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14. ABSTRACT Murine cortical neurons were cultured via standard methodologies on multi-electrode arrays, which interfaced with MatLab software. Cultures displayed synchronous, spontaneous synaptic signals, indicating that despite the apparent random connectivity inherent in synaptogenesis in dissociated neuronal cultures, neurons retain the ability to transmit information in an organized manner. Thinner cultures displayed rapid, bipolar, high-amplitude individual spontaneous signals ("spikes") with no apparent temporal regularity. By contrast, "thicker" cultures					
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Final Progress Report
W911NF-08-1-0222 "Optimization of Neuronal-Computer Interface"
Thomas B Shea, PhD

Foreward

We undertook in this STIR to optimize our signaling methodology, in order to characterize further how to modulate the relative contribution of inhibitory and excitatory neuronal populations to downstream signal output. We set forth the following Specific Aims:

- Plating and analyses of less dense cultures, and cultures from which glia have been eliminated, which will allow us to achieve more precise signaling among neurons.
- Optimization of the biological signal amplitude, duration and inter-signal interval in order to maximize our ability to stimulate selectively excitatory and inhibitory neurons.
- Characterization the extent of contribution of inhibitory neurons to spontaneous and induced signaling in these long-term cultures
- Characterization of the nature and extent of learning and its reversion

Summary of Findings

• Cultures displayed synchronous, spontaneous synaptic signals, indicating that despite the apparent random connectivity inherent in synaptogenesis in dissociated neuronal cultures, neurons retain the ability to transmit information in an organized manner.

•The pattern of spontaneous signals differed dramatically between thinner” cultures (i.e., cultures plated with a sparser number of cells) and “thicker” cultures. Thinner cultures displayed a large number of bipolar, rapid, high-amplitude individual signals (which we term “spikes) with no apparent temporal regularity. By contrast, “thicker” cultures instead displayed clusters of signals at regular intervals (which we term “bursts”). This differential signaling was observed even within thinner and thicker areas of the same culture.

•Inhibitory neurons (approximately 25% of the total neurons in our cultures) mediated the differential signal patterns observed above, since addition of the inhibitory antagonist bicuculline eliminated the bursts of thick cultures, and induced the irregular spikes characteristic of thinner cultures. These findings indicate that a balance of excitatory and inhibitory signaling is essential for organized transmission of information.

•Cultures were capable of “learning” (synaptic signal strengthening) as demonstrated by increases in duration and amplitude of signals following repetitive stimulation with a localized “biological signal” (i.e., a snippet of a spontaneous synaptic signal from at “thick” culture). Inhibitory neurons were essential for this phenomenon as inclusion of bicultulline during training periods prevented alterations in signaling.

•Glial cells were important for long-term survival of cultures, and modulated the number and pattern of signals. This could in part be surmounted with GCM.

•Due to the robust data received during the above analyses, we have not yet undertaken the goal of optimizing the biological signal. This will be carried out during the course of ongoing work.

BACKGROUND and SIGNIFICANCE

System Characteristics We have refined a system for neuronal culture that allows regional stimulation and recording. Primary murine embryonic cortical neurons harvested at day 17 of gestation from C57B/6 mice were plated and maintained as in our prior studies (e.g., Ho et al., 2002) in a commercial Multi-Electrode Array (MEA) culture chamber (Multichannel Systems; Fig. 1A). Cultures are monitored and stimulated via an amplifier/computer configuration (Fig. 1; Serra et al., 2008a,b) modified from prior studies (Kamioka et al., 1996; Wagenaar et al., 2006). Consistent with these prior studies signals are routinely detected as soon as a network of neurites is visible (≤ 2 weeks; Fig. 1B). Cultures were routinely maintained for 1 month prior to experimentation to allow for stabilization of connections (Kamioka et al., 1996). To confirm synaptic origin of signals, a cocktail of the excitatory synaptic antagonists kynurenic acid (3mM), 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX; 40 μ M), and D-2-Amino-5-phosphonovaleric acid (AP-5; 400 μ M; all obtained from Sigma Aldrich), was added for 2 additional hours prior to recording (Birch et al., 1988; Li et al., 2007; Wagenaar et al.,

2004, 2006). Following data acquisition, all blockers were removed by replacing the medium with fresh medium lacking blockers. A final set of recordings was obtained 2hrs later to confirm the return of activity. The lack of signals in cultures lacking neurons, coupled with reversible cessation of signals following addition of a cocktail of excitatory blockers neuronal origin of signals (Fig. 1C).

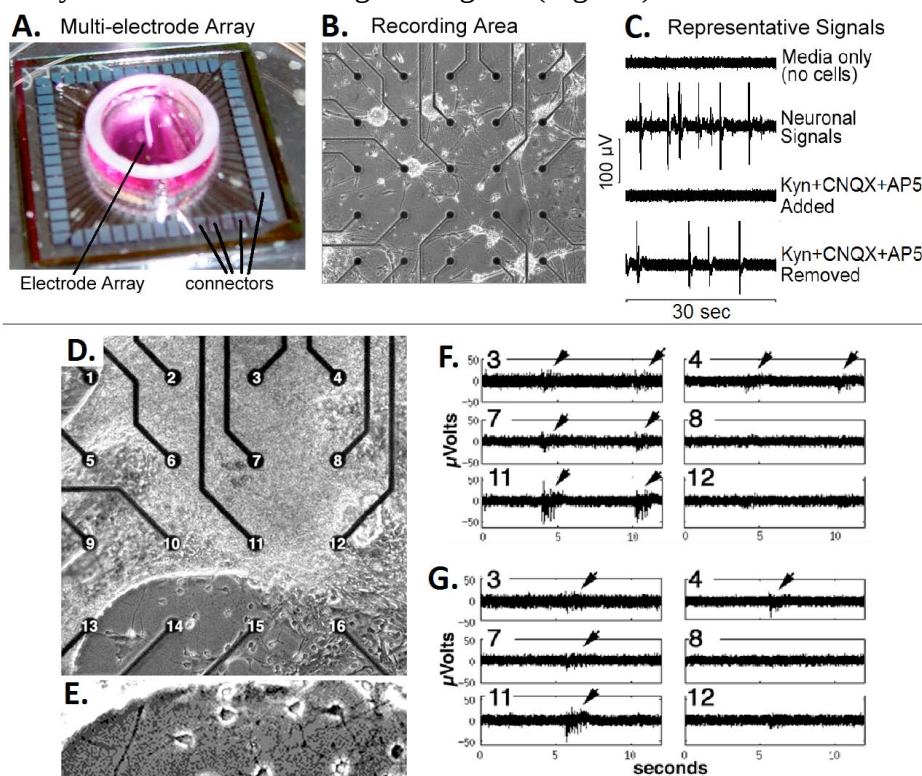


Fig. 1: Characterization of cultures and their spontaneous signaling patterns

Panel A presents a MEA. The chamber is permanently mounted on a glass square the edges of which are lined with a total of 60 “amplifier connection pads.” Sixty circular electrodes within the culture dish are connected to these pads by insulated wires etched onto the glass. The amplifier unit interfaces with each connection pad for recording and stimulation; Panel B depicts embryonic cortical neurons after 14 days in culture; note the elaborate axonal network interconnecting clusters of cells. Electrodes and wires appear as black dots and lines, respectively. Panel C presents representative recordings. Baseline fluctuations are present in the absence of neurons, while signal activity was only observed in the presence of neurons (“neuronal signals”). Note that signaling was reversibly blocked by addition and removal of a cocktail of excitatory synaptic blockers as indicated. Panel D presents a representative 2-month old neuronal-glial co-culture; electrodes have been annotated 1-16 for reference. Glial proliferation typically obscures the neuronal network in large areas of the culture at these later intervals. Panel E presents a higher magnification of the area of the culture encompassed by electrodes 9 to 15; the neuronal network is more clearly revealed in this area due to lack of glial cells. Panels F and G present two sets of spontaneous recordings from the indicated electrodes; recordings in each set were captured simultaneously. Arrows denote the presence of clear spontaneous signals. Note synchronous signaling at multiple electrodes within the culture.

In ongoing studies, we have maintained cultures for several months. In efforts to achieve the most natural and therefore potentially favorable environment for long-term neuronal and maximal signaling, the chamber surface was coated with laminin (Ho et al., 2003), and we made no efforts to eliminate glial cells. As a result, their continued proliferation during our extended culture intervals encompassed neuronal clusters and their interconnecting axons. Thus, direct microscopic observation of neuronal connections is eventually precluded in many areas of these co-cultures. (Fig. 2A; e.g., compare the region of the culture over electrodes 1-12, where glia have proliferated, versus the area encompassed by electrodes 9 to 15, where neurons are clearly visible). Notably, prevention of glial proliferation is readily accomplished by a short treatment with cytosine arabinoside (which selectively eliminates dividing cells yet is benign to post-mitotic neurons) as in our prior studies (e.g., Shea et al., 1984). The impact of glial depletion on neuronal signaling will be discussed further below.

Spontaneous, synchronous signaling in long-term cultures As described in prior studies from other laboratories (Madhavan et al., 2007; Rolston et al., 2007; Wagenaar et al., 2006), long-term cultures developed spontaneous signals that radiate across large areas of the culture, as evidenced by their simultaneous appearance at multiple electrodes (Fig. C, D). Analyses of multiple recordings demonstrated that these spontaneous signals exhibited a regular amplitude ($40.0 \pm 5.0 \mu\text{V}$), duration ($0.8 \pm 0.2 \text{ sec}$, mean $\pm$ standard deviation, $n = 20$) and periodicity (with intervals of $4.2 \pm 0.3 \text{ sec}$ between events ($n = 7$; Table 1). ***These data support the notion that, despite the apparent random connectivity inherent in synaptogenesis in dissociated neuronal cultures, neurons retain the ability to transmit information in an organized manner (Wagenaar et al., 2006).***

Stimulus	Signal Amplitude (μV)	Signal Duration (sec)	Interval between Signals (sec)	Timing post stimulus (sec)
None (spontaneous)	40.0 ± 5.0	0.8 ± 0.2	4.2 ± 0.8	n/a
Single Square	20.5 ± 2.4	0.6 ± 0.3	3.9 ± 0.2	0.43 ± 0.05
Triplet Square	23.7 ± 1.6	0.5 ± 0.2	Not recorded*	0.37 ± 0.4
Biological	26.4 ± 5.5	0.7 ± 0.1	4.2 ± 0.6	0.29 ± 0.13
After Biological	32.0 ± 7.3	0.7 ± 0.2	5.3 ± 1.4	n/a

Table 1: Characterization of spontaneous and invoked signals See text for further description.

Stimulation of signaling We next examined whether or not, or how, our cultures would respond to external stimuli. We first applied a biphasic “square wave” signal ($+350\text{mV}$ for $400\mu\text{sec}$ followed immediately by -350mV for $400\mu\text{sec}$ for a total of $800\mu\text{sec}$), with a grounding electrode located distally from the signal array; this large signal with distal ground was intended to stimulate the entire electrode field simultaneously, which has been the “classical” method of studying responses to stimuli (e.g., Wagenaar et al., 2002, 2004; Fig. 2A). Stimulation with this biphasic signal elicited a 2.7-fold increase in signals vs. those generated spontaneously within approx. 0.4sec after stimulation (Fig. 2B; Table 1). The amplitude of signals elicited in response to stimulation was reduced as compared to spontaneous signals (Table 1), as previously reported (Maeda et al., 1998; Wagenaar et al., 2006).

We considered that more complex signals might evoke more complex or stronger responses. To examine this possibility, we first applied the above square wave 3 times in rapid sequence with 358msec between pulses, with 2.8 sec between triplets (Fig. 2B). Signals elicited by the triplet signal were of the same amplitude and duration as those elicited by a single stimulating signal, and exhibited the same delay post stimulus (Table 1). However, this “triplet” signal induced a 1.5 fold increase in number of signals versus those elicited by a single square wave signal ($p < 0.05$; $n = 5$ independent recordings). The period encompassed by application of the triplet signal unfortunately precluded quantification of the interval between the elicited response and subsequent resumption of spontaneous signals within our 12-sec recording intervals.

We next carried out several modifications of our system with the intent of generating a more physiological situation. We considered that neurons might respond more favorably to an actual neuronal signal rather than a square wave (or a series of 3 square waves) as above. We also considered that localized stimulation of neurons with a less robust signal might provide more information, and ultimately allow more precise monitoring of interaction of particular neurons, than could be obtained by the above large-scale simultaneous stimulation of the entire culture. We therefore selected one of our recorded signals to utilize as a stimulation signal, which we refer to as a “biological signal” (Fig. 2C,D). Finally, since prior studies indicated that improved response was observed by sequential stimulation at 2 adjacent electrodes (Eytan et al., 2003; Sharaf and Marom, 2001), we applied the biological signal at 2 sets of adjacent electrodes (electrodes 15 and 12; and electrodes 12 and 7), and recorded responses from a third electrode situated midway between these pairs (electrode 11). We also utilized an adjacent electrode pads (electrode 10) as a ground electrode (Fig. 2C). This setup allowed analyses regional stimulation. This biological signal had a recorded amplitude of approx. $70\mu\text{V}$. We first applied the biological signal with only slight amplification (to $100\mu\text{V}$); we received no apparent response, and spontaneous signals continued at their regular interval (not shown). We next amplified the biological signal to 1mV , which invoked response signals within the same time interval. Since our biological signal was not a simple biphasic signal, we

also applied the biological signal in an inverted form. Application of the biological signal at electrodes 15 and 12 did not invoke a response, but did evoke a response when applied at electrodes 12-7. By contrast, the inverted biological signal invoked a stimulus when applied at electrodes 15-2 but not at electrodes 12-7 (Fig. 2D). These differential responses from the same electrodes rule out any underlying electrical artifact, since inversion of the signal altered whether or not a response was observed.

In those instances where the biological signal invoked a response, it occurred within a similar interval as was observed with either single or triple square wave pulses, and spontaneous signaling resumed within a similar interval from both square wave pulses and the biological signal (Table 1). However, in those instances where a response was not observed, we noted a significant ($p < 0.03$) delay in resumption of spontaneous signals, which did not return until 8.1 ± 1.2 sec (approx. twice the 4.2 ± 0.6 sec delay observed following all other stimulation conditions; Table 1). This suggests generation of an inhibitory signal under some conditions, and indicates that, depending upon signal orientation and location, the biological signal can invoke inhibitory or excitatory responses that alter downstream neuronal signaling. Moreover, since application of the biological signal at an electrode pair invoked a response at a downstream electrode, while application of the inverted signal inhibited spontaneous signaling at the same downstream electrode, *populations of excitatory and inhibitory neurons co-exist within culture regions* (Fig. 4). These findings collectively demonstrate the physiological relevance of this culture system, suggest that the nature and distribution of spontaneous signaling patterns may derive from a combination of excitatory and inhibitory signals; e.g., some regions display simultaneous bursts while adjacent electrodes remain quiescent (Fig. 3). They further demonstrate that signaling in these cultures can be modulated. The ability to modulate signals is further supported by attenuation of response signal amplitude by all 3 types of stimulating signals (Table 1), as reported previously following square wave pulse stimulation (Wagenaar et al., 2006).

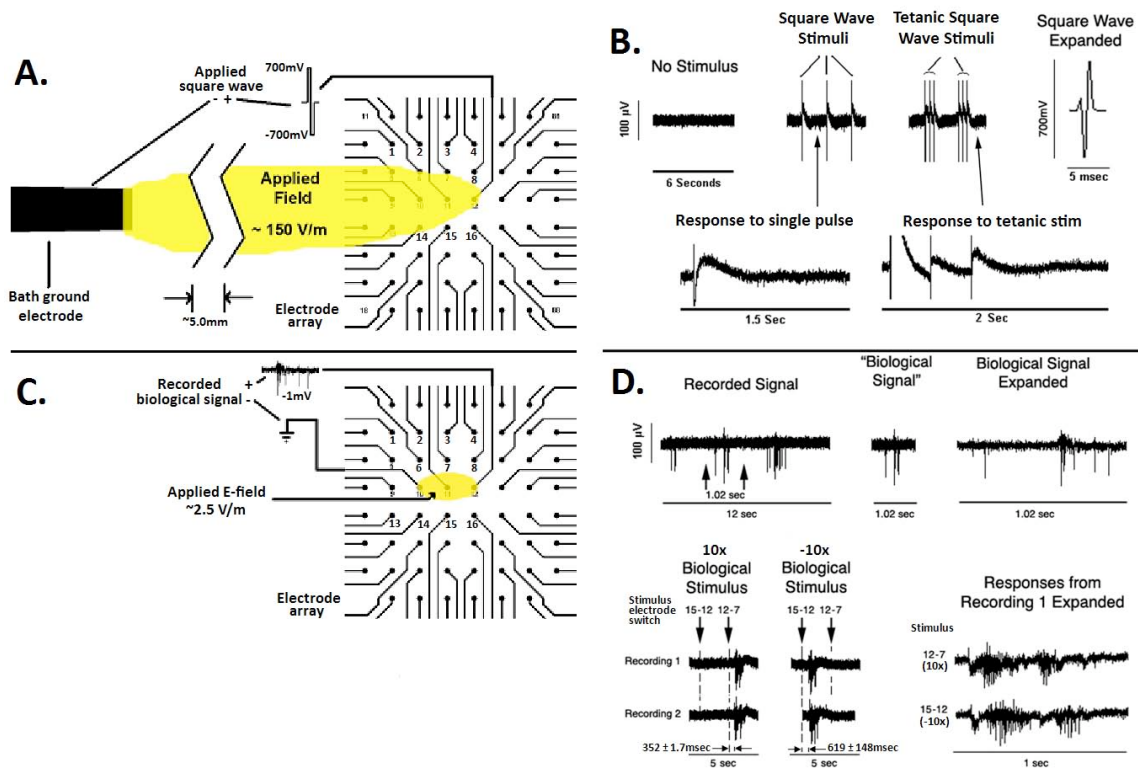


Fig. 2: Different stimulation patterns induce varied responses Panel A presents the approximate electric field distribution (yellow region) using the classical stimulation method with a “full-culture” stimulation. Electrodes numbered 1 through 16 indicate the 16-channel recording area of our MEA system. Panel B presents the response to a single biphasic square ± 700 mV stimulus and a triplet of this stimulus. Panel C presents the approximate electric field (yellow region) utilizing our localized stimulus approach. Arrows point towards response signals; these regions are also presented at higher magnification. Background “noise” (No Stimulation) is presented for reference; see also Fig. 1. Panel D presents the 12 sec recording from which a 1.02 sec portion was selected and defined as the “biological signal;” this signal profile is included in the upper left of panel C. Two representative recordings from electrode 11 are presented. This signal was then applied to electrode pairs. Note that when this signal was applied at electrodes 15-12 it did not invoke a response,

but did do so when applied at electrodes 12-7. By contrast, the inverted biological signal (see text) invoked a stimulus when applied at electrodes 15-2 but not at electrodes 12-7. The biological signal and responses obtained are presented at higher magnification to highlight their complexity.

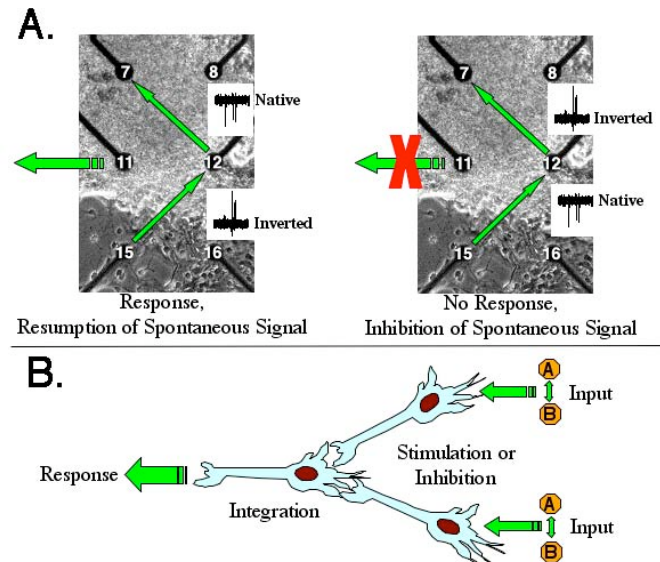


Fig. 3: Diagrammatic representation of signaling following application of biological signal. Panel A presents the signaling patterns observed after application of the biological signal in native or inverted form. Note that the native signal invokes responses at electrode 11 when applied across electrodes 12-7, but does not provoke a response, and inhibits downstream signaling when applied across electrodes 15-12; the inverted biological signal has the opposite effects. Panel B presents a schematic showing the convergence of excitatory and inhibitory neurons, which can be selectively stimulated via orientation of the biological signal, on a downstream population from which signals were recorded.

Cultures contain inhibitory and excitatory neurons Cessation of signaling following addition of the excitatory inhibitor cocktail (Fig. 1) confirmed the presence of excitatory neurons. The above alterations in signaling following regional stimulation suggested the presence of inhibitory neurons. To confirm whether this was or was not the case, we carried out immunofluorescent examination of cultures. To accomplish this, cultures were probed with an antibody directed against nonphosphorylated epitopes of neurofilaments as a general neuronal marker, and with a second antibody directed against the receptor for the inhibitory neurotransmitter gamma-aminobutyric acid (GABA) receptor subunit as a general marker of inhibitory neurons (Beck et al., 1993). These analyses confirmed the presence of GABA-positive neurons (Fig. 4).

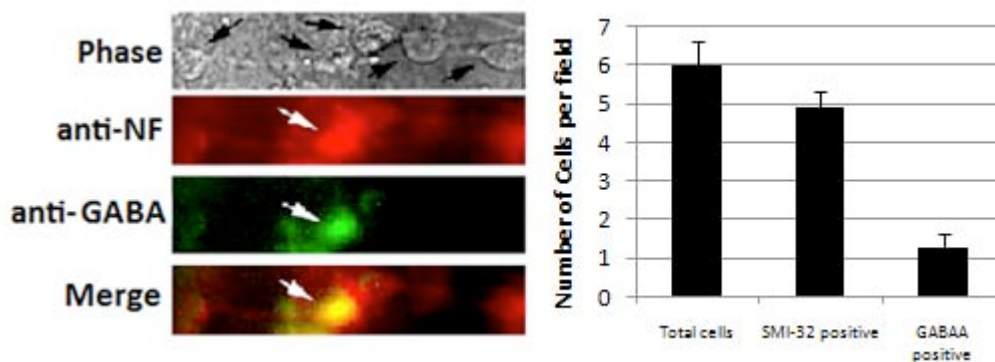


Fig 4: Cultures contain inhibitory neurons. Cultures were subjected to double-immunofluorescent analyses for neurofilaments (anti-NF) using monoclonal antibody SMI-32 and a polyclonal antibody directed against the GABA receptor (anti-GABA), followed by Texas Red- and fluorescein-conjugated secondary antibodies, respectively. All neuronal profiles observed in phase-contrast microscopy (black arrows) displayed neurofilament immunoreactivity, while a subset (one, in this particular image) also displayed GABA immunoreactivity (white arrows in merged image). The accompanying graph depicts quantification of total and GABA-positive neurons in 3 such cultures.

Inhibitory neurons contribute to the signal profile in “thick” cultures

Our prior studies indicated that inhibitory neurons were capable of preventing spontaneous signals (Fig. 2). We therefore considered that counter-intuitive reduction in total signaling and/or signal amplitude observed in

thick versus thin cultures may be due to activity of inhibitory neurons. We therefore examined the impact of bicuculline on overall signaling in thick cultures. Addition of bicuculline fostered an increase in signaling, indicating that inhibitory neurons were responsible for restricting overall spontaneous activity. (Fig. 5).

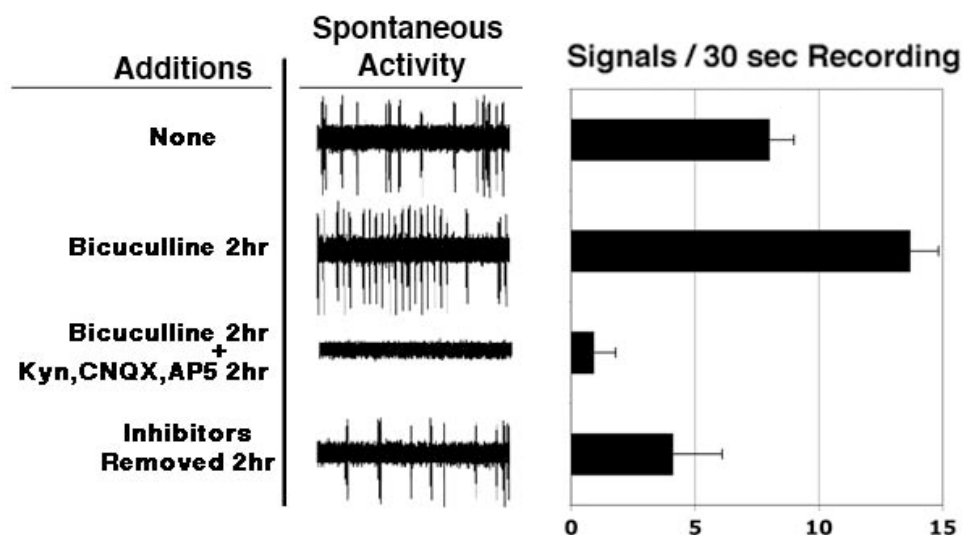


Fig. 5: Inhibitory neurons restrict spontaneous signaling. Recordings (30 sec) of representative spontaneous signals obtained prior to and 2hr following addition of bicuculline, 2hr after the excitatory inhibitor cocktail in the continued presence of bicuculline, and 2hr following removal of both (“blockers removed”; accomplished by replacement of medium with medium lacking inhibitors). Note the relative increase in signaling following addition of bicuculline. Cessation of all signals accompanied addition of excitatory inhibitors. Note further the restoration of signaling following removal of inhibitors. The accompanying graph presents quantification of these events for 5 cultures.

Signaling can be modulated following stimulation: “learning in culture” We examined more closely the nature of responses following stimulation with our biological signal. While the amplitude (i.e., the highest peak) of signals was reduced during stimulation (Table 1), inspection of signals prior to, during, and following stimulation with a biological signal suggested that a marked increase overall spikes were present during stimulation than prior to stimulation, and furthermore that this apparent increase was retained following stimulation (Fig. 6). We therefore quantified the total amount of signaling from 4 different electrodes prior to, during 3 rounds of stimulation 1 sec apart with the biological signal (1mV), and 3.4hr following stimulation. We observed a significant ($p < 0.02$) increase in signaling during stimulation, the majority of which was retained ($p < 0.06$) for at least 3.4hr following stimulation (the latest time tested; Fig. 6). In addition, while synaptic bursts were $14.7 \pm 0.6\%$ longer during stimulation ($p < 0.11$ vs. spontaneous bursts; $n = 6$), they were $37.1 \pm 0.7\%$ longer 3.4hr later ($p < 0.002$ vs. spontaneous bursts; $n = 8$). Moreover, bursts 3.4hr later were significantly longer ($p < 0.03$) than those recorded during stimulation (e.g., Fig. 6). We also observed alterations in signaling amplitude during and following stimulation, and examined these alterations over a total of 6 days, with a sustained change in signaling over this interval. Prior to stimulation, cultures displayed a mixture of low- ($< 50\mu V$) and high amplitude ($> 50\mu V$) signals. Stimulation initially induced an increase in low-amplitude signals, which was followed 3.5 hrs later by a shift to larger amplitude signals reappeared less often than prior to stimulation. When the same cultures were subjected to the same stimulation regimen on day 5, the immediate response (i.e., increase of low-amplitude signals) was less evident than on day 1, while 3.5hr later, signals again shifted to a majority of high-amplitude signals. Spontaneous signals on day 6 more closely resembled those obtained following stimulation on days 1-5, with less of a total change following stimulation than observed on prior days. The demonstrated of a shift towards longer and higher-amplitude signals over time resembles classical learning *in situ*. Paired-pulse stimulation of presynaptic and postsynaptic neurons with intracellular or patch electrodes can induce long-term potentiation and long-term depression in cortex (Bi et al., 1998; Markram et al., 1997). As in the prior studies of Wagenaar et al. (2006), it is impossible in our relative dense cultures (especially following glial proliferation), to determine which neurons were stimulated and/or inhibited. However, our ability to evoke increased, sustained bursting following sequential stimulation of series of electrodes, is consistent with potentiation.

Addition of the inhibitory antagonist bicuculline prevented these alterations; we quantified the percentage of electrodes demonstrating the above alterations in signaling following stimulation: under normal conditions, $28.1 \pm 4\%$ of electrodes in multiple cultures displayed alterations following stimulation. By contrast, when bicuculline was present during stimulation, only $3.1 \pm 1.8\%$ of electrodes displayed alterations ($p < 0.001$), ***These findings indicate that inhibitory activity was essential for retention of information imparted by stimulation.***

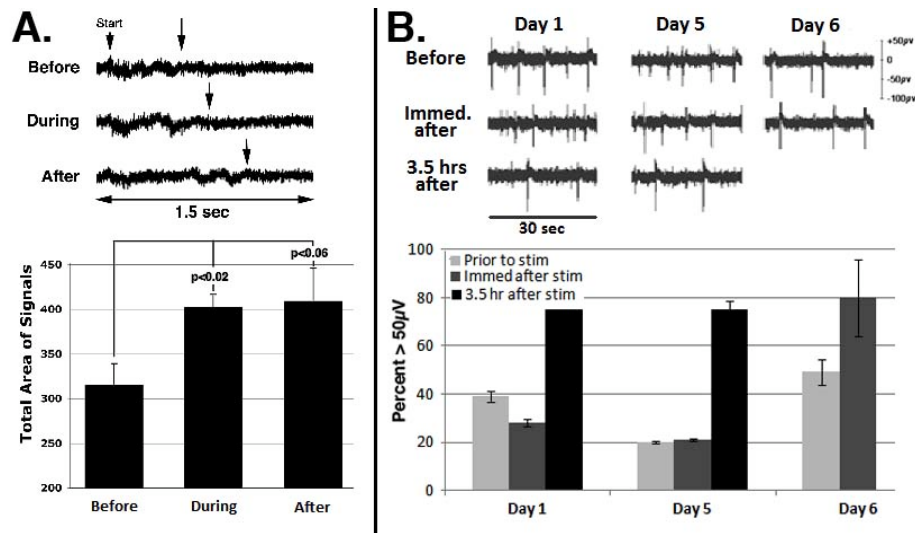


Fig. 6: Signaling undergoes a sustained increase following stimulation. Panels present representative signals before stimulation, during/immediately after stimulation with the biological signal, and 3.5hr after stimulation with the biological signal. Panel A presents quantification of signals generated following stimulation; note that the total time is only 1.5sec, which allows detailed analyses of individual responses. The upper images depict representative signals. The arrowhead labeled “Start” denotes the onset of response in the aligned signals. Arrows denote cessation of variation from baseline. The accompanying graph presents quantification of signal area (mean total pixels $\pm$ SEM deviating from baseline in 12-15 recordings at each time point from 4 electrodes). Note the significant increase in total signal area following stimulation, and retention of this increase 3.4 hr later. Panel B presents the alteration in signaling obtained following repeated stimulation of the same cultures with the biological signal over a total of 6 days. The same stimulus applied after 1, 5 and 6 days resulted in signal organization immediately following stimulation. Prior to stimulation, cultures displayed a mixture of low- ($<50\mu V$) and high amplitude ($>50\mu V$) signals. The number of low-amplitude signals increased immediately after the stimulation regimen. By contrast, 3.5 hrs later, low-amplitude events had declined and larger amplitude signals reappeared less often than prior to stimulation. On day 5, the immediate response (i.e., increase of low- and increase of high-amplitude signals) was less evident than on day 1. Spontaneous signals on day 6 more closely resembled those obtained following stimulation on days 1-5, with less of a total change following stimulation than observed on prior days. The accompanying graph quantifies the percentage of low- and high-amplitude signals generated at each time point.

Culture density modulates spontaneous signaling: The above studies utilized cultures in which a relative large density of cells was plated. We next set out to establish neuronal networks of a minimal density, with the thought in mind that a simple “loop” of individual neurons might provide more regular and malleable signaling patterns. We therefore established less dense cultures (by seeding fewer cells) and monitored spontaneous signaling one month after plating as above. Microscopic examination revealed the formation of a neuronal network within one month, as with “thicker” cultures; these “thinner” cultures were not as quickly overrun by glial proliferation (Fig. 7). Somewhat unexpectedly, however, a significantly great number of spontaneous signals were elicited by thinner cultures (Fig. 7). Moreover, the amplitude of spontaneous signals was significantly greater in thinner cultures; spontaneous signals in thinner cultures displayed a biphasic symmetry; thicker cultures tended to display signals with a more robust negative than positive phase (Fig. 7). These differential signaling patterns observed for thick and thin areas of the same culture (Fig. 8).

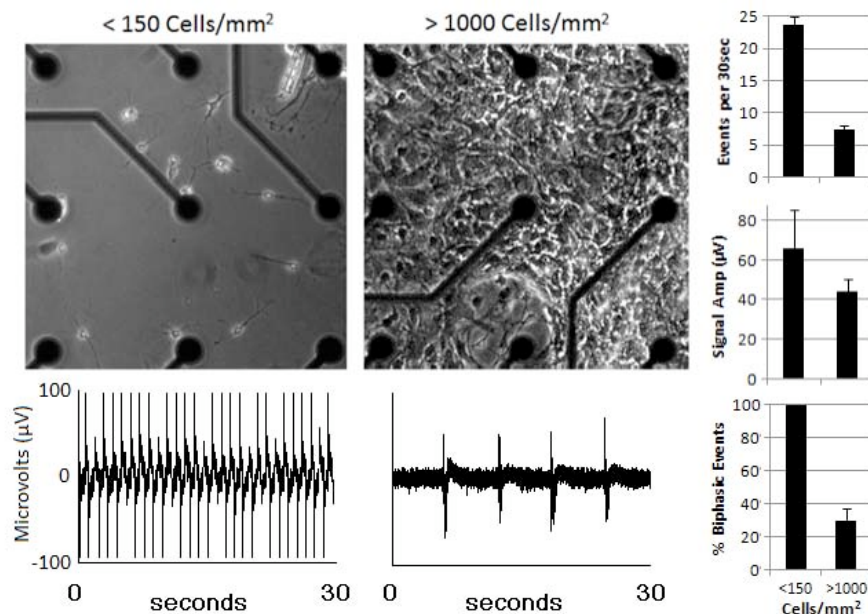


Fig. 7: Culture density modulates signaling patterns. Cultures were arbitrarily designated as “thin” if they displayed an average of <150 cells per mm² as shown, and “thick” if they displayed >1000 cells per mm²; cultures of intermediate density were not quantified. Representative thin and thick images are presented, along with representative recordings of spontaneous signals. The accompanying graphs present quantification of signal number, amplitude and whether or not they were equally biphasic (see text). as indicated. Note the relative increase in total signals of larger amplitude and biphasic symmetry observed in thin versus thick cultures.

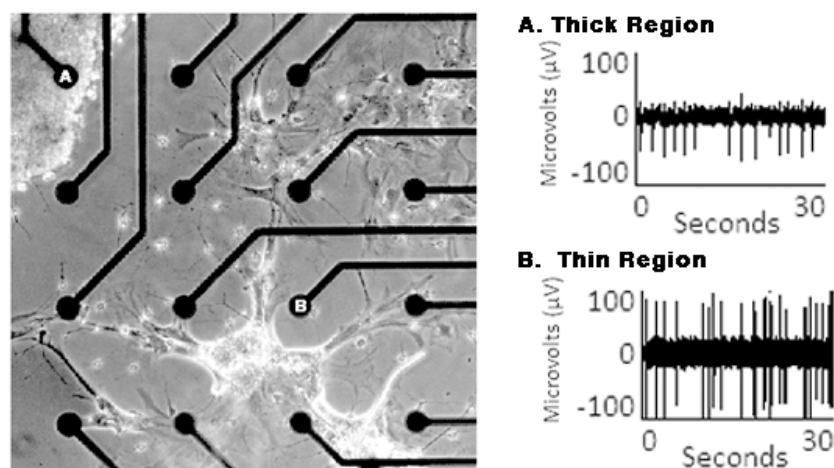


Fig. 8: Different signaling in thin and thick areas of the same culture. Spontaneous signals were recorded from electrode A, which was under a relatively thick region of the culture and electrode B, which was under a relatively thin area. Distinct signaling was observed from these two regions. Note that the pattern from electrode A resembled that recorded within thick cultures, while that at electrode B resembled that recorded from thin cultures (Fig. 6).

Inhibitory neurons modulate signaling patterns Our findings above demonstrated that inhibitory neurons regulated spontaneous and stimulated signals. We therefore examined whether or not they played a role in the differences observed in signaling patterns of thick versus thin cultures. Addition of bicuculline to a thick culture altered the pattern of spontaneous signals to one that resembled the pattern routinely obtained from thin cultures (Fig. 9). Prior examination of thick cultures demonstrated that approximately $24.6 \pm 7\%$ of neurons were inhibitory (Fig. 4). We have not examined whether or not the percentage of GABA-positive neurons in thinner cultures is or is not reduced. However, we consider that the simple likelihood of fewer total synaptic connections per neuron within thinner cultures would be sufficient to reduce functional inhibitory activity below a critical threshold.

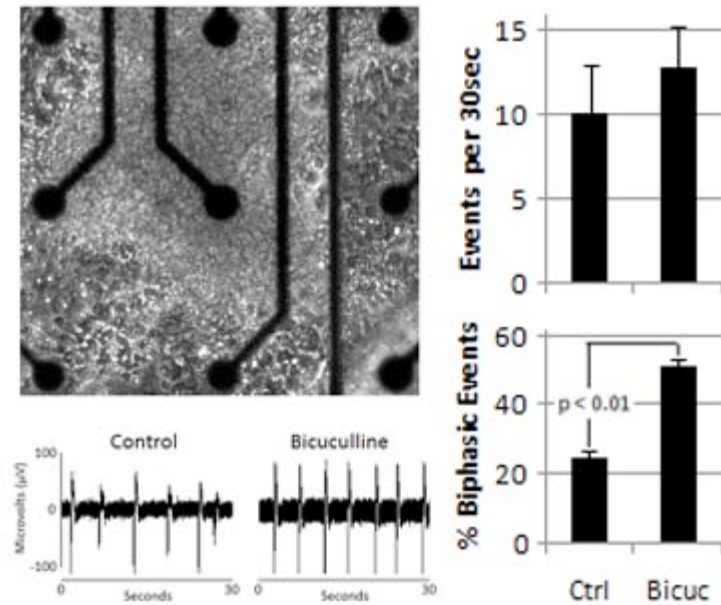


Fig. 9: Bicuculline alters signaling patterns Panels present a representative thick culture and spontaneous signals recorded from this region of the culture prior to (control) and 2hr after addition of bicuculline. The accompanying graphs present quantification of signal numbers and the percentage of signals that displayed biphasic symmetry (see text) from multiple cultures. Note the increase in total and biphasic signals following bicuculline treatment.

Glial cells modulate neuronal survival and signaling We carried our limited analyses of the impact of astroglial cells on neuronal cultures. Glial cells were abundant within our thicker cultures, and, as seen above, generated a monolayer that obscured the view of the neuronal network. We treated cultures with cytosine arabinoside shortly after plating to eliminate dividing cells. Notably, thin cultures did not survive cytosine arabinoside treatment or, if they did survive, elaborate as dense a neuronal network as did thick cultures (not shown). In continued studies, we examined the impact of glial-conditioned medium (GCM) on signaling in those thin cultures that did survive cytosine arabinoside treatment. GCM was obtained by removing medium from confluent neonatal astroglial monolayers (which are largely devoid of neurons) after 3 days, and mixing it 50-50 with fresh medium (Shea et al., 1992). Spontaneous signals were recorded prior to and following a medium change, in which some cultures received GCM or fresh medium mixed 50-50 with 3-day old medium from thick neuronal cultures. GCM stimulated signaling. *The positive impact of GCM suggests that; if necessary to maintain or optimize robust neuronal signaling, glia can be cultured separately and resultant GCM can be added to neuronal cultures as a source of essential glial-derived growth factors.*

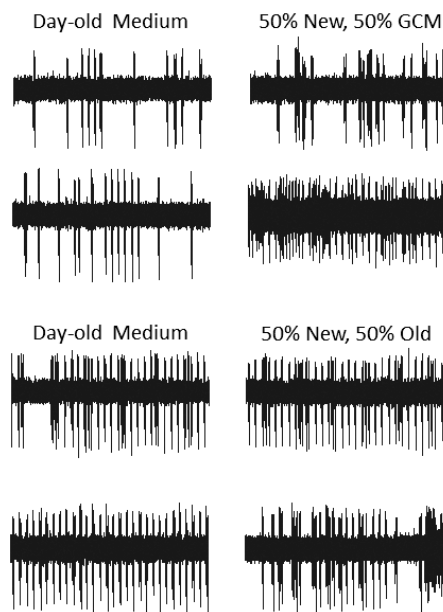


Fig. 10: Glial-conditioned medium stimulates signaling. Spontaneous signaling was recorded one day after receiving fresh medium ("day-old medium") then again one day after receiving either 50% GCM/50% fresh medium, or 50% fresh medium/50% medium that had been in contact with neurons for 3 days. Two examples of each are presented. Note that GCM increased neuronal signaling.

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