Cortical Networks Grown on Microelectrode Arrays As a Biosensor for Botulinum Toxin

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ABSTRACT: Botulinum toxin (BoNT) is a potent neurotoxin produced by toxigenic strains of *Clostridium botulinum*. Botulinum toxin poses a major threat since it could be employed in a deliberate attack on the U.S. food supply. Furthermore, BoNT may be liberated in any insufficiently processed food containing a reduced oxygen atmosphere. Hence, rapid and reliable detection of BoNT in foods is necessary to reduce risks posed through food contamination. We present a BoNT biosensor employing living neural cultures grown *in vitro* on microelectrode arrays (MEAs). An MEA is a culture dish with a grid of electrodes embedded in its surface, enabling extracellular recording of action potentials of neural cultures grown over the array. Pharmaceutical grade BoNT A was applied to the media bath of mature cortical networks cultured on MEAs. Both spontaneous and evoked activities were monitored over 1 wk to quantify changes in the neural population produced by BoNT A. Introduction of BoNT A resulted in an increased duration and number of spikes in spontaneous and evoked bursts relative to control cultures. Increases were significant within 48 h of BoNT A dosage ($P < 0.05$). Application of BoNT A also induced unique oscillatory behavior within each burst that is reminiscent of early developmental activity patterns rather than the mature cultures used here. Three or more activity peaks were observed in 50% of the BoNT dosed cultures. Control cultures exhibited only a single activity peak. Thus activity of these cortical networks measured with MEAs could provide a valuable substrate for BoNT detection.

Keywords: assay, biosensor, botulinum, detection, toxin

Introduction

Efficient detection of foodborne toxins is necessary to secure the food supply from both intentional and unintentional contamination. Botulism toxin, associated with foodborne botulism, is a potent neurotoxin that may be liberated in any unprocessed or insufficiently processed food containing an oxygen deficient atmosphere. Furthermore, BoNTs may be used as a bioweapon in a deliberate attack on the U.S. food supply. Currently, 7 antigenic strains of botulism toxin (A-G) have been identified, which are produced by the anaerobic bacteria *Clostridium botulinum*. Botulism is extremely toxic, with an estimated oral lethal dose of as little as 70 μg of botulism toxin type A (BoNT A) for a 70 kg human (Arnon and others 2001). Containment of a botulism outbreak is dependent upon rapid identification of the toxin in suspect foods. While there are many assays available for detecting specific toxins, toxin fragments, and DNA of causative organisms, the FDA continues to primarily rely on the "mouse bioassay" for the detection of botulinum toxicity in foods (Solomon and Lilly 2001). The mouse bioassay detects the presence of toxins by observing death rates of animals injected with suspect food samples. Identification of toxin serotypes, determined through neutralization assays using equine antisera, requires additional time and animal use. The mouse bioassay is extremely sensitive, detecting as little as few picograms of toxin, and capable of identifying the presence of virtually any type of biologically active toxin (Barr and others 2005). It is also time consuming, prohibitively expensive due to the large number of animals required, and available at only a limited number of facilities nationwide. Alternative assays tend to focus on specific toxins and may provide false positive indications when fragments of inactive toxin proteins or DNA are present (Scarlatos and others 2005).

Cell-based assays are a promising alternative to the mouse bioassay. Agents in suspect samples can be identified through changes in cellular physiology. One potential cell-based assay employs a neural culture grown microelectrode array (MEA). An MEA is a tissue culture dish with a grid of 60 independent extracellular electrodes embedded under the surface. Each electrode measures the electrophysiological activity (that is, action potentials) of the neurons (Figure 1) for extended periods of hours to weeks (Potter and DeMarse 2001). Microelectrode arrays have been employed to measure the activity from a variety of electrically active cell lines such as cortical, hippocampal, and spinal neurons (Marom and Shahaf 2002; Segev and others 2002; Eytan and others 2003; Selinger and others 2004; Wagenaar and others 2006a, 2006b), cardiac myocytes (Reppel and others 2007), and retinal ganglion cells (Meister and others 1994, 1995).

The use of cortical cultures grown on MEAs for detection of environment threats is an application currently in development at a number of labs (Gross and others 1997; Gholmieh and others 2001; Keefer and others 2001; Stenger and others 2001; Gholmieh and others 2003; Pancrazio and others 2003; Gramowski and others 2004; Selinger and others 2004). MEAs have thus far been used to detect a variety of environmental threats, including mercuric chloride, sodium arsenate, phosdrin, chlordimeform, strychnine, and brevetoxin (O'Shaughnessy and others 2004; Kulagina and others 2006; Xiang and others 2007). These threats come from a broad assortment of contaminants, including heavy metals, organophosphates, insecticides, and marine neurotoxins. In addition, MEAs have been used with a variety of pharmacological agents, including bicuculline, valproate, atropine, and methanandamide (Morefeild and others 2000; Cadotte 2004; Gramowski and others 2004; Cadotte and DeMarse 2005).

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These biosensors typically use changes in the spatial and temporal patterns of activity across a network of neurons as an indicator of toxicity. One of the most prominent patterns of activity in these cultures, known as population bursting or bursts, consists of network wide synchronous volleys of action potentials (that is, spikes) observed on nearly all electrodes that last anywhere from 100 ms to several seconds. Both spiking and bursting activity are highly sensitive to environmental changes, including the introduction of chemical agents to the culture. Moreover, measuring network-wide activity enables the detection of subtle changes in neuronal dynamics that may be missed by other single neuron based assays. Hence, monitoring changes in the dynamics of these neural cultures following application of test agents could provide a valuable diagnostic tool and practical biosensor for a variety of neurologically active agents.

In this article, we assess the potential of cortical networks cultured on MEAs as a biosensor for BoNT A by bath application of the toxin. We then measure the activity of these cortical networks over the course of 1 wk to characterize changes in the spatiotemporal patterns of activity relative to untreated controls indicative of BoNT A.

Botulism toxin (BoNT A)

Botulism toxins inhibit presynaptic release of neurotransmitters through cleavage of SNARE proteins essential for vesicle fusion docking. Specifically, BoNT A, C, and E cleave SNAP-25, BoNT G, D, E, and F cleave synaptobrevin (VAMP), and BoNT C1 cleaves syntaxin (Arnon and others 2001).

Flaccid paralysis resulting from botulism intoxication is attributed to blockage of acetylcholine release at the neuromuscular junction. While the neuromuscular junction is the primary target of BoNTs *in vivo*, BoNTs have also been shown to act on the central nervous system *in vitro*, including spinal cord cultures, hippocampal slice cultures, cerebellar tissue, and cortical neurons (Verdio and others 1999, 2006; Foran and others 2003; Luvisetto and others 2003; Keller and others 2004; Sutton and others 2004; Costanin and others 2005). In addition, it has been shown that BoNTs block release of a variety of neurotransmitters, including acetylcholine, glutamate, glycine, noradrenaline, dopamine, serotonin, and neuropeptides (Verdio and others 1999, 2006; Keller and others 2004).

Materials and Methods

Dissociated neural cultures

Detailed methods used for neuronal culture have been previously described (Potter and DeMarse 2001). Briefly, cortical hemispheres from E17–E18 Wistar rat embryos (Brain Bits, Springfield, Ill., U.S.A.) were dissociated using papain and mechanical trituration. After dissociation, approximately 50000 cells (neurons and glia) were placed in the center of an MEA. Surface treatment with polyethyleneimine (PEI) (Sigma-Aldrich, St. Louis, Mo., U.S.A.) was used to make the surface preferable for cell adhesion and laminin (Sigma-Aldrich) was used to promote neurite outgrowth. The neurons were cultured in medium consisting of 90% Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlesbad, Calif., U.S.A.) and 10% equine serum (Hyclone, Logan, Utah, U.S.A.), which was equilibrated to 5% carbon dioxide and 35.5 °C. The medium for each culture was exchanged twice per week and the cultures were incubated continuously in 5% CO_2 at 35.5 °C.

Neurons began producing spontaneous action potentials within the 1st few days after plating within the MEA. Cultures were considered mature at 4 wk *in vitro*, the point at which the neurons express the full complement of receptors (Habets and others 1987; Muramoto and others 1993; Marom and Shahaf 2002). All cultures used were older than 30 d *in vitro* to allow the cultures to be considered fully developed. Recordings were conducted within an environmental chamber which was maintained at 35.5 °C and 5% CO_2 to duplicate the environment within the incubator in which the cultures were maintained.

Microelectrode arrays (MEA)

The MEAs (Figure 1) used in this experiment were obtained from Multichannel Systems GmBH (Reutlingen, Germany). Electrodes were arranged in an 8 × 8 grid to measure neural activity over a 1.6 mm^2 area. Electrode traces were insulated using silicon nitride (SiN). Titanium nitride (TiN) was used at the electrode sites to form a low impedance interface between the recording amplifiers and culture media. Each electrode records changes in ionic voltage within an approximate radius of 60 μm. Activity was sampled and digitized at 25 kHz using Multichannel Systems MEA1060 amplifier and A/D hardware.

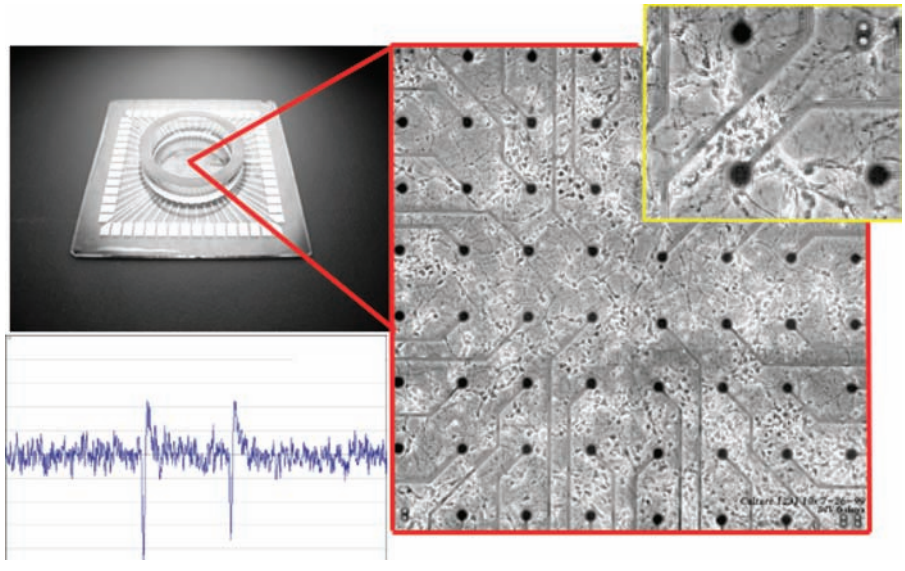

Figure 1 — Upper left: a microelectrode array (MEA) from multichannel systems. An 8 × 8 grid of electrodes 200 μM apart (right panel) enables the recording of changes in extracellular voltages produced by activity (action potentials, lower left) from cortical networks grown *in vitro*.

Procedure

Drug application. Lyophilized BoNT A complex (BOTOX®, Allergen Pharmaceuticals) was reconstituted in 0.9% sterile saline at a concentration of 200 U/mL (approximately 10 ng/mL). Each vial of BOTOX contains 100 U of botulinum toxin type A, 0.5 mg of human albumin, and 0.9 mg sodium chloride (Allergen Pharmaceutical Ireland 2004). Twenty microliters of toxin solution (4 U of toxin) were applied directly to the 1 mL culture bath. For safety reasons, the concentration chosen was well below human lethality limits. In addition, it was sufficiently low to demonstrate sensitivity of the networks for BoNT A. This is particularly important since dilution of crude samples will likely be necessary prior to dosage of networks in future studies. Toxin carrier solution, consisting of albumin diluted in 0.9% sterile saline to a concentration of 1 mg/mL, was applied to control cultures.

Experimental protocol. Ten MEAs were randomly assigned to either a BoNT A group ($N = 5$) which received the toxin or a control group ($N = 5$) without toxin. Twenty-minute spontaneous neural activity, recorded prior to treatment with either BoNT or no BoNT, was used as a baseline activity measurement. Activity was then recorded immediately following application of BoNT for the BoNT A group and during a similar period for the control group. This was followed by 20-min spontaneous activity recordings at 12, 24, 36, 48, and 72 h and 1 wk after treatment.

Following each recording, 5 of the 60 channels were stimulated in a randomized order to measure any changes in the amount of evoked activity between the BoNT and control group. Each stimulation consisted of a single 600 mV, 200 μS, biphasic pulse that would typically evoke a short burst of activity for 100 ms or more (Jimbo and others 1999). The 5 stimulation sites were selected prior to the 1st recording with the criterion that a single stimulation pulse would produce activity on at least 50% of the channels. Each site was stimulated with 5 pulses at 0.10 Hz. The stimulation order was randomized during each stimulation session.

At the end of the week of recordings, MEAs in the control group that had not been dosed with BoNT A were then dosed with BoNT and recorded for a 1-wk period similar to the BoNT group. One of the control cultures during this reversal was removed from the experiment due to infection. The results from the BoNT-dosed control dishes were used to confirm trends observed during the initial experiment.

Analysis and statistics. Action potentials (that is, spikes) produced by neurons grown on the surface of the array were detected as voltage deviations greater than 5 standard deviations above or below the mean noise level for each electrode. Activity was also stimulated through the application of 600 mv, 200 μS, biphasic voltage pulses at select electrode sites (Wagenaar and others 2004). Spike events were extracted and stored online for further offline processing to identify burst events. Burst events were detected by smoothing the number of spike times from all channels into 0.5 ms bins. Consecutive bins that exceeded a threshold number of spikes based upon the mean bin size were then identified as bursts. The end of each burst was detected as a 10- to 20-ms period in which no activity was detected. Data are presented as a mean percentage of the baseline activity ± S.E.M. The separate variance t-test was used to establish significance between control and BoNT-dosed groups (cultures) with $P < 0.05$ considered as significant. The paired Student's t-test was used to compare posttreatment activity to baseline (within groups). The paired Student's t-test was used as well as to compare BoNT-dosed control dishes from the reversal to control activity obtained the week prior. In this case, results were confounded with blocks (weeks 1 and 2).

Results

Spontaneous activity

Application of the toxin resulted in significant changes in the spontaneous activity before against after treatment in the BoNT group and relative to the control group. In 89% of BoNT treated cultures, application of BoNT resulted in an increase in duration of spontaneous bursts and the number of spikes within those bursts with respect to activity recorded during baseline (before the toxin had been delivered). Figure 2 shows the mean duration of each burst (panel A) and average number of spikes per burst (panel B) in the BoNT and control groups from 12 h to 1 wk following BoNT application. Dishes dosed during the control reversal also had a significant increase in burst duration and spikes per burst (data not shown). Increases were observed within 12 to 24 h after BoNT dosing and continued to increase until 72 h. Increases were significant 48 h after dosage with BoNT A ($P < 0.05$). There was, however, no significant difference between spike rates during each burst or interburst interval immediately following the application of the toxin (data not shown in the figure, $P > 0.05$). Finally, there was no significant difference between the BoNT and control group prior to the application of the toxin for spike rate, burst rate, interburst interval, and burst duration ($P > 0.05$).

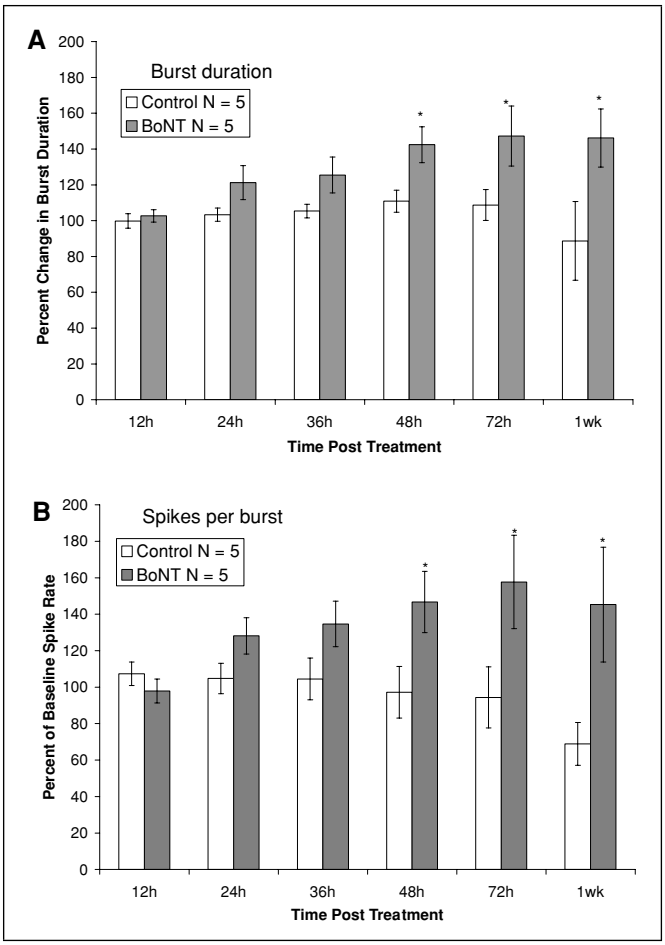

Figure 2—(A) Average burst duration as a percentage of baseline activity. (B) Average spikes per burst as a percentage of baseline activity. *Indicates a significant difference between control and BoNT A dosed tissue cultures (Student's *t*-test, $P < 0.05$), control $N = 5$, BoNT $N = 5$.

Intraburst neural dynamics

Application of the toxin not only changed the number of spikes in a burst and the burst duration but also affected the distribution of spikes within each burst event. To illustrate this change, return plots were generated in which the activity during a burst was plotted as a series of points comparing the rate of activity at time *t* to the activity observed 50 ms in the past. In a normal culture, a typical burst would consist of tonic spiking observed across the majority of the array in which a rapid increase in activity peaks within 50 ms followed by a slowly decay appearing in a return plot as a loop beginning at the origin (Kamioka and others 1996; Jimbo and others 2000; Segev and others 2001; Tabak and Latham 2003; van Pelt and others 2004, 2005; Wagenaar and others 2006b).

Figure 3 shows return plots for the control group (left column) and BoNT group (right column) for 3 subjects during baseline, 24, 48, and 72 h. The normal burst patterns are illustrated in the left column of the figure for 2 subjects in the control group. A similar pattern can also be seen for the baseline activity in the BoNT group before the application of the toxin (right column, blue line). In contrast, the shape of the trajectories during each burst was altered after delivery of the toxin in the BoNT group (right column). After 24 h, slight oscillations in the rate of activity during the burst begin to appear that became more pronounced over time. The oscillatory behavior represents the appearance of multiple peaks of activity (3 or more) within each burst. This was true particularly after 48 and 72 h and was apparent in 50% BoNT cultures, but never observed in controls.

Note that the scale of the left upper panel of Figure 3, labeled as control 7097, is nearly an order of magnitude smaller than the other panels. This is because the network covered a much smaller portion of the array, approximately 50%. However, despite the much smaller magnitude observed in the burst trajectory, the shape of the burst trajectory remained stable over the 72 h shown.

Evoked response following the toxin

The effect of the toxin on spontaneous activity was also compared with activity evoked through a series of 5 brief biphasic pulses after each recording session. Application of the toxin resulted in significant changes to the overall rate of spiking during the burst and the pattern of spiking relative to the control group. Figure 4 shows an example of the action potentials (plotted as points) produced for each channel following a single stimulation pulse (panels A, B) and the average activity across channels (panels C, D). There was no significant difference in the number of evoked spikes between the BoNT and control group during baseline or 12 h after toxin delivery. However, there was an increase in the number of spikes evoked in the BoNT against control group 24, 48, and 72 h following the toxin, which persisted up to 1 wk. The increase in spike activity was most apparent in the late phase of the evoked burst (100 to 300 ms

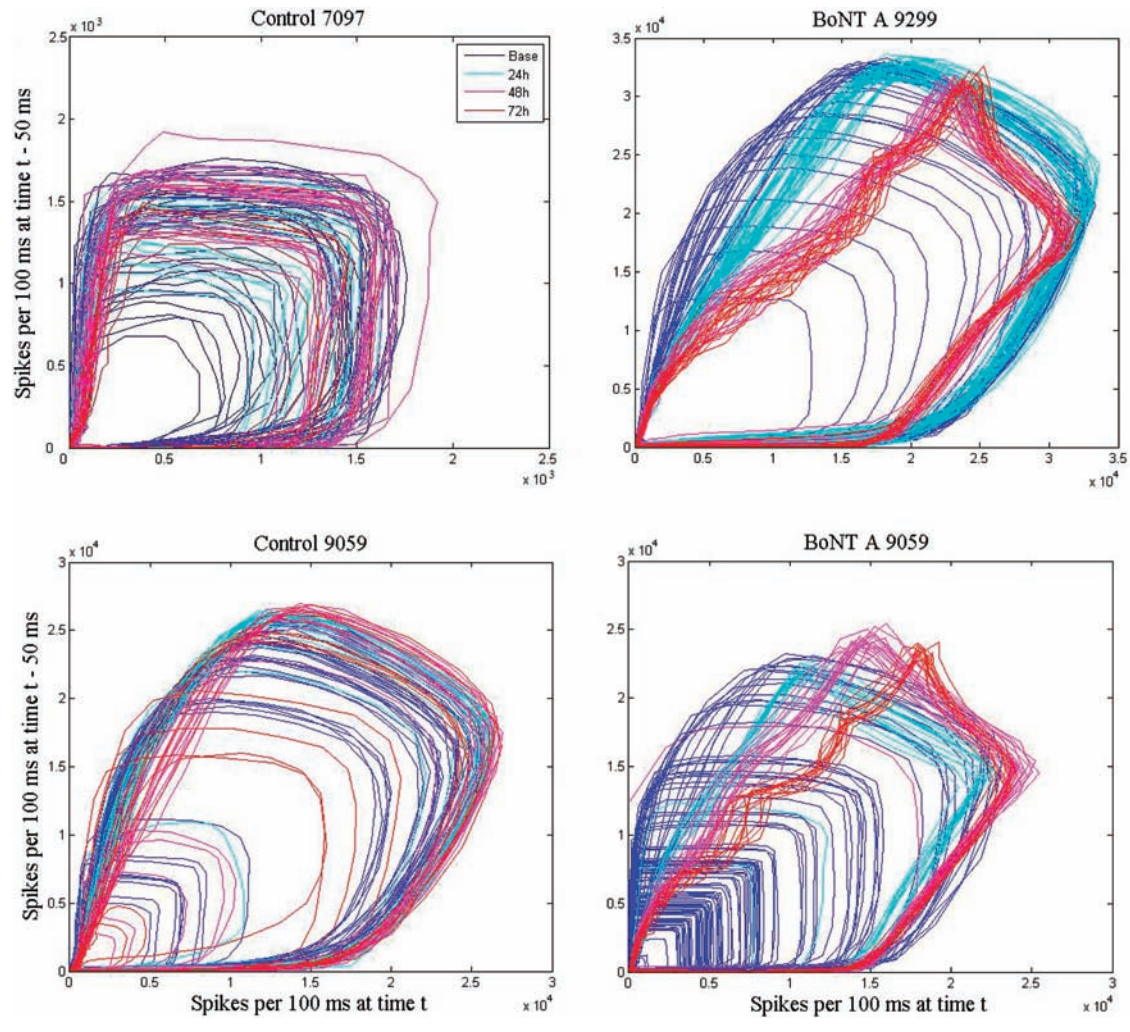

Figure 3 — Return plots generated by sorting spikes into 100 ms bins and plotting bins at time *t* against bins at time *t* – 50 ms. Responses are from baseline recordings (dark blue), as well as 24 h (light blue), 48 h (pink), and 72 h (red) in 2 carrier dosed cultures (left column) and 2 BoNT dosed cultures (right column). The bottom row is the same tissue culture with and without BoNT treatment.

poststimulation). Figure 5 shows the average number of late phase spikes evoked through stimulation before and 12, 24, 48, 72 h, and 1 wk after toxin delivery in the BoNT and control groups. There was no significant difference between the number of stimulations that resulted in a burst (control 20.0 ± 0.91, BoNT 21.0 ± 0.92).

A comparison between the pattern of evoked activity 300 ms following stimulation in the control group (top panel) against the BoNT group (bottom panel) is shown in Figure 6. There was no significant change in the activity observed in BoNT A dosed cultures during baseline or 12 h following administration of the toxin. However, beginning at 24 h, 8 of the 9 BoNT-dosed cultures displayed elevated activity during the later part of the elicited burst (> 100 ms poststimulation), which continued up to 1 wk following toxin delivery. Interestingly, after 36 h, multiple peaks in the rate of activity were observed in 5 of the 9 BoNT-dosed cultures and 8 of the 9 cultures at 72 h recording. The initial peak rate of activity observed in both the experimental and control groups was followed by a series of smaller peaks in the BoNT group but not the control group. Each peak in the rate of activity in the BoNT group was slightly smaller and slightly longer in duration than the previous peak, lasting 50 to 100 ms and spaced 50 to 100 ms apart. This is similar to the effect observed during spontaneous recordings. Moreover, this oscillatory effect was still apparent even after 1 wk following the toxin in the BoNT group.

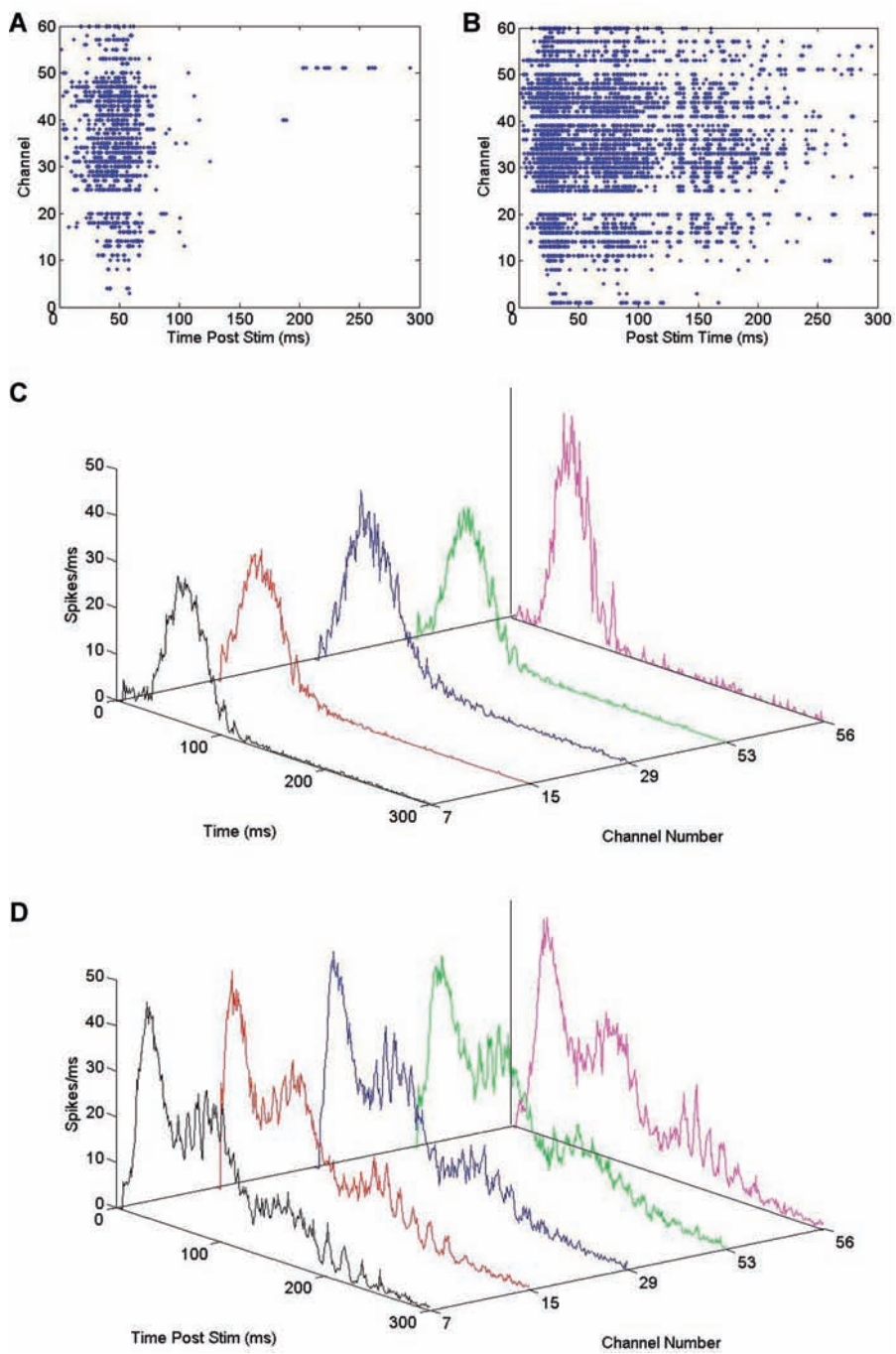

Figure 4—Stimulation response for cortical culture grown on MEA 9053 during the baseline recording (A, C) and 72 h after treatment with 200 pg BoNT A (B, D). Raster plot of activity in cortical culture after a single stimulation (top panels), and average stimulation response for each stimulation channel (bottom panels).

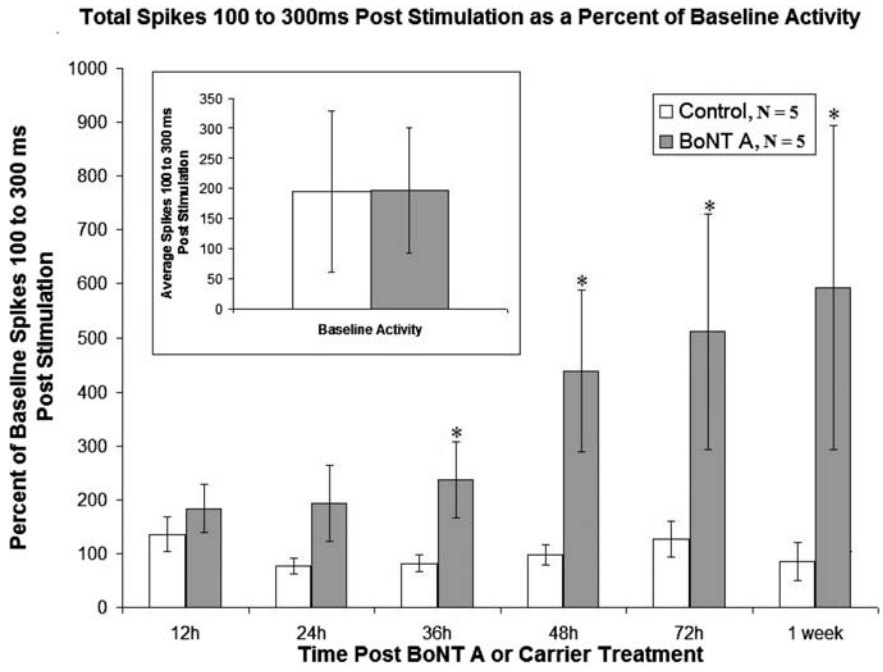

Figure 5—Mean change in the number of evoked spikes 100 to 300 ms poststimulation in the BoNT (closed bar, $N = 5$) and control cultures (open bar, $N = 5$). Due to culture-to-culture variability the data were normalized as a percent of baseline activity, where 100% indicates no change in activity. The baseline average of spikes 100 to 300 ms poststimulation for control and BoNT groups is shown in the panel inset. There was no significant difference between baseline activity of BoNT A dosed and control cultures. *Indicates a significant increase in late phase response (< 100 ms) with respect to control cultures using the Student's *t*-test ($P < 0.05$).

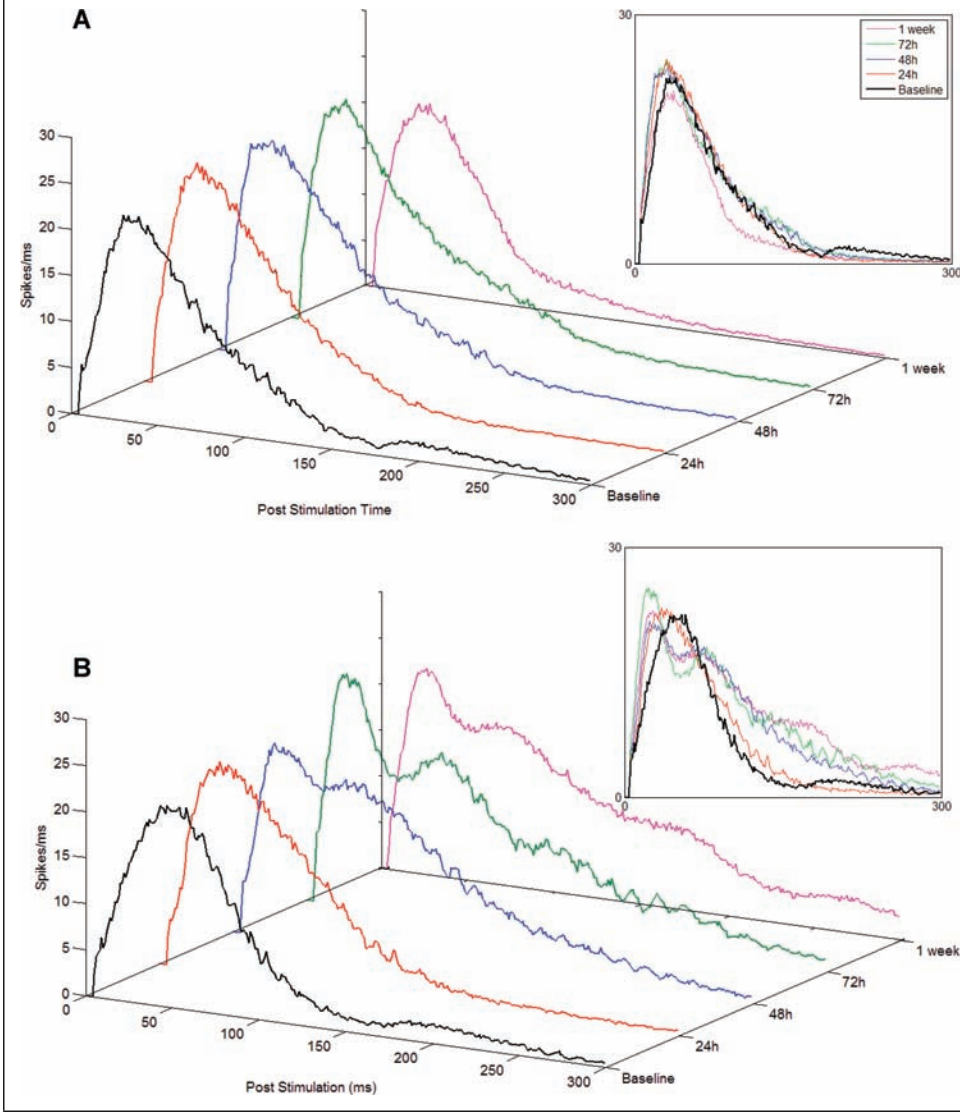

Figure 6—Average stimulation response for control (panel A, $N = 5$) and BoNT dosed (panel B, $N = 5$) tissue cultures. Note the increase in spike rate during the latter portion of the stimulation response (>100 ms) as well as the emergence of oscillatory behavior most prominent at 72 h.

Discussion

Use of cultured neural networks as a biosensor depends upon identification of distinctive modifications to native activity, which can serve as a fingerprint for biological agents of interest. A variety of inherent network properties have been used to measure the changes in activity. Some of the more common methods used include changes in burst rate, spike rate, burst duration, mean spikes per burst, interspike interval, burst amplitude, and spike amplitude (Keefer and others 2001; Pancrazio and others 2003; Gramowski and others 2004). We have examined the effects on neural activity of an important foodborne toxin, BoNT A. Application of BoNT A to the culture media resulted in an increase in the number of spikes per burst within 24 h after BoNT dosage. Furthermore, an oscillatory change in bursting activity was observed, which to our knowledge has not been previously reported with other biological agents. These changes are present not only in spontaneous bursts but also in bursts elicited through stimulation.

As shown in Figure 3, the size and shape of spontaneous burst activity produced in a single recording session can be highly variable (Wagenaar and others 2006b). Thus a long recording period is necessary to obtain a representative sample of activity. However, elicited bursts tend to be less variable in size and shape and are produced reliably over time. Thus the amount of recording time necessary to obtain a representative sample of activity is much shorter during stimulation experiments than spontaneous recordings. Therefore, it is anticipated that monitoring stimulated burst activity will greatly aid in the detection of BoNT A by reducing variability and time. Additionally, return plots of spike activity are a simple method to rapidly visualize changes in burst activity. These plots can easily be obtained during online data processing since they do not require prior knowledge of burst activity. Thus, they may be a valuable tool for visually identifying changes in activity associated with drug application.

Why this oscillatory behavior appeared following the toxin during both spontaneous and evoked bursts is not clear. Previous studies have demonstrated that the primary target of BoNTs in the hippocampal and cortical tissue are upon excitatory synapses, while GABAergic synapses remain much less affected (Verdio and others 2006). For example, BoNT has been shown to block presynaptic release of not only acetylcholine, but glutamate, glycine, noradrenaline, dopamine, serotonin, and neuropeptides also (Verdio and others 1999, 2006; Keller and others 2004). Factors such as structural connectivity, synaptic strength, and the balance between excititory and inhibitory contributions within these cultures have been shown to produce a profound effect on the pattern of activity observed, especially during development (Nakanishi and Kukita 1998; Streit and others 2005; Wagenaar and others 2006b). Thus, it is not surprising that this toxin would alter the pattern of activity within each burst.

In this experiment, at least 24 h after toxin exposure was required before a significant change in activity could be observed. This is similar to the mouse bioassay, which requires from 12 to 24 h for BoNT detection, perhaps reflecting the slow toxin uptake (Scarlatos and others 2005). In addition, ongoing changes in activity observed after the 24-h recording may be a result of further SNAP-25 cleavage after BoNT uptake with the maximum level of SNAP cleavage not occurring until 48 to 72 h posttreatment. Reducing the time required for BoNT detection by increasing the rate of BoNT uptake would increase the efficiency of this technique. Recent studies have indicated that botulism toxin uptake is coupled to vesicle recycling (Dong and others 2006; Verdio and others 2006). Therefore it may be possible to elevate the rate of vesicle recycling, increasing uptake and hence decreasing the detection time for this toxin. For example, Keller and others (2004) used elevated potassium media for studies of BoNT uptake in spinal cord cultures. In this case, optimal BoNT A uptake was achieved after 4 min of toxin exposure in elevated potassium media with a significant level of SNAP-25 cleavage demonstrated 2.5 h after toxin exposure. While less than 20% of SNAP-25 was cleaved in media containing 3 mM KCl, more than 50% was cleaved in media containing 50 to 80 mM KCl. However, concentrations of BoNT A used in the current study were 2 to 4 orders of magnitude smaller than concentrations used by Keller and others (2004). By increasing potassium in the media during toxin dosing and subsequently increasing native activity in our cultures, the time for toxin detection may be drastically reduced.

Alternatively, repeated stimulation may also increase the rate of vesicle recycling. Not only would this decrease the latency to detect the toxin, the repeated measures provided by more stimulations would provide a more stable and higher resolution measure than that used in this study. It is also possible that by further refining the fingerprint for BoNT toxin with a more detailed analysis of, for example, functional connectivity within the network, a more reliable and faster detection time can be achieved.

This assay has been tested against a simplified food matrix comprising only the toxin carrier solution. However, it is likely that changes in observed activity will be detectable in the presence of food matrices. Phosphate buffered saline will likely be used to extract toxin from food samples, and thus its effect on network activity would need to be characterized. Furthermore, dilutions will likely be necessary to minimize alterations of activity such as was demonstrated with seawater (Kulagina and others 2006). Although dilutions might limit the sensitivity of this assay, it will likely remain applicable at concentrations relevant to human safety.

Conclusions

This study demonstrated that cortical tissue grown on MEAs provides a suitable substrate for BoNT A detection. Use of living neuronal tissue enables only toxin, which is capable of internalizing and cleaving SNARE proteins, to be detected. Thus, this assay may be suitable for the evaluation of the biological activity of the toxin as well as toxin detection similar to the mouse bioassay. While this study is currently limited to exploring the effect of BoNT A on network activity, this technique could also be useful for detecting and perhaps even differentiating the effects of other BoNT strains (types B to G). Finally, the mouse bioassay requires multiple animals for a single assay. With this technique, a single animal could provide tissue for multiple neural network-based assays on MEAs. Furthermore, development of MEAs with multiple chambers would enable simultaneous recordings of test samples along with controls, further reducing time, cost, and animal use. While a great deal of work remains before this assay will be suitable for evaluation of suspect food samples, it is conceivable that an assay based on this technique could provide a powerful method for detecting a wide variety of foodborne agents, including both known and perhaps even unknown threats.

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