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Chronic Network Stimulation Enhances Evoked Action Potentials

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Abstract

Neurons cultured on multielectrode arrays almost always lack external stimulation except during the acute experimental phase. We have investigated the effects of chronic stimulation during the course of development in cultured hippocampal neural networks by applying paired pulses at half of the electrodes for 0, 1, or 3 hr/day for 8 days. Spike latencies increased from 4 to 16 ms as the distance from the stimulus increased 200–1700 μm, suggesting an average of 4 synapses over this distance. Compared to no chronic stimulation, our results indicate that, chronic stimulation increased evoked spike counts per stimulus by 50% at recording sites near the stimulating electrode and increased the instantaneous firing rate. On trials where both pulses elicited responses, spike count was 40–80% higher than when only one of the pulses elicited a response. In attempts to identify spike amplitude plasticity, we found mainly amplitude variation with different latencies suggesting recordings from neurons with different identities. These data suggest plastic network changes induced by chronic stimulation that enhance the reliability of information transmission and the efficiency of multisynaptic network communication.

Keywords

Electrode array; MEA; Paired-pulse; Chronic stimulation; Plasticity

1. Introduction

Neural networks develop in the mammalian hippocampus with daily external inputs that modulate excitatory and inhibitory connections, partially due to Hebbian mechanisms (Hebb, 1949) and homeostatic plasticity (Turrigiano and Nelson, 2004), but monitoring the effect of external input on development is difficult at best. Multi-electrode arrays (MEAs) (Thomas et al., 1972; Gross, 1979; Pine, 1980) provide the potential to determine the effect of frequent excitation over periods of days to weeks simultaneously on a large number of neurons, but experiments to date are usually conducted without the continuous inputs present in vivo. Somewhat surprisingly, traditional live neuronal networks that are created by plating embryonic rodent brain neurons onto MEA's are able to develop their synapses and show spontaneous activity without external inputs. They have proven to be highly valuable in studying cellular and synaptic changes over the course of the development

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exhibiting complex network dynamics like synchronized network bursts (Maeda *et al.*, 1995; Van Pelt *et al.*, 2004; Van Pelt *et al.*, 2005; Chiappalone *et al.*, 2006) and self-organization (Beggs and Plenz, 2003; Pasquale *et al.*, 2008). Moreover, stimulation does induce plasticity in these neuronal networks. More recently, long-term plasticity was investigated by comparing spontaneous activity before and after tetanic stimulation (Maeda *et al.*, 1998; Wagenaar *et al.*, 2006; Chao *et al.*, 2007; Madhavan *et al.*, 2007; Vajda *et al.*, 2008). Evoked responses showing a pathway-specific potentiation and depression were investigated in pioneer work by Jimbo *et al.* (1999). Furthermore, a form of learning (Shahaf and Marom, 2001; Marom and Shahaf, 2002; Eytan *et al.*, 2003) and use as a pattern recognition device (Bonifazi *et al.*, 2005; Ruaro *et al.*, 2005) were investigated in vitro. However, the usefulness of MEAs to study slowly developing forms of plasticity may be limited by their lack of external inputs that occur normally during development (Wagenaar *et al.*, 2005). In one sense, the literature has largely reported responses of sleeping networks without inputs. Turrigiano's group have shown in rats in vivo that monocular deprivation greatly inhibits synaptic development and that restoration of visual input leads to scaled development of both excitatory and inhibitory synapses in the ocular cortex (Maffei *et al.*, 2006). We know of no studies where a stimulation protocol was applied during the course of development to a neuronal network, although directed training effects have been reported for periods of several hours (Eytan *et al.*, 2003; Stegenga *et al.*, 2009). Usually, the probe stimulation is applied only in the day of the recording (no more than 40 min), not before. Here we chronically stimulated arrays for 0, 1 and 3 hr/day, during 8 days before the recording day in order to investigate the long-term effects that external inputs can provide to the network compared to the standard unstimulated condition.

In order to increase the probability of excitation without overstimulation, we applied a paired-pulse stimulation paradigm. Previous experiments have shown that facilitation was achieved by inter-pulse intervals (IPI) of 20–50 ms that facilitate the EPSP amplitude (Hama *et al.*, 2004; Mori *et al.*, 2004). The first pulse activates presynaptic voltage-gated Ca^{2+} channels causing Ca^{2+} influx; a sufficiently fast arrival of the second pulse increases the calcium concentration before its removal (Zucker and Regehr, 2002). The result is an increased fusion of transmitter vesicles to deliver more transmitter. Thus, pairs of stimuli with IPI of 50 ms evoke larger postsynaptic currents with the 2^{nd} than the 1^{st} pulse. Here, we determine whether this stimulus paradigm has an additional effect on action potential amplitude as it has been demonstrated for tetanic stimulation (Bakkum et al., 2008).

We were also interested in the larger effect on the network to determine how chronic stimulation affected network elements at a distance. For this reason, we stimulated only half of the array to be able to detect responses as an explicit effect of distance from the stimulation site. We have extended Potter's concept of acute stimulation of the network on one day (Bakkum *et al.* 2008) to 8 days to determine longer term effects of chronic stimulation on the efficiency of communication in the network.

2. Methods

2.1. Neuron culture

E18 rat hippocampal neurons were plated as 500 phase-bright, trypan-blue excluding cells/mm^2 on poly-D-lysine coated MEA's in NbActiv4™ medium that was optimized for higher synapse density and spike rate (Brewer *et al.*, 2008) (BrainBits, Springfield, IL), incubated at 37°C, 5% CO_2, 9% O_2 and saturating humidity (Thermo-Forma, Columbus, OH). Arrays with fixed glass rings and Teflon film covers were used to minimize leaks and evaporation. Every 4–5 days, ½ of the culture medium was removed and replaced with the same volume of fresh NbActiv4. At the recording day of 3 weeks, the culture was comprised of about 67% neurons and 33% astroglia (Brewer *et al.*, 2008).

2.2. Multi-electrode arrays (MEA)

The MEA's from Multichannel Systems (MCS, Reutlingen, Germany) consist of 59 TiN electrodes with diameters of 30 μm, spacing of 200 μm and SiN_3 insulation, plus one ground or reference electrode. The activity during stimulation on the MEA's was measured using an MCS 1100x amplifier at 40 kHz sampling with a hardware filter of 8–3000 Hz at 37°C under continuous flow of hydrated, 5% CO_2, 9% O_2, balance N_2 (AGA custom, Springfield, IL). Electrical stimuli were delivered by a stimulus generator (MCS STG2004). A zebra strip (Fujipoly America Corporation, Carteret, NJ) was used on arrays to reduce noise. MCRack software was used for data recording and software filtering at 200 Hz (High Pass Butterworth). Offline data analysis was performed in MATLAB (The Mathworks, Natick, MA) on data imported in MCD format from MCRack.

2.3. Probe and chronic stimulation

Stimulation trains included two groups of 30 μA constant current paired-pulses (biphasic, 100 μs/phase duration beginning positive; 50 ms ISI between individual stimuli), Figure 1A. Constant current stimulation has the advantage of consistent charge injection with variable electrode impedance, and stimulation is theoretically believed to be consistently correlated with current. However, there are competing reasons that lead others to find constant voltage stimulation preferable (Wagenaar *et al.*, 2004). The stimulus intensity of 30 μA used in our experiments has been shown to be in the range that maximizes the reliability of an evoked response for cultures with the low cell density used here. A paired pulse paradigm with delay of 50 ms was chosen as a minimum number of stimuli that could avoid short-term plasticity while enhancing the probability of postsynaptic potentials (Soleng *et al.*, 2004). Between pairs, there was a wait period of 5 seconds a time determined to be just long enough to preclude most effects of the prior stimulus. An automatic stimulation program was created in Microsoft Visual C++ 2008 that stimulated the entire top half (30 electrodes) of the MEA in a pseudorandom sequence (Figure 1B). The pseudorandom design avoided stimulation of any two adjacent electrodes consecutively to minimize plasticity from stimulation of adjacent electrodes. To reduce spillover of evoked bursts, stimuli were delayed for 1 sec after switching to a new channel. The bottom half of the array was never stimulated to serve as a within-array control for recording sites more than 400 μm from a stimulating electrode. Non-stimulated experiments were arrays that did not receive any stimulation during their developmental phase in vitro (0 hr/day chronic stimulation, n=8 arrays). On the other hand, stimulated experiments involve arrays that were chronically stimulated for 1 (n=8) or 3 (n=12) hour(s)/day at 7, 11, 12, 14, 18, 19, and 21 days in vitro (DIV). Thus, each of 30 electrodes received 0, 2100 or 6300 stimuli/day x 8 days. Since stimulation began at 7 days in culture, before much activity, sites were stimulated without regard to active or non-active electrodes. Analysis covered all electrodes active or not and stimulated or not. The 28 arrays studied were the result of 26 different animal dissections. Activity levels between the networks on arrays plated from the same animal were no more similar than others from different animals.

Recordings started after 21 DIV, allowing functional and structural maturation of the synapses (Muramoto *et al.*, 1993; Kamioka *et al.*, 1996) and stabilization of synchronized network bursting activity (Van Pelt *et al.*, 2004). On the day of recording (day 20–27), arrays were submitted to the following protocol: *(i)* chronic stimulation for 0, 1 and 3 hour(s); *(ii)* spontaneous activity was recorded for 3 minutes; *(iii)* probe stimulation (each electrode in the top half received a train of 10 paired-pulses, a total of 600 stimuli); *(iv)* spontaneous activity was recorded for 3 min. Arrays that did not receive any chronic stimulation (0 hr) were initially recorded for 3 minutes to obtain spontaneous activity then received only a probe stimulation. With the probe stimulation, activity was recorded 10 ms before stimulation and 40 ms after the stimulus was delivered. Stimulus artefacts were

suppressed by a blanking circuit in the MCS amplifier and by discarding any spike detected 2 ms after the stimulus was delivered. A companion paper reports the results of the spontaneous activity (Brewer *et al.*, 2009b). Here we report only evoked responses during the probe stimulation (2–40 ms).

2.4. Optimizing Spike Detection

We investigated the optimal threshold setting (multiples of the standard deviation (SD) of the noise measured before stimulation for each electrode) for detecting action potentials on the basis of peak-to-peak amplitude (Maccione *et al.*, 2009). Standard deviation of the noise was evaluated 10 ms before and 30 ms after each pulse (1 ms bins). The median standard deviation of these 20 bins was calculated to avoid outlier effects. Spikes were detected 2–40 ms after each pulse in a 2 ms sliding window and 1 ms refractory period. Measures taken include number of detected spikes and number of apparently active electrodes as a function of the SD multiple (ranging from 8 to 13), both before and after the addition of a cocktail [final concentrations] of NMDA receptor antagonist APV (5 μM), AMPA receptor antagonist CNQX (10 μM), and Na+ channel blocker TTX (1 μM), administered as a half solution change. Three arrays were recorded.

2.5 Amplitude of the First Evoked Spike

Once the optimal threshold was determined, responses to the stimuli were recorded with each action potential's post-stimulus time and amplitude. We accumulated statistics on the amplitude of the first spike in response to each pulse for comparison purposes.

2.6. Reconstruction and Evaluation of Compound Action Potentials from Pairs of Action Potentials

A simple test can illustrate the likelihood that a single large spike is actually the sum of a pair of spikes from different neurons. Assuming independent recording of the two individual spikes, computer summation at all reasonable time offsets produces compound action potential templates that can be compared numerically (rms error) to the recorded putative compound spike.

2.7. Statistics

Statistical analyses were performed by with Matlab software as Two-Sample Student's T-test for data with normal distributions, with a cuttoff at $p<0.05$ for significance. Data not normally distributed were tested with a non-parametric Kruskal-Wallis test. All graphs display mean ± S.E.

3. Results

3.1. Optimal spike detection threshold

We chose to detect spikes as events whose voltage extremes exceeded a threshold within a 2 ms window, set as a multiple of the standard deviation (SD) of the noise. To optimize, we detected spikes using thresholds varying from 8 to 13 SD, followed by a repeated measure on the same network after inhibition of synaptically induced spiking activity and low level biological noise with an APV/CNQX/TTX cocktail to block NMDA and AMPA receptors and sodium channels, respectively. We measured the difference in the number of apparently active electrodes before and after drug application at each threshold setting, looking for the maximum difference (Figure 2A), finding that a multiplier of 11 was optimal. Further we measured the ratio of total spikes detected after to before drug application, looking for a near zero asymptote (the lowest SD that was not significantly different from zero), which occurred at 11 SDs. Thus, a setting of 11 SD permits a maximal number of evoked channels

and spikes to be detected, but a minimal number of operationally false positive noisy or indirectly active channels and events. We note this is approximately equivalent to a zero to peak threshold of 5.5 x S.D. for a symmetric bipolar spike.

3.2. Effects of chronic stimulation and distance from stimulus on active channels

Figure 3A provides an example of the average number of channels that evoked a response to the stimulus (S). Figure 3B shows that an average of 2.7 channels per stimulus was activated for each distance (200–1721 μm), which suggests that 16 channels on average were activated every time a stimulus was delivered, averaged over all chronic stimulation conditions. Based on all arrays tested, Figure 3C shows that the fraction of channels recording any evoked activity during the entire probe recording (~17 min) was 45–50% with no significant increase due to chronic stimulation. In contrast, this response rate was two-fold higher than spontaneous activity without chronic stimulation and 30% higher than spontaneous activity with chronic stimulation (Brewer *et al.*, 2009b). The percent active electrodes could surely be increased by increasing the density of plated neurons or by addition of extra astroglia (Boehler *et al.*, 2007).

3.3. Effects of chronic stimulation and distance from stimulus on evoked activity

Figure 4 shows the neuron distribution on a MEA at 21 DIV cultured in NbActiv4 medium, for each condition (0, 1 and 3 hr/day chronic stimulation). Chronic stimulation did not change their morphological properties or cause neuron death. Figure 4A also provides examples of evoked activity recorded 2–40 ms after the stimulus was delivered to electrodes located close (283 μm) and far (1721 μm) away from the stimulation site. Notice three parameters changed with distance from the stimulus: *i)* the probability of an evoked spike decreased, *ii)* latencies increased and *iii)* evoked spike amplitudes decreased.

We first determined how the Euclidian distance from a recording to a stimulating electrode affects the probability of a response to the stimulus. Note that the distances between closest, diagonally adjacent, and most widely separated pairs of electrodes are 200 μm, 283 μm and 1721 μm, respectively (Figure 1B). Chronic stimulation (1 or 3 hours) led to higher response rates (50% or 35% vs. no chronic stimulation) at recording electrodes near the probe electrode (≤283 μm away) (Figure 5A). Response rates decreased 3 fold and monotonically with distance to the furthest electrode (Figure 5B). Electrodes in close proximity to the stimulus (283 μm) recorded 3-fold more spike responses than the ones at longest distance (1721 μm). During 1 hr/day chronic stimulation, the number of spikes per stimulus, for all distances, was 10–15% higher than with no chronic stimulation, with the values for 3 hr/day stimulation falling in between. The results show both enhancements due to chronic stimulation and connectivity that declines with distance.

3.4. Effects of chronic stimulation and distance from stimulus on first spike latency

We tested the hypothesis that neurons in close proximity to the stimulus tend to be activated more often than the distant ones, consequently evoking more spike responses. Figure 6A (*i–iv*) illustrates that, near the stimulating electrode, it is common to record spikes at short latency and uniform amplitude, suggesting reliable, direct or at most monosynaptic excitation of the same neuron. In contrast, at maximal distance, Figure 6A (*v–viii*) shows a variety of spike latencies and amplitudes, suggesting activation of different paths and recorded neurons. Figure 6B shows that the coefficient of variation of the latency (mean/SD) increases with distance (from 0.8 to 1.6), largely independent of time of chronic stimulation, indicating greater dispersion with distance from the stimulus site. The latencies summarized in Figure 6C suggest near linear propagation with distance, equivalent to a speed of 0.1 mm/msec. If one extrapolates to zero distance, the latency is 1.2 msec, which may be the time to activation of a directly stimulated neuron. Alternatively, the graph appears to asymptote at 4

msec, the same as might be inferred as the time to activation of a directly responding neuron (Bakkum *et al.* 2008). Therefore, at maximum distance of 1721 μm, an average delay of 16 ms suggests either 3 or 4 synapses.

3.5. Effects of chronic stimulation and distance from stimulus on spike amplitude evoked by the 2nd pulse

Even if extracellular recordings are not the best choice to assess changes in amplitude, we examined evidence for spike amplitude plasticity because the developmental accumulation of sodium channels at the axon initial segment might be influenced by chronic stimulation. We also know that our recordings come from multiple neurons, so any change in amplitude could be from neurons with different identities. Spike sorting was considered problematic due to the huge variety of spike shapes observed in our cultures, especially for overlapping waveforms during bursts, as pointed out also by others (Eytan and Marom, 2006; Rolston *et al.*, 2007; Chiappalone *et al.*, 2008).

To test for spike amplitude plasticity caused by paired pulse or chronic stimulation, we compared first spike latency and amplitude responses from the 1st to those from the 2nd pulse. We found no differences in the probability of the number of responses or spike latency (data not shown). However, Figure 7A summarizes a remarkable 4-fold increase in the evoked spike amplitude of the 2nd pulse minus that evoked by the 1st pulse.

To determine whether distance factored into this change in spike amplitude, Figure 7B shows examples of responses from a chronically stimulated culture for each of the paired pulses at distances close and far from the stimulating electrode. In this case, notice that near the stimulus, spike amplitudes to the paired pulses are similar (Bi-ii), and that far away the spike amplitude is much larger for the 2nd pulse (Biv) compared to the 1st pulse (Biii). Our observations are summarized in Figures 7C–E, indicating the general decline in amplitude with distance. Remarkably, the condition of 3 hr/day chronic stimulation (7E) resulted in an enhancement of the amplitude of the initial spikes to the 2nd vs. the 1st of the probing pulse pairs. Indeed, the 2nd pulse response did not decline with distance. Figure 7F shows the histogram for the evoked spike amplitudes. For amplitudes lower than 50 μV, both distributions were similar; however, the 2nd pulse elicited more responses with higher amplitudes (see the insets). Several possible mechanisms could explain the enhancement in spike amplitude by the 2nd pulse: a) spillover of a burst elicited by the 1st pulse into the recorded response to the 2nd stimulus; b) different neurons being recruited each time; c) compound action potentials; or d) spike amplitude plasticity.

3.6. Spikes are larger in bursts than isolated spikes: spike spillover

To determine which of these mechanisms was responsible for the spike amplitude increases, we examined individual responses to stimuli. Figure 8Ai-ii shows an example of a delayed burst evoked by the 1st pulse at 1721 μm distance. At this distance, average latency was 16 ms (Figure 6C). Therefore, we chose a window of latencies longer than 10 ms for the 1st pulse and shorter than 10 ms for the 2nd pulse to examine the responses to the 2nd pulse that could be spillover of burst firing into the 2nd window. We found that in response to the 1st pulse, 65% of the records contained spikes after 10 ms, which could possibly spillover into the window of the 2nd pulse. Also, in response to the 2nd pulse, 45% of the records evoked a spike before 10 ms. These large percentages suggest a high frequency of burst continuation or spillover from the 1st to the 2nd window. If the first spike after a pulse occurs during a burst, its amplitude is likely to vary considerably according to the identity of the neuron, position of the spike within the burst, and partial or complete overlap of spikes from the 1st pulse, complicating interpretation. These factors precluded further analysis but indicate a

large fraction of spikes during the time window following the 2nd pulse were due to spillover of burst activity evoked by the 1st pulse.

If burst spikes evoked by the 1st stimulus spillover into the time window of the 2nd stimulus, then spike frequency might increase in the window of responses to the 2nd pulse. Since spontaneous burst duration was 300 ms in these same networks (Brewer *et al.*, 2009b), if a burst was initiated during the 50 ms following the 1st pulse, it would likely continue or spillover into the window that follows the 2nd pulse. One characteristic of a burst is a higher spike rate. In Figure 8B, we calculated the inter-spike interval (ISI) of evoked responses and represented it in terms of an instantaneous firing rate (1/ISI). Note that the frequency is highest for the 3 hr/day condition, indicating a higher spike rate within bursts for this chronic stimulation. Figure 8C, shows a stimulation-dependent distribution of firing rates with rates higher than 600 Hz only for the 3 hr/day condition.

We next determined whether chronic stimulation affected spike amplitude either for isolated spikes or spikes within a burst. The spontaneous activity from the same arrays (Brewer *et al.*, 2009b), exhibited a mean intra-burst frequency of 50 Hz, about 2 spikes in our 2–40 ms window for the evoked activity. Considering this criterion for possibly evoked ‘bursts’, in Figure 8D we sorted evoked responses into singles (1 spike only, 60%) and ‘bursts’ (≥2 spikes, 40%). We found lower spike amplitudes in the single response category, medium spike amplitudes in mid-burst (all spikes but the first) and largest spike amplitudes in the first spike of a burst. This decrease in mid-burst spike amplitudes is similar to rundown, evidence of short-term depression when the first spike in a burst is larger than mid-burst spikes, traditionally explained by a depletion model (Liley and North, 1953). Note this type of response for 0 and 1 hr/day chronic stimulation, but the 3 hr/day condition had the same amplitudes for first spike and mid-burst. This can partially explain the larger amplitudes with chronic stimulation, but again, within the resolution of our recordings, overlapping spikes and neurons with different identities are involved.

3.7. Compound Action Potentials from Overlapping Spikes

Even when action potentials appear non-overlapping and distinct, overlap is still possible. Figure 9A shows an example where two spikes appear in response to the 1st pulse with 12 and 14 ms delay (Ai), whereas a single, larger spike appears in response to the 2nd pulse (Aii). Using computer addition of the first two smaller spikes at variable offsets shows that they can be matched very well to the later large spike (6 μV rms error) (Aiii). This strongly suggests that the change in apparent amplitude is due to serendipitous alignment of action potentials from two neurons detected at one electrode. Figure 9B shows an analogous case with two closely spaced spikes in response to the 1st pulse (Bi) but only one for the 2nd pulse (Bii). Superposition of waveforms (not shown) was unsatisfactory (rms error > 10 μV), suggesting that the disappearing waveform was due to failure of the neuron to fire at 13 ms rather than overlap. While selective testing of the compound action potential-overlap hypothesis can be convincing, systematically quantifying the contribution of overlap to the overall amplitude change was judged too cumbersome due to the thousands of possible spike combinations.

3.8. Most examples of short-term spike amplitude plasticity arise from spikes of differing latencies

We examined whether spike responses that occurred at the same time could arise from the same unit with a possible plastic gain in amplitude in response to the 2nd stimulus possibly enhanced by chronic stimulation. In order to search for short-term spike amplitude plasticity as a possible explanation for differences in amplitude seen with chronic stimulation, we further separated the records into those where the first spikes had nearly the same latency in

response to the 1st and 2nd pulses (differences < 0.5 ms). These comprised 45–50% of spikes. Figure 10 shows that there were no significant changes in amplitude for spikes of the same latency, regardless of chronic stimulation or distance from the stimulus. The changes in amplitude were confined to the population of spikes with different latencies which were positively affected by chronic stimulation, consistent with Figure 7C–E. For 0 and 1 hr/day chronic stimulation, the slopes were similar with a 0.8 μV change in amplitude per μm distance, while the 3 hr/day condition evoked a 60% higher slope of 1.3 μV/μm. This suggests that the amplitude effect was caused by different identities and numbers of units being recorded with different latencies rather than spike amplitude plasticity of the same unit with the same latency.

3.9. Pulse order effects on evoked spikes

We found no difference in the number of responses evoked by the 1st or 2nd pulses. However, there were notable differences in the statistics for records in which there were evoked responses to both pulses (50%) or to only one of the pulses (50%). Figure 11Ai-vi illustrates three cases we distinguished. Figure 11B shows that, when both pulses were effective, more spikes were elicited (2.5 spikes/stimulus) than when only one of the pulses elicited spikes (1.5 spikes/stimulus). The 3 hr/day chronic stimulation further increased these rates. In addition, Figure 11C shows that spike amplitudes were 40–80% higher for responses to both pulses of the paired stimulus compared to responses to only one of the stimuli.

4. Discussion

External stimulation during development increases evoked responses

Here, we investigated the extent to which in vitro cultured neural networks coupled to MEAs were affected by chronic stimulation and the relationship to the distance from the stimulus. Our results provide evidence that networks exposed to external paired-pulse chronic stimulation can increase their evoked responses almost 50% in comparison to networks cultured in the absence of external stimuli. We also observed an increase in spike frequency in evoked responses. Others have observed that networks cultured in vitro present more inhibitory GABAergic synapses (Liu *et al.*, 2000) than excitatory connections (Vogt *et al.*, 2005), which could result from the absence of external stimulation. This could reduce responsiveness by long term depression of excitatory synapses (Kirkwood *et al.*, 1996; Rittenhouse *et al.*, 1999) and enhancement of inhibitory synapses (Duffy *et al.*, 1976). In vivo, visual deprivation in rats left excitatory connections in cortical layer 4 unaffected, but markedly potentiated inhibitory feedback (Maffei *et al.*, 2006). In another work, sensory deprivation caused long-term depression of responses to the stimulus by trimming two whiskers from postnatal rats (Rema *et al.*, 2003). Finally, (Glazewski and Fox, 1996) reduced the complement of vibrissae on one side of the muzzle to a single whisker and found a linear decline of responses to the stimulus with time of sensory deprivation (7, 20 or 60 days). Thus, the opposite effect from our chronic stimulation suggests that the lack of sensory inputs can cause a decrement in spike activity. This correlates well with the rise in evoked responses in our chronically stimulated cultures, compared to the unstimulated condition. It suggests that cultured neuronal networks benefit from external stimulation during development to facilitate a higher probability of an evoked response.

This rise of evoked activity by chronic stimulation can be associated with the activation of silent synapses (Nakayama *et al.*, 2005; Sivakumaran *et al.*, 2009), which are believed to represent the majority of glutamatergic synapses caused by the lack of functional AMPA receptors and low glutamate release (Gasparini *et al.*, 2000). In our previous work with developing unstimulated neuronal networks (Brewer *et al.*, 2009a), we found an exponential

increase in spike rates with development correlated with a linear increase in synapses. The synaptic receptors NR1, GluR1 and GABA-A scaled linearly with development, but the rate of increase was faster for excitatory than inhibitory receptors. It will be interesting to determine the effects of chronic stimulation during development on excitatory and inhibitory receptors.

Stimulation is more effective and reliable at short distances but spike frequencies and 2nd pulse spike amplitudes are enhanced at distances corresponding to 4 synapses

Distance from the stimulus did correlate with number of evoked responses and timing, consistent with some data in Bakkum *et al.* (2008). Our findings show that electrodes in close proximity to the stimulus evoked 3-fold more responses than the ones at long distance. Early responses (~4 ms) at short distances strongly suggest a direct activation (monosynaptic) of neurons whose identity is determined by the stimulating electrode position. In contrast, at long distances the increase in both latency (~16 ms) and coefficient of variation (Figure 6B–C) suggests propagation of signals through an average of 4 synaptic pathways in the network. The general increase in variance with distance indicates a variety of routes for every stimulus. But with 3 hr/day chronic stimulation, the variance at long distance was 40% less, suggesting more common pathways for this condition. This finding correlates well with the work of Jimbo, where evoked responses were categorized by two distinct phases, an early phase that lasts ~25 ms after the stimulus and a late “reverberating” phase that can last hundreds of milliseconds. Both early and late responses seem to be related to the mechanisms that control glutamatergic synapses (Jimbo *et al.*, 2000). The average delay of 16 ms also suggests 3 or 4 synapses, or 430 μm/synapse, which is in the 80–585 μm range found in the developing rat neocortex by (Markram *et al.*, 1997). The higher probability of response at short latency can be related to the development of functional connections. Reliable and monosynaptic responses within 30 ms are believed to belong to a period of functional strengthening of network connections (le Feber *et al.*, 2009). We also observed a general decline in spike amplitude with distance from the stimulus. However, chronic stimulation caused spike frequencies and 2nd pulse spike amplitudes to be enhanced relative to 1st pulse spike amplitude, especially at distances corresponding to 4 synapses. If response probability increases with higher amplitude spikes, then this suggests that chronic stimulation causes more efficient network signalling. However, some caveats follow.

Neurons with different identities make plasticity interpretations difficult

Network plasticity without external stimulation occurs spontaneously with development (Van Pelt *et al.*, 2004). (Wagenaar et al., 2005) also demonstrated short-term plastic inhibition of bursts by tetanic stimulation. We hypothesized that chronic stimulation by paired pulses would increase response rates to probe stimulation. Greater network activity was evident from as many as 3 spikes/stimulus and a higher spike rate within bursts for the 3 hr/day condition. We cannot distinguish higher rates of firing in individual neurons from recruitment of activity of multiple neurons at the recording site. In other work in the same arrays, we found that chronic stimulation increased the spontaneous spike frequency within a burst (Brewer *et al.*, 2009b). Spontaneous and evoked responses have been reported to activate different populations of glutamate receptors (Atasoy *et al.*, 2008). (Stegenga et al., 2009) have interpreted spontaneous changes in network instantaneous firing rates as evidence for development of stable firing characteristics amenable to modification through learning.

Our findings regarding the enhancement of spike amplitudes by the 2nd pulse provided evidence of both short-term spike amplitude facilitation and depression. However, within the resolution of our recordings, we cannot prove the direct effects of these phenomena, but

only the indirect effects due to greater network activity. Reasonable explanations for the difference in amplitude arise from overlapping spikes of several different neurons and spillover of frequent bursting activity. Studies in vivo have shown short-term plasticity with a paired-pulse stimulation protocol, wherein the 2nd pulse enhanced spike amplitudes due to a higher density of Na channels in the axon initial segment (Kole *et al.*, 2008). Compound action potentials are more likely generated when the axon initial segments from two neurons are within the range of detection of a single recording electrode. (Soleng et al., 2004) found a hyperpolarization-activated current that prolonged excitability in CA3 of hippocampal slices. The paired pulse stimulation resulted in up to a 30% increase in the compound action potential when the 2nd pulse followed the 1st by 8–128 ms. Finally, feed-forward activation of interneuron release of GABA largely activates hyperpolarizing GABA-A channels, but spike intervals <40 ms predominantly activated depolarizing GABA-B channels (Scanziani, 2000). Thus, the amount of depolarization from GABA spillover increases as spike frequency increases. Furthermore, burst frequency is more dependent on the timing of GABA-A currents than excitatory currents because these GABA-A currents last longer than the excitatory currents to promote bursting (Aradi and Maccaferri, 2004).

Finally, paired-pulse stimulation was more effective when neurons responded to both pulses evidenced by increasing frequency and spike amplitudes. A possible scenario is that the stimulus is propagating through several axonal branches and synapses converging onto the same neuron near the recording electrode. The arrival of several stimuli through different branches would increase neuron excitability, thus enhancing either dendritic spiking or more spikes from the axon initial segment. This kind of excitability is often related to associative learning and memory processes. Intrinsic excitability allows or facilitates modifications of synaptic strength in a defined time window following training (Daoudal and Debanne, 2003), in our case, chronic stimulation for 8 days.

Patterned networks to unravel uncertain paths

As discussed before, neurons with different identities make specific interpretation of results difficult. One solution is to employ spike sorting (Eytan and Marom, 2006; Rolston *et al.*, 2007; Chiappalone *et al.*, 2008). Traditional MEAs provide adequate temporal resolution to investigate the overall network activity, but the low spatial resolution is still a bottleneck to exploit the full electrophysiological capability of such arrays. In part, this problem can be addressed by patterning the growth of the neurons (Corey *et al.*, 1991; Branch *et al.*, 1998; Chang *et al.*, 2003). Patterning provides the opportunity to extract more precise pathway information and also to determine the source of signals. Other developments of potential utility include application of tension forces on the position of neurons, in which single cells were confined by specially constructed cages or wells (Maher *et al.*, 1999; Claverol-Tinturé *et al.*, 2007), microelectronic circuits with microdrop delivery systems (Macis *et al.*, 2007), high density microelectrode arrays (Berdondini *et al.*, 2008), MEAs with PDMS microtunnels connecting different wells (Dworak and Wheeler, 2009), carbon-nanotube MEAs (Greenbaum *et al.*, 2009) and three-dimensional MEAs (Musick *et al.*, 2009).

In conclusion, paired-pulse chronic stimulation of hippocampal networks cultured on MEAs results in an enhancement of the probability of evoked spikes at short distances and a higher spike rate for 3 hr/day chronic stimulation for the entire network. Apparent increases in spike amplitudes could be accounted for by incommensurate neuron identities, overlapping spikes and burst activity. Burst activity is of higher amplitude than isolated spikes and is associated with higher response probabilities to both pulses in the paired stimulus. These data suggest plastic network changes induced by chronic stimulation that enhance the reliability of information transmission and the efficiency of multisynaptic network communication.

Acknowledgments

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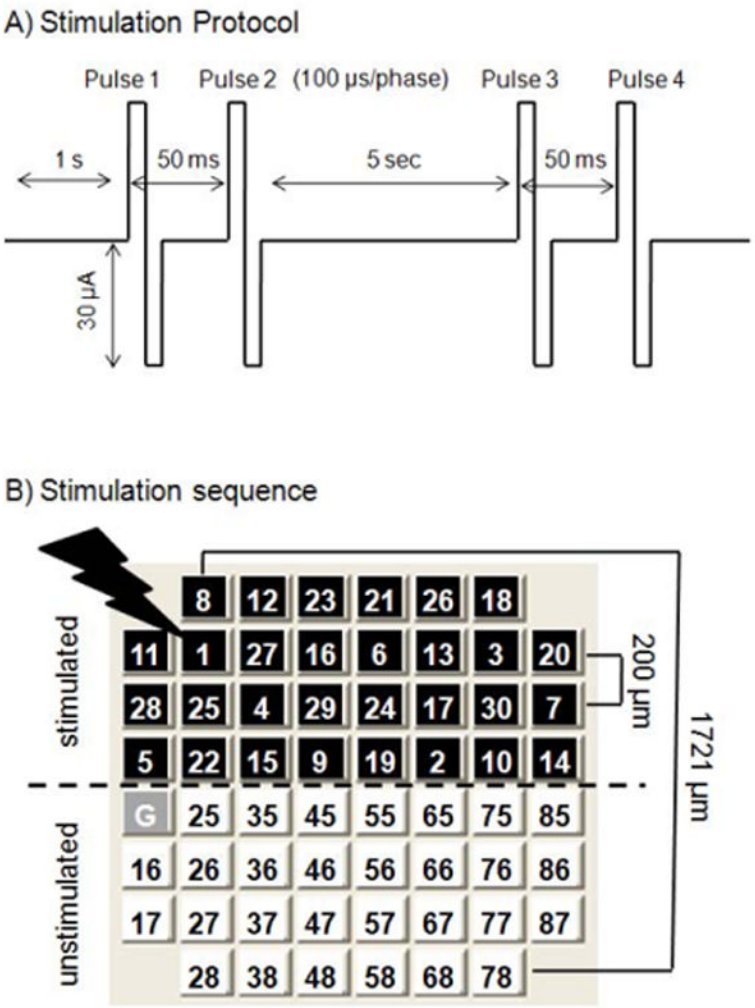

Figure 1. Stimulation and probe protocol

(A) Trains of 2 paired-pulse stimuli, separated by 5 s, 30 µA amplitude (positive first), 50 ms interpulse interval and 1 sec for switch time between electrodes. (B) The bottom half electrodes (unstimulated) are numbered according to column and row, G is the ground. The top half electrodes (stimulated) are numbered according to the fixed but pseudorandom stimulation sequence chosen to avoid stimulation of any two adjacent electrodes consecutively. The shortest distance between two electrodes is 200 µm (vertical or horizontal) and the furthest is 1721 µm.

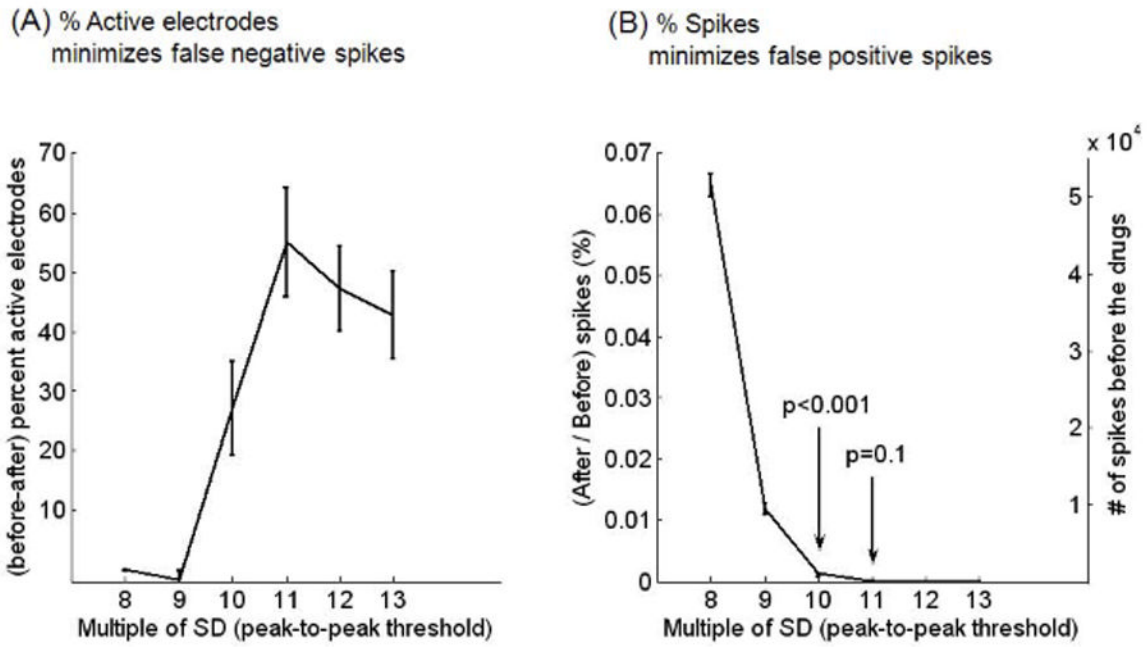

Figure 2. Pharmacological analysis suggests that 11 x S.D. peak to peak is the best threshold for spike detection

Recordings were made before and after the addition of drugs to block synapses and sodium channels (APV, CNQX and TTX). Offline, the threshold for spike detection was varied as a multiple of S.D. of the noise. (A) Difference between active channels before/after drugs found substantial false positive detections when the threshold is 8–9 x S.D., but is reduced greatly at 10 x S.D. and maximally at 11 x S.D. (B) Percent false positive spikes were defined as those still seen after inhibitory drugs divided by those detected before drugs at each multiple of detection sensitivity (S.D.). As multiples increased, the first multiple with spike counts not significantly different from zero was at 11 x S.D., which maximized the number of true positives spikes and minimized false positives spikes. n=3 arrays.

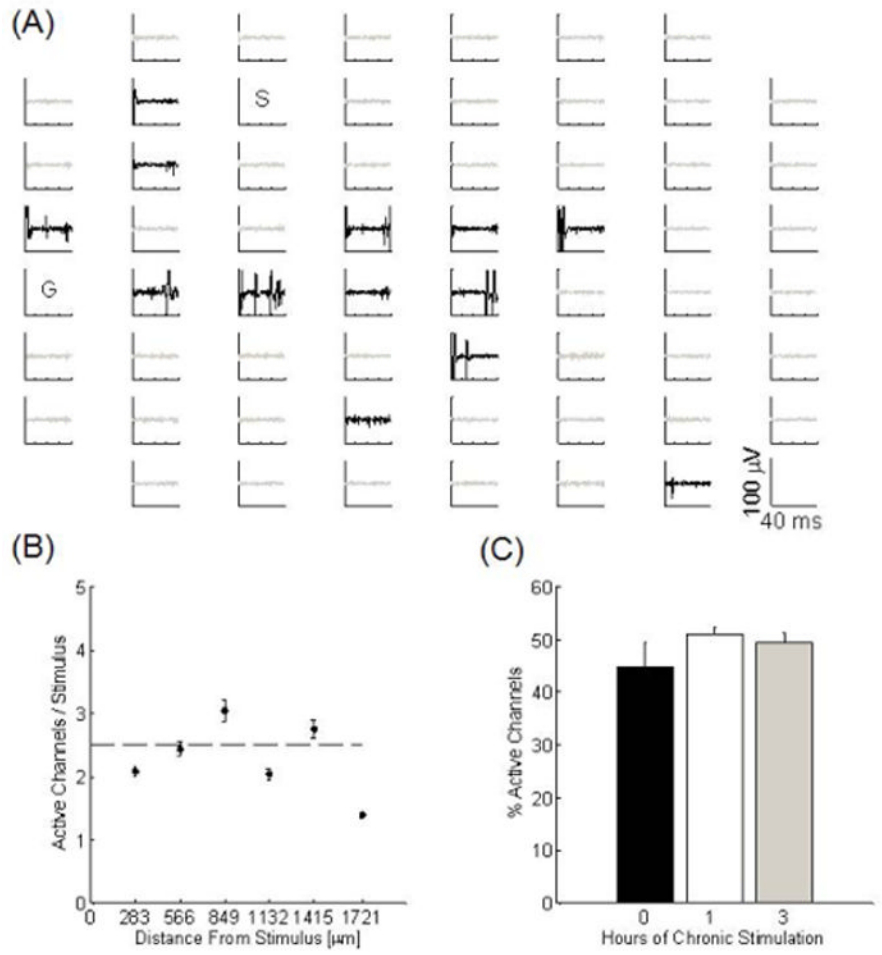

Figure 3. Average number of active channels does not change with either chronic stimulation or distance from the stimulus

(A) Example of recordings from active channels during the probe stimulation; *G* is the grounding electrode; *S* is the current stimulating electrode. Channels in gray are inactive electrodes and channels in black are active (at least 1 evoked spike). (B) An average of 2.7 channels/stimulus (--) are active for each distance (200–1721 µm), independent of chronic stimulation (n=28 arrays). (C) Overall percent active channels for the 600 stimuli per array were 45–50% for 0 hr/day (n=8 arrays), 1 hr/day (n=8 arrays) and 3 hr/day (n=12 arrays).

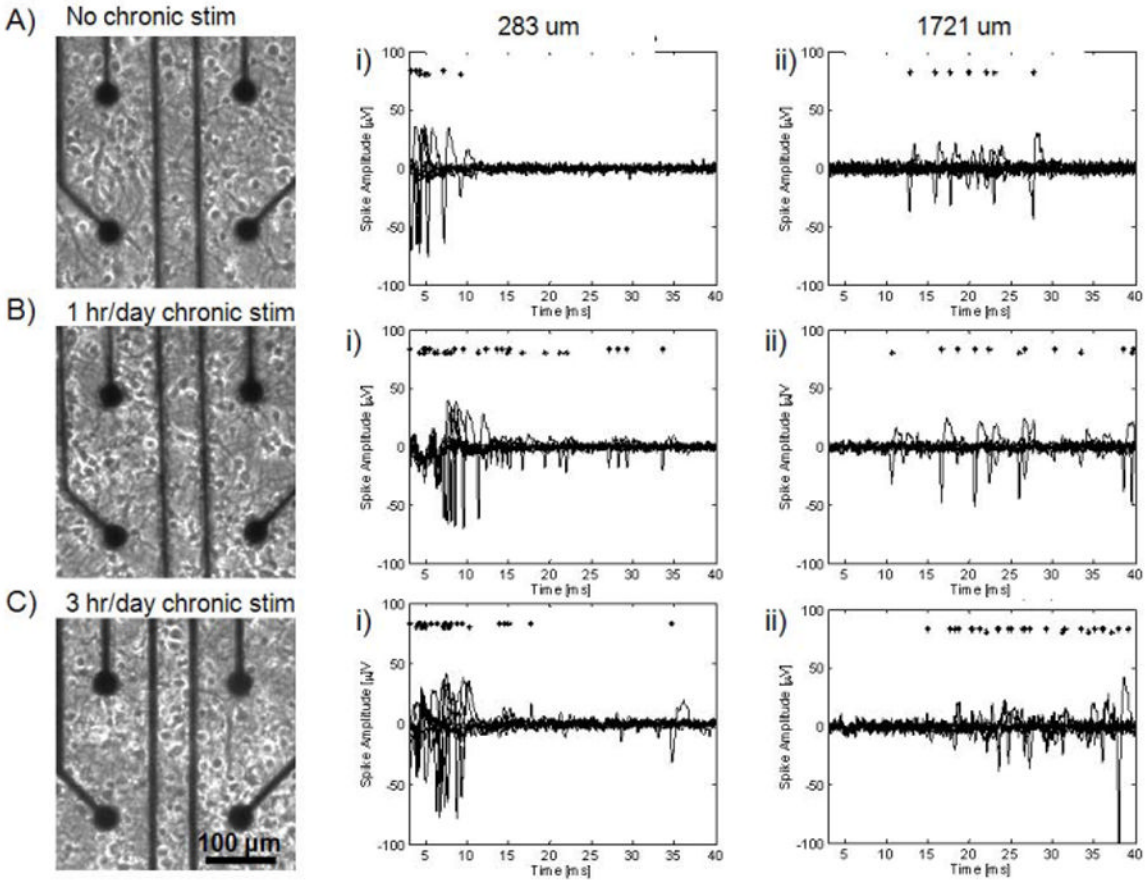

Figure 4. In comparison to the unstimulated condition, chronic stimulation increases the probability of response without changing morphological properties of neurons cultured on MEAs

(A) Neuron distribution on an MEA after 21 days of culture in NbActiv4 for the unstimulated condition; (B) 1 hr/day chronic stimulation; and (C) 3 hr/day chronic stimulation. Corresponding data traces (n=10 overlapping traces each) are shown to the right according to the distance from the stimulation site. At short distance (283 µm), spike amplitudes are higher and latencies are shorter, when compared to responses at long distance (1721 µm). Stars (*) on the top of each trace indicate the spikes detected at the corresponding time.

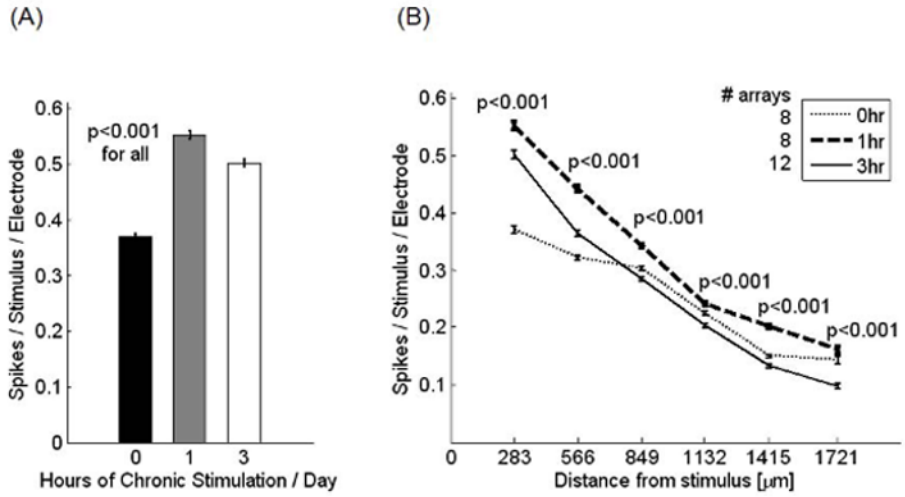

Figure 5. Probability of an evoked response increases with chronic stimulation and decreases with distance from the stimulus

(A) Within 283 μm distance from the stimulus, 1hr/day chronic stimulation elicits 50% more responses than unstimulated condition and 35% for 3hr/day. (B) Probability of evoking a response decreases with distance from the stimulus. n ≥ 10,000 stimuli for each distance.

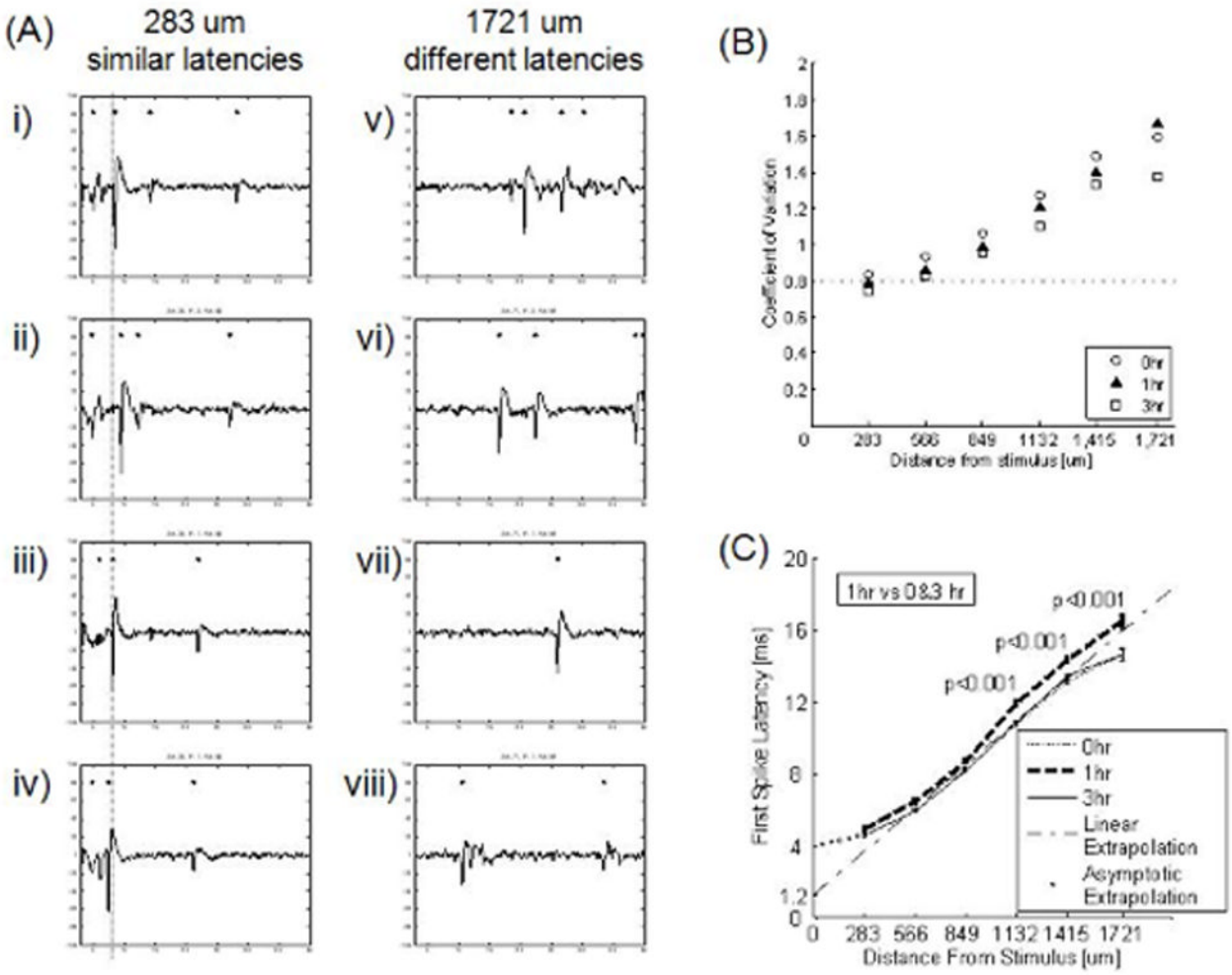

Figure 6. First spike latency increases with distance from the stimulus

(A) Examples of evoked responses at distances of 283 μm (left column) and 1721 μm (right column) from the stimulus. Responses at electrodes near the stimulus have similar latencies (i–iv, respectively for pulses 1–4 in sequence), while the ones localized far away have different latencies (v–viii, in sequence). Stars (*) on the top of each trace indicate the spikes detected at the corresponding time. (B) Coefficient of variation of latency with distance from the stimulus. Independent of chronic stimulation there is 2x more variability at long distances. 14000 > n > 800 for each distance. The n decreases when distance from the stimulus increases. (C) Overall average of first spike latency with distance from the stimulus. An asymptotic extrapolation shows that responses at electrodes within 283 μm of the stimulus first occur on average with a 4 ms delay (activation time for 1 synapse), and 1.2 ms delay for linear extrapolation (spike activation time). Electrodes at 1721 μm responded with a delay of 16 ms, suggesting 4 synapses.

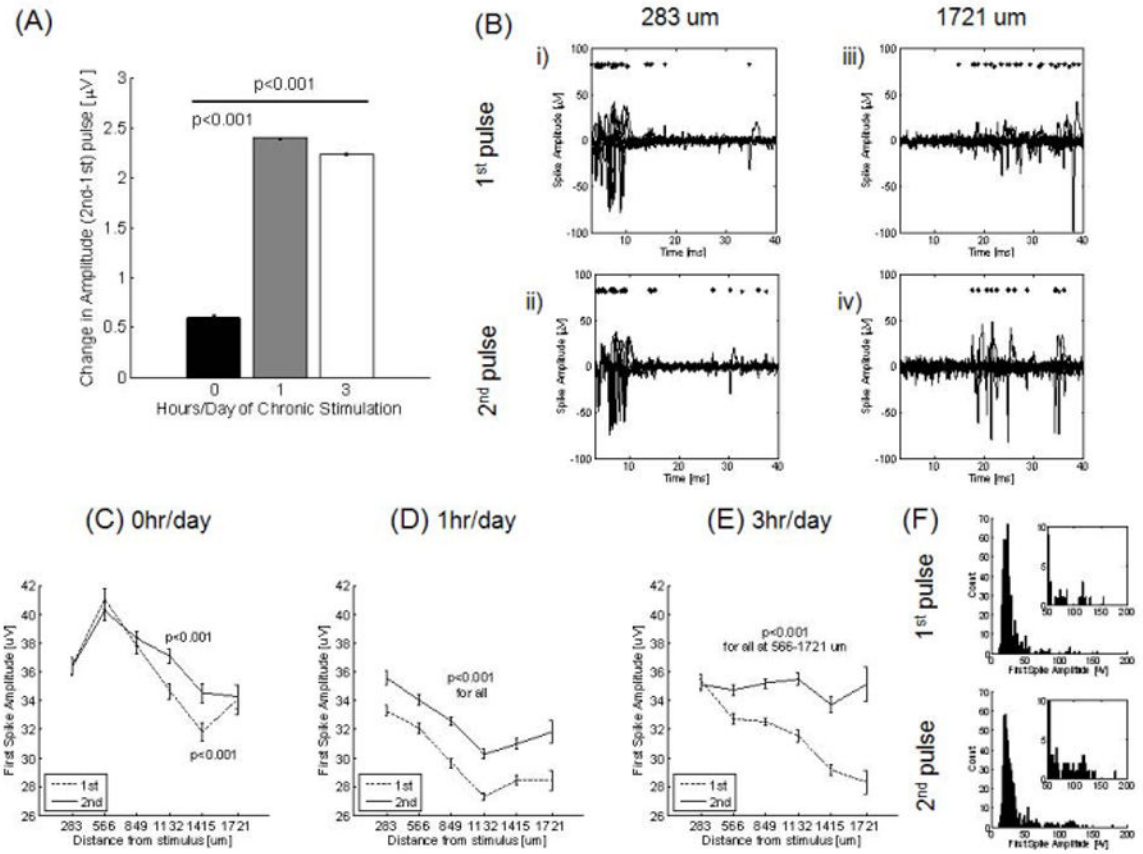

Figure 7. The 2nd pulse increases spike amplitudes during chronic stimulation

(A) The change in the average amplitude of the first spike evoked by the 2nd pulse minus the corresponding average in response to the 1st pulse for 0, 1 and 3 hr/day chronic stimulation. (B) Examples of evoked responses (n=10 traces each) for 3hr/day chronic showing the lower amplitude for the evoked responses to the 1st pulse at 1721 μm distance from stimulus. Stars (*) on the top of each trace indicate the spikes detected at the corresponding time. (C) 0 hr/day: there is no difference between responses to the 1st & 2nd pulse. (D) 1hr/day, spike amplitudes elicited by the 2nd pulse are 10–15% higher than for the 1st pulse. (E) 3hr/day: responses to the 2nd pulse are unchanged across distance, despite a 25% amplitude decline with distance for responses to the 1st pulse. 8000 > n > 300 for each distance. The n decreases with distance from the stimulus. (F) Histogram of spike amplitudes evoked by the 1st (i) and the 2nd (ii) pulse at 1721 μm distance indicates a greater number of larger amplitude spikes. Insets: blow up from 50–200 μV.

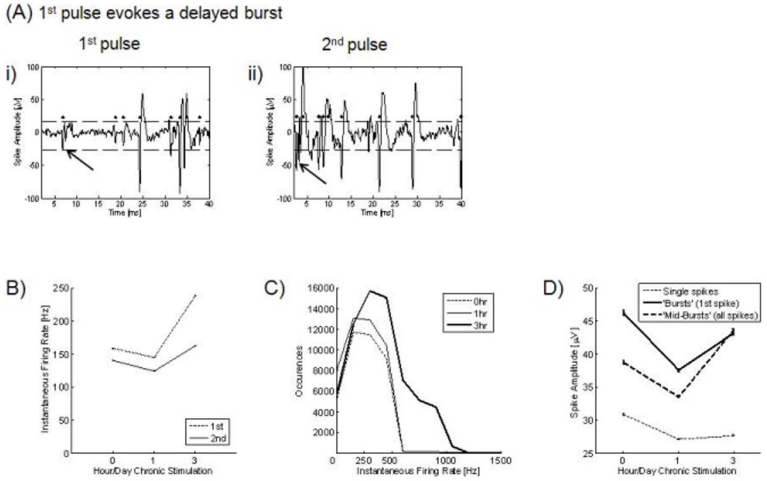

Figure 8. Firing rate and spike amplitudes following the 2nd pulse are higher due to spillover from evoked bursts with higher amplitudes than those of isolated spikes

(A) The 1st pulse evoked a burst that began at 24–31 ms (Ai) and co+ntinues so as to overlap the arrival of the 2nd pulse at 53 ms (3 ms in Aii). Stars (*) indicate the position of the spikes detected in the corresponding traces; arrows (↑) indicate the first evoked spike; and the dashed lines (--) indicate the peak-to-peak amplitude of the first spike evoked by the 1st pulse. (B) Chronic stimulation increases instantaneous firing rate and (C) evokes more spikes at higher frequency (bin size 150 ms). (D) Spike amplitudes are larger in the first spike of ‘bursts’ than in single responses or mid-bursts (all spikes but the first).

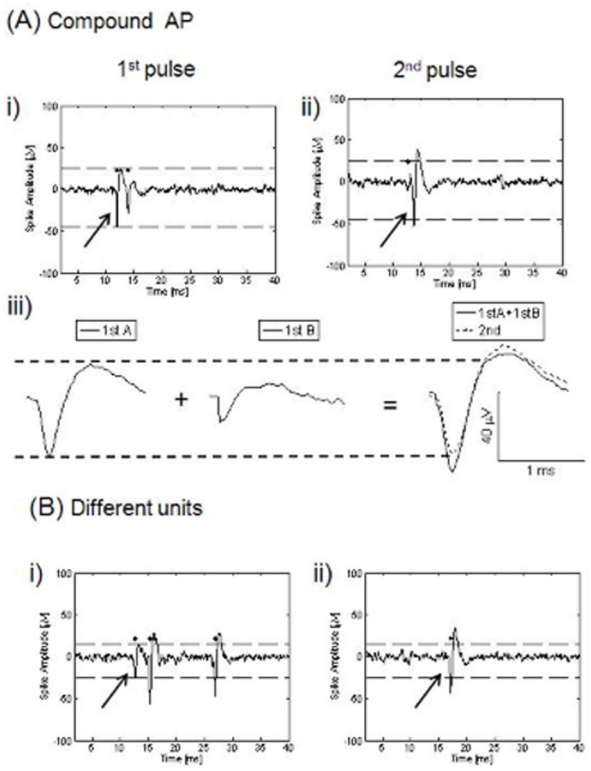

Figure 9. The 2nd pulse evokes responses with larger spike amplitudes due to the recruitment of different units and compound action potentials

(Ai-ii) The 2nd pulse evokes a larger amplitude spike, possibly arising from the sum of the two resolved spikes in Ai. Stars (*) indicate the position of the spikes detected in the corresponding traces; arrows (↑) indicate the first evoked spike; and the dashed lines (--) indicate the peak-to-peak amplitude of the first spike evoked by the 1st pulse. (Aiii) Comparison of the sum of the spike profiles in Ai at optimal time offset to the profile of the spike in Aii. (B) First spikes are recorded from different units, characterized by different latencies and spike shapes that do not sum as well.

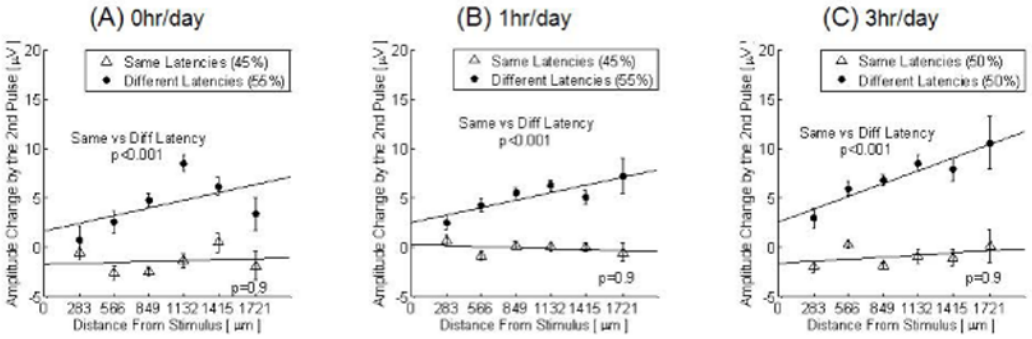

Figure 10. The 2nd pulse does not induce spike amplitude plasticity, independent of the chronic stimulation and distance from the stimulus

Amplitude change when comparing evoked responses by the 1st to the 2nd pulse with distance from the stimulus, for (A) 0hr/day, (B) 1hr/day and (C) 3hr/day chronic stimulation. Evoked responses ($60 < n < 2500$) are separated by type, either same (within 0.5 ms)(△) or different latencies (>0.5 ms)(●). Evoked responses with same latencies did not have a significant change in amplitude, while the ones with different latencies presented an increase in the 2nd spike amplitude response with distance.

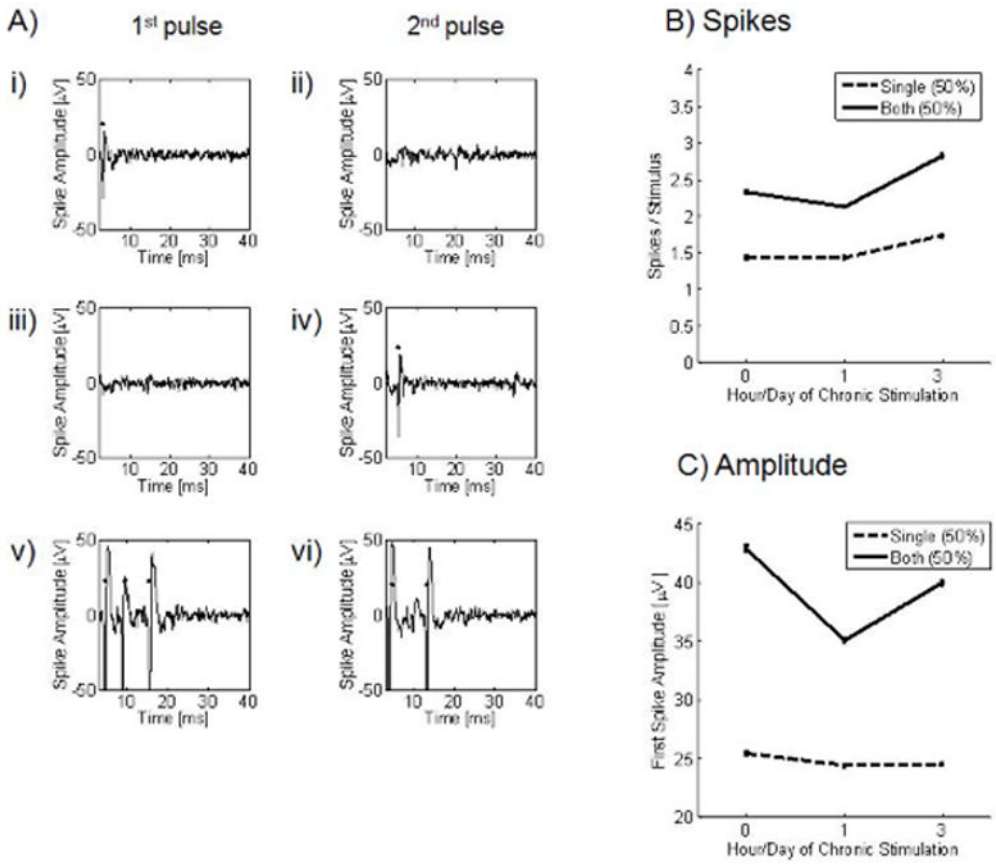

Figure 11. When 1st & 2nd pulses both evoke a response, more spikes with higher amplitudes are seen than when a pulse evokes only one response

(A) Examples of responses evoked by the 1st pulse only (i–ii), by the 2nd pulse (iii–iv) only, and by both pulses (v–vi). Stars (*) indicate the position of the spikes detected in the corresponding traces. (B) The number of evoked spikes (n > 10,000) and (C) the evoked spike amplitudes are higher when there are responses to both 1st and 2nd pulses.