded fewer bursts than slow stimulation, not just per stimulus, but per unit time: stimulation at 5 stim/s or more reduced burstiness to below spontaneous levels. Distributing the stimuli across 20 or more electrodes proved highly effective to reduce it even further, and with 50 stim/s distributed across 25 electrodes, bursting was suppressed completely in all cultures tested, independent of the selection of electrodes. However, such a high stimulation rate may be undesirable in some applications, or that many electrodes may not be available. When the number of electrodes used for burst control was limited to 10, stimulating with closed-loop feedback was found to be the optimal solution: this protocol completely suppressed bursts in over 50% of cultures using 10 stim/s distributed across randomly selected groups of 10 electrodes. With careful selection of electrodes, fixed-voltage stimulation through single or multiple electrodes suppressed bursting in a similar fraction of cultures as feedback stimulation. However, feedback stimulation was far more robust: in 30% of cultures it worked regardless of electrode selection. Electrode independence was never seen for fixed-voltage stimulation at 10 stim/s. To extend burst control beyond 5-minute runs, such robustness is highly desirable.

Synchronized bursting is fundamentally a network phenomenon, emerging from the synaptic interactions between a large number of cells. Whether these cells would endogenously burst in the absence of synaptic input is probably not essential for this phenomenon. The cellular and network mechanisms of bursting and burst suppression are not yet understood in detail. There is some controversy about the origin of the refractory periods for spontaneous bursts: Opitz et al. (2002) reported synaptic depression immediately after population bursts, while Darbon et al. (2002) found no evidence of synaptic depression: no depletion of vesicles, and no desensitization of post-synaptic receptors.

It has been suggested that the persistence of global bursting in mature cultures is evidence that such

(A)



(B)

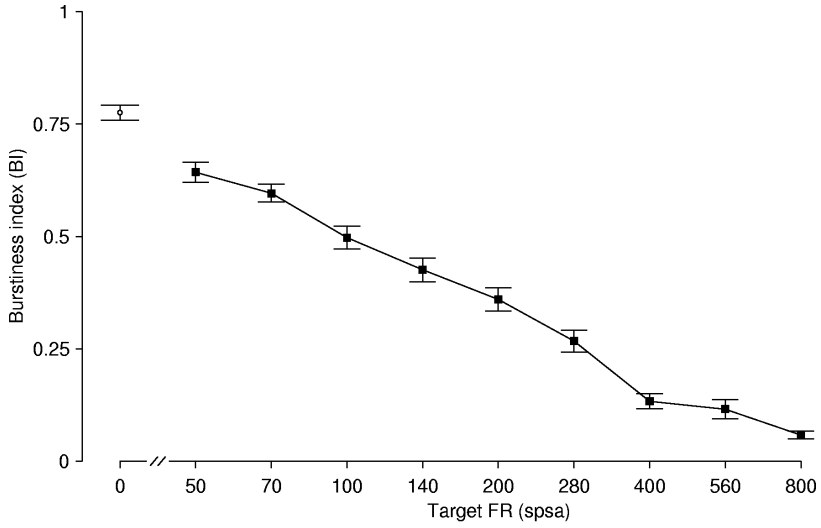


Figure 8: Burstiness during closed-loop control of tonic firing rate. (A) After an initial period of about 15 s during which the feedback algorithm settles, burst control was perfect at the higher target firing rates. From a culture at 43 div. (This culture was not tested at 800 spsa.) (B) Burstiness index decreased monotonically with the target rate, and was always below the spontaneous level (open circle). Values are mean $\pm$ SEM from $N = 85$ runs using different sets of electrodes, on 10 cultures.

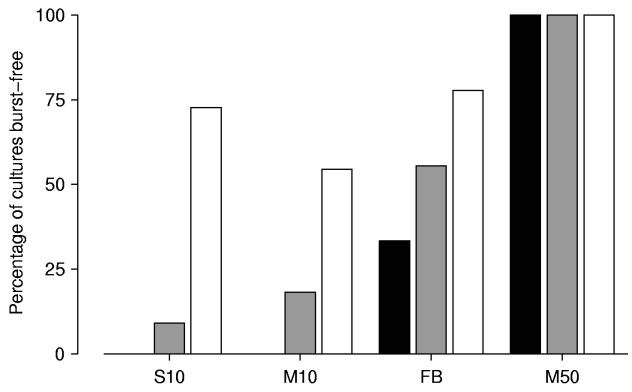


Figure 9: Assessment of the success rate of different burst suppression protocols. Bars show the percentage of cultures in which each protocol successfully suppressed bursting, by random electrode selection (grey), by at least one of 10 selections tested (white), or by all of 10 selections tested (black). None of the cultures used in these experiments were burst-free in spontaneous activity. Protocols compared are: protocol 'S' at its optimal stimulation rate (10 stim/s, 'S10'); protocol 'M' at the same rate ('M10'); protocol 'FB' at its optimum (target 800 spsa, 'FB'); and protocol 'M' at its optimum (50 stim/s distributed across 25 electrodes, 'M50').

cultures are in a state of arrested development as a result of lack of sensory input. Our experiments support this view, since we found that substituting for thalamic inputs with distributed electrical stimulation reduced bursting dramatically. Given that the developmental fine structuring of several primary cortical sensory areas *in vivo* is known to be determined by the pattern of inputs, it is tantalizing to ask whether persistently applied stimulation *in vitro* might similarly influence network topology.

In contrast to burst suppression by (partially) blocking excitatory synaptic transmission, e.g. using AP5, CNQX (Jimbo et al., 2000), magnesium or kainic acid (Furshpan and Potter, 1989), distributed stimulation does not reduce the ability of the culture to respond to additional stimuli. Continuously applying distributed stimulation to suppress bursts is thus compatible with studies of use-dependent modification of activity in cultured networks. Additional stimuli can be superimposed on a background of burst-quieting stimuli, to tetanize particular pathways or to probe network activity. Moreover, distributed stimulation mimics more natural modes of activation in which sensory signals are continuously coming in to the network. Bursts are known to have an effect on tetanus-induced synaptic plasticity (Maeda et al., 1998). Therefore, we suggest that burst suppression may lead to more stable connections, and more predictable results of tetanization (Z. C. Chao, Wagenaar, and Potter, 'Random external background stimulation helps maintain network synaptic stability after tetanization: a modeling study', *submitted*). We expect that burst control will make these networks more useful for the study of distributed information processing, robotic control, and network plasticity related to learning and memory (Potter, 2001; DeMarse et al., 2001; Potter et al., 2004).

If distributed stimulation so effectively reduces bursting *in vitro*, it might also work *in vivo*. Epileptic seizures in human cortex, while probably due to very different causes, have a strikingly similar phenomenology: ensemble bursts extending over large areas of neural tissue (Lopes da Silva et al., 2003). Electrical stimulation has been used in several experimental therapies for epilepsy; stimulation of the vagus nerve is the most well-known example (Penfield and Jasper, 1954; Hammond et al., 1995; Ben-Menachem et al., 1994; Fisher et al., 1997; Handforth et al., 1998; Koo, 2001). Alternatively, animal and modeling studies suggest that focal stimulation at the site of the seizure can terminate seizures after they have started (Lesser et al., 1999; Franaszczuk et al., 2003; Slutzky et al., 2003). In humans, focal stimulation in the cortex or hippocampus has indeed been found effective in a number of studies (Cooper et al., 1973, 1976, 1977; Lüders et al., 1988; Shulz et al., 1997; Velasco et al., 2000, 2001; Motamedi et al., 2002; Vonck et al., 2002). Stimulation through a single electrode offered protection against seizures, but only if the stimulus was strong enough that the entire seizure-prone area was reached (Motamedi et al., 2002; Kellinghaus et al., 2003),

which was difficult in practice. Distributing stimulation across multiple electrodes might be attractive for several reasons. Firstly, the amplitude of pulses delivered to each electrode could be much lower, reducing the risk of side effects (Wheless, 2001; Schachter, 2002), tissue damage (Shepherd et al., 1991; Tehovnik, 1996), or electrode damage commensurately. Secondly, the system would be more fault tolerant (Davis, 2000), as losing one or two electrodes from a large ensemble would hardly compromise efficacy. Thirdly, unlucky placement of a single electrode can result in poor burst control, while with multi-electrode stimulation, the result is much less dependent on exact placement. Finally, if the electrodes were connected to a recording system equipped with seizure prediction software, stimulation parameters could be tailored to the predicted locus of impending seizures (Iasemidis, 2003). Our real-time controlled stimulator (Wagenaar and Potter, 2004) could be a starting point for developing such a system.

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