Neuroscience 171 (2010) 50–61

MINIMUM NEURON DENSITY FOR SYNCHRONIZED BURSTS IN A RAT CORTICAL CULTURE ON MULTI-ELECTRODE ARRAYS

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Abstract—To investigate the minimum neuron and neurite densities required for synchronized bursts, we cultured rat cortical neurons on planar multi-electrode arrays (MEAs) at five plating densities (2500, 1000, 500, 250, and 100 cells/mm^2) using two culture media: Neuron Culture Medium and Dulbecco's Modified Eagle Medium supplemented with serum (DMEM/serum). Long-term recording of spontaneous electrical activity clarified that the cultures exhibiting synchronized bursts required an initial plating density of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum. Immediately after electrical recording, immunocytochemistry of microtubule-associated protein 2 (MAP2) and Neurofilament 200 kD (NF200) was performed directly on MEAs to investigate the actual densities of neurons and neurites forming the networks. Immunofluorescence observation revealed that the construction of complicated neuronal networks required the same initial plating density as for synchronized bursts, and that overly sparse cultures showed significant decreases of neurons and neurites. We also found that the final densities of surviving neurons at 1 month decreased greatly compared with the initial plating densities and became saturated in denser cultures. In addition, the area of neurites and the number of nuclei were saturated in denser cultures. By comparing both the results of electrophysiological recording and immunocytochemical observation, we revealed that there is a minimum threshold of neuron densities that must be met for the exhibition of synchronized bursts. Interestingly, these minimum densities of MAP2-positive final neurons did not differ between the two culture media; the density was approximately 50 neurons/mm^2. This value was obtained in the cultures with the initial plating densities of 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum.

Key words: dissociated culture, neuronal network, immunocytochemistry, fluorescence microscopy, neuronal survival.

Dissociated neurons cultured *in vitro* autonomously form complicated networks that spontaneously show synchronized bursting (Kamioka et al., 1996; Opitz et al., 2002). Studies using multi-electrode arrays (MEAs) have demonstrated that synchronized bursts are highly variable in terms of their spatio–temporal firing patterns but highly correlated among neurons (van Pelt et al., 2004; Chiappalone et al., 2006; Wagenaar et al., 2006). Furthermore, recent studies of synchronous activity have revealed that cortical cultures *in vitro* showed scale-free topology of connectivity (Eytan and Marom, 2006), precisely timed activity (Rolston et al., 2007), and neuronal avalanches (Pasquale et al., 2008). Bursting activity has also been observed during mammalian development *in vivo* and is thought to be involved in the formation of neuronal circuits (Ben-Ari, 2001; Zhang and Poo, 2001; Khazipov et al., 2004). Therefore, neuronal cultures *in vitro* using MEAs are a useful tool for modeling the generation and control of bursts (Maeda et al., 1995, 1998; Wagenaar et al., 2005; Ham et al., 2008).

Spontaneous synchrony is a remarkable phenomenon of multi-neuronal activity (Mao et al., 2001; Cossart et al., 2003). Although numerous studies have been performed using dense cultures on MEAs to record the electrical activity of neuronal ensembles (Jimbo et al., 1999; Marom and Shahaf, 2002), there have been few studies on sparse cultures that do not show synchronized activities. Wagenaar et al. (2006) performed rat cortical cultures of various densities and showed that ultra-sparse cultures exhibited tiny or no bursts. However, it has not been clarified whether or not there is a minimum boundary density for synchronized bursts.

The cerebral cortex contains not only neurons, but also other cells such as glial cells and fibroblasts. Immunocytochemical methods have been widely used to identify neurons and network distributions both *in vivo* and *in vitro* (Storm-Mathisen et al., 1983; Ren et al., 1992; Voigt et al., 1997, 2001). Thus, direct immunocytochemical analysis of neurons on MEAs is thought to be effective for quantifying the actual neuron number and evaluating the contribution of spatial distribution to electrical activity. These observations of network morphology on MEAs using immunocytochemistry have got started to analyze spatial factors that affect neuronal dynamics (Ito et al., 2010).

In the present study, we performed cortical cultures *in vitro* at five different plating densities on MEAs for more than 1 month using Neuron Culture Medium (details were described in *Cell culture on MEAs at five different densities*). For comparison with the conventional culturing condition, we prepared other cortical cultures using Dulbecco's Modified Eagle Medium supplemented with serum (DMEM/serum). Electrical recording of the entire course of network maturation using MEAs demonstrated that overly sparse cultures did not show synchronized bursts. We then performed an immunocytochemical analysis of the neurons,

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Abbreviations: DIV, days *in vitro*; DMEM/serum, Dulbecco's Modified Eagle Medium supplemented with serum; MAP2, microtubule-associated protein 2; MEA, multi-electrode array; MED, multi-electrode dish; NF200, neurofilament 200 kD; PBS, phosphate-buffered saline.

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doi:10.1016/j.neuroscience.2010.08.038

neurites, and cell nuclei in each culture directly on MEAs and quantified the spatial distribution of the networks. The results revealed that ultra-sparse cultures were not able to form a sufficient density of networks to produce synchronized bursts. In addition to collecting the results of electrical recording, we determined the boundary density of the surviving neurons required for synchronized bursts using immunocytochemistry. The immunocytochemical observation of the cultures clarified that the synchronized burst occurred suddenly only when the density of surviving neurons surpassed the minimum threshold density.

EXPERIMENTAL PROCEDURES

Cell culture on MEAs at five different densities

Cortical cell cultures were prepared from Wistar rats at embryonic day 17 using the Nerve-Cell Culture System (Sumitomo Bakelite Co., Tokyo, Japan) as described previously (Mizuno et al., 2004; Banno et al., 2005; Takeuchi et al., 2005). Cortices were dissociated into single cells using dissociation solution (mainly papain), and they were resuspended in Neuron Culture Medium (serum-free conditioned medium from 48-h rat astrocyte confluent cultures based on Dulbecco's modified Eagle's minimum essential medium/F-12 with N2 supplement, Sumitono Bakelite; Banno et al., 2005; Takeuchi et al., 2005). After the cell density was determined using a haemocytometer, dissociated cortical neurons were diluted at final concentrations of 5×10^5, 2×10^5, 1×10^5, 5×10^4 and 2×10^4 cells/mL in a Neuron Culture Medium. A 100 μl aliquot of each cell suspension was then plated onto a poly(ethylenimine)-coated multielectrode dish (MED) probe (Alpha MED Scientific, Osaka, Japan), which consisted of 64 planar microelectrodes (Kudoh et al., 2007; Hosokawa et al., 2008). The size of each electrode was 50×50 μm^2 and the electrode spacing was 150 μm. To avoid cell attachment onto reference electrodes, we used a cloning ring with an inner diameter of 5 mm and a total area of 19.6 mm^2 (Honma et al., 1998). The rings were removed after 3 h. These procedures yielded cultures with nominal densities of 2500, 1000, 500, 250 and 100 cells/mm^2. Fig 1 shows three examples of cultures at 2500, 1000 and 100 cells/mm^2. The cultures were incubated with the above-described Neuron Culture Medium in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C. Half of the culture medium was renewed once a week over 1 month.

For the comparison with conventional culture conditions, we prepared the other cultures using common medium consisting DMEM (Invitrogen-Gibco, Carlsbad, CA, USA) supplemented with 5% fetal bovine serum (Invitrogen-Gibco), 5% horse serum (Sigma-Aldrich, St. Louis, MO, USA), 25 μg/mL insulin (Invitrogen-Gibco), 100 U/mL penicillin and 100 μg/mL streptomycin (Invitrogen-Gibco). After 3 days' culture using Neuron Culture Medium, half of the culture medium was replaced with fresh DMEM/serum medium. Then, half of the culture medium was renewed twice a week for 1 month. The cultures were incubated under the same conditions as the cultures using Neuron Culture Medium in a humidified atmosphere containing 5% CO_2 and 95% air at 37 °C.

Recording of electrical activity

The spontaneous electrical activity of each cortical culture was recorded using a MED64 extracellular recording system (Alpha MED Scientific) with a sampling rate of 20 kHz for 300 s. A/D conversion was performed by MED64 conductor software (Alpha MED Scientific). Because a humidified atmosphere affects the contact between the MED probe and MED connector, all recordings were conducted in an incubator at 37 °C (non-humidified). To avoid the contamination and evaporation of culture medium during the recording procedure, MED probes of cortical cultures were completely filled with each culture medium, which had been preserved overnight in a CO_2 incubator, and then sealed with a sterile coverslip and silicone grease (Haga et al., 2005; Nagayama et al., 2007). After the spontaneous electrical activity was recorded, the coverslip was removed and each culture was kept in a humidified atmosphere containing 5% CO_2 at 37 °C. After 3 days of culture *in vitro*, the activity was recorded for each day *in vitro* (DIV) for more than 1 month (which varied from 34 to 38 DIV).

Spike detection

Extracellular potential traces usually contain biological and thermal noises of about 10–20 μV. In this study, the spikes were determined when their amplitude exceeded a noise-based threshold within a window of 1 ms. To determine the threshold, we first made histograms of the field potential distribution using all the recorded data for the experiment. Then, we searched for the point at which the tangent slope reached the horizontal along the histograms. This point was set as the threshold for detecting spikes.

Synchronized burst detection

Synchronized bursts were detected using a method described in Mukai et al. (2003). In brief, the time window was set to 100 ms. Then the spikes (the total for all electrodes) in the window were counted. By shifting the window, a histogram of the change in the firing rate across time was obtained for each culture. Anything above the threshold was defined as a synchronized burst. In this study, the threshold was set to 100 spikes per window.

Immunocytochemistry and fluorescence microscopy

Immediately after the final recording at about 5 weeks, immunocytochemistry of the cultures on MEAs was performed. The cultures were washed twice with phosphate-buffered saline (PBS;

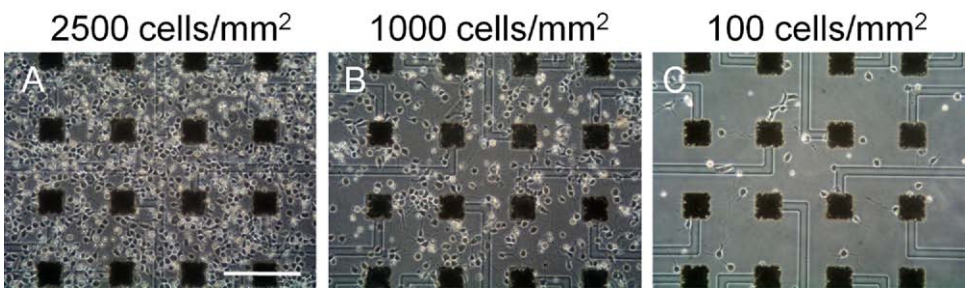

Fig. 1. Phase-contrast micrographs of typical cortical cultures at each of three densities (2500, 1000, 100 cells/mm^2) on MEAs at 1 DIV. Each photograph shows the central area of MEAs. (A) Dissociated density of 2500 cells/mm^2. (B) 1000 cells/mm^2. (C) 100 cells/mm^2. The scale bar represents 150 μm. Photographs of 500 and 250 cells/mm^2 are omitted. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

Invitrogen-Gibco) and fixed with 4% formaldehyde in PBS for 10 min, then washed twice more with PBS at room temperature. The fixed cultures were permeabilized with 0.5% Triton X-100 in PBS for 10 min and washed twice with PBS, then incubated with PBS containing 10% goat serum and 0.01% Triton X-100 for 30 min. The permeabilized cultures were incubated with primary antibodies in PBS containing 10% goat serum overnight at 4 °C and were rinsed with PBS for 10 min three times. The cultures were then incubated with secondary antibodies in PBS containing 10% goat serum for 1 h and were rinsed three additional times. Microtubule-associated protein 2 (MAP2) was detected by using anti-MAP2 mouse IgG (1:200; Sigma-Aldrich) as the primary antibody and 0.4% Alexa Fluor 488-labeled anti-mouse IgG (Molecular Probes, Eugene, OR, USA) as the secondary antibody. Neurofilament 200 kD (NF200) was detected using anti-NF200 rabbit IgG (1:500; Sigma-Aldrich) as the primary antibody and 0.4% Alexa Fluor 546-labeled anti-rabbit IgG (Molecular Probes) as the secondary antibody. To observe the cell nuclei, cultures were incubated with 2 μg/mL Hoechst 33342 (Dojindo Laboratories, Kumamoto, Japan) for 10 min after incubation with secondary antibodies. Fluorescence observation was performed using an epifluorescence microscope (IX71; Olympus, Tokyo, Japan). The images were captured through an Olympus 10× or 20× objective lens and collected with a charge-coupled device (CCD) camera (DP71; Olympus).

Image analysis

Image analysis was performed by using ImageJ 1.41 software. The number of neurons was determined by counting MAP2-stained somata manually using cell counter plugins. The area of neurites was determined by transforming NF200-stained images into black-and-white images, which were then transformed into binary files, and the thresholds were automatically set using an Otsu filter. The cell nuclei were counted by the same procedure as used to determine the area of neurites using Hoechst 33342-stained images. Objects in the binary images that were slightly overlapped were separated by Watershed separation using ImageJ 1.41 software. The nuclei were counted automatically after they were determined to be between 5 and 200 mm^2. Four microscopic images captured through a 10× objective lens (total 2.30 mm^2) in the center area of the cultures were analyzed for each MED plate.

RESULTS

Recording of electrical activity

Developmental changes in the spontaneous array-wide firing rate, that is, the number of spikes per second summed over all electrodes (also called the array-wide spike rate), were recorded for each plating density on each day from 5 DIV to 38 DIV (Fig. 2A). The activity was recorded from about 5 DIV in cultures of 2500 cells/mm^2 and was found to increase from 2 to 3 weeks in Neuron Culture Medium (left in Fig. 2A). This activity lasted for up to 5 weeks but varied in intensity from day to day. The 1000 and 500 cells/mm^2 cultures showed a delayed increase in the firing rate, but a similar change compared to the 2500 cells/mm^2 cultures. Six of the seven 250 cells/

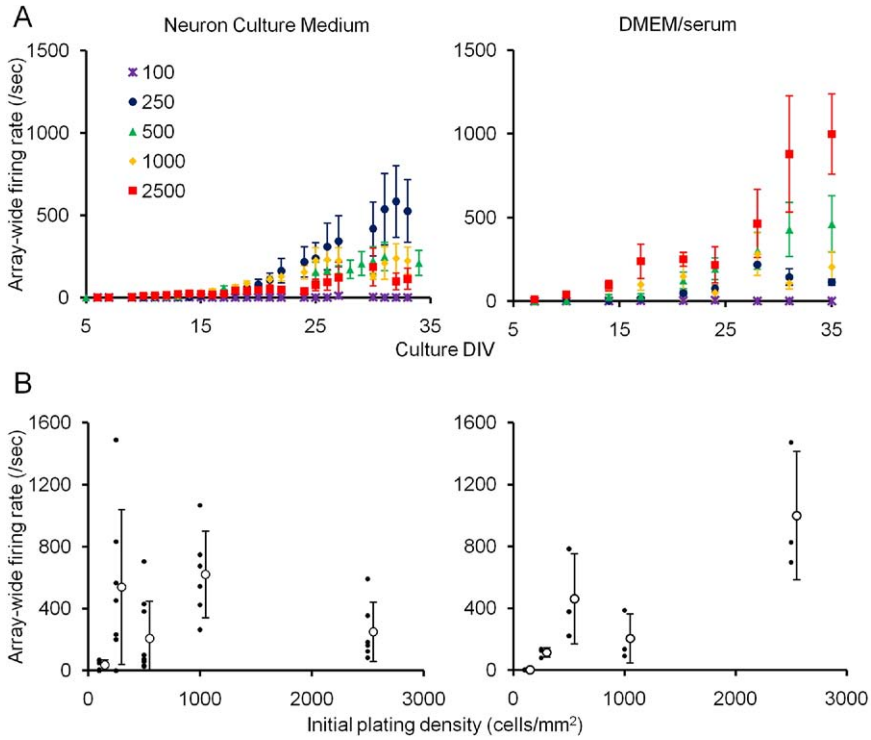

Fig. 2. The spontaneous array-wide firing rate at each of the five plating densities in cultures using Neuron Culture Medium (left) and DMEM/serum (right). (A) Developmental changes in the array-wide firing rates at five different densities. Each symbol represents the firing rate at different culture DIVs. Red squares indicate the cultures of 2500 cells/mm^2, yellow rhombuses those of 1000 cells/mm^2, green triangles those of 500 cells/mm^2, blue circles those of 250 cells/mm^2, and purple asterisks those of 100 cells/mm^2. n=6, 6, 7, 7, 7 for Neuron Culture Medium and n=3, 3, 3, 3, 3 for DMEM/serum, respectively. Data are presented as the mean±SEM. (B) Array-wide firing rate of each culture recorded on the final day (which varied from 34 to 38 DIV). The filled circles represent each culture, whereas the open circles represent the mean of each density (horizontal jittering was performed for visual clarity). Data are presented as the mean±SD. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

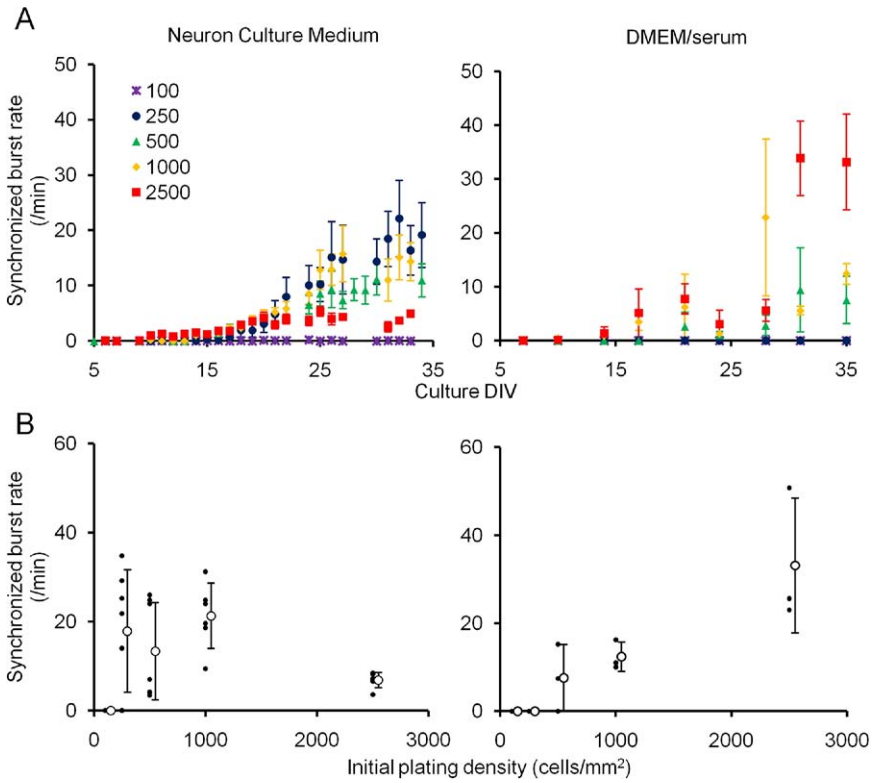

Fig. 3. Synchronized burst rate at each of the five plating densities in cultures using Neuron Culture Medium (left) and DMEM/serum (right). The data were calculated by burst detection methods (see *Synchronized burst detection*) using the data on the array-wide firing rate. (A) Developmental changes in the synchronized burst rate. Each symbol represents the synchronized burst rate at different culture DIVs. Red squares represent the cultures of 2500 cells/mm^2, yellow rhombuses those of 1000 cells/mm^2, green triangles those of 500 cells/mm^2, blue circles those of 250 cells/mm^2, and purple asterisks those of 100 cells/mm^2. n=6, 6, 7, 7, 7 for Neuron Culture Medium and n=3, 3, 3, 3, 3 for DMEM/serum, respectively. Data are presented as the mean±SEM. (B) Synchronized burst rate of each culture recorded on the final day (which varied from 34 to 38 DIV). The filled circles represent each culture, whereas the open circles represent the mean of each density (horizontal jittering was performed for visual clarity). Data are presented as the mean±SD. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

mm^2 cultures began firing later than the dense cultures, but their firing rates were dramatically elevated at about 4 weeks. The other 250 cells/mm^2 culture showed no firing. In all seven 100 cells/mm^2 cultures, there were very faint spikes on any DIV. In the cultures using DMEM/serum, the cultures with more than 250 cells/mm^2 groups showed spontaneous firing (right in Fig. 2A). However, the 2500 cells/mm^2 cultures showed higher activity and the 250 cells/mm^2 cultures showed lower activity than the cultures using Neuron Culture Medium. None of the 100 cells/mm^2 cultures showed spontaneous electrical activity in DMEM/serum. Fig 2B shows the array-wide firing rate at each plating density for all experiments recorded on the final day (which varied from 34 to 38 DIV). In the case of cultures in which more than 250 cells/mm^2 were plated, the firing rate was highly variable in Neuron Culture Medium (left in Fig. 2B). However, the cultures with 100 cells/mm^2 showed a lower firing rate and differed from the denser cultures with regard to the level of spontaneous electrical activity. In the cultures using DMEM/serum, more than 500 cells/mm^2 cultures showed variable activity, whereas the 250 cells/mm^2 cultures and 100 cells/mm^2 cultures showed lower firing rates (right in Fig. 2B).

Using a burst synchronization detection method (see *Synchronized burst detection*), we evaluated developmental changes in the synchronized burst rate in cultures at five different densities over a 5-week culture period (Fig. 3A). The change in the burst rate resembled the changes in the firing rate (Fig. 2A). In the cultures using Neuron Culture Medium, the 2500 cells/mm^2 cultures began to exhibit synchronized bursts at around 10 DIV, and the rate increased until 3 weeks, after which it did not change significantly until 5 weeks (left in Fig. 3A). As the plating density became more sparse, the initial detection of bursts became later and the burst rate increased up to 5 weeks. Five of the seven 250 cells/mm^2 cultures showed a higher burst rate than the dense cultures. However, none of the 100 cells/mm^2 cultures showed any bursts throughout the entire culture period. In the cultures using DMEM/serum, the cultures with more than 500 cells/mm^2 showed synchronized bursts within 2 to 3 weeks, whereas neither the 250 cells/mm^2 nor 100 cells/mm^2 cultures exhibited synchronized burst activity (right in Fig. 3A). The synchronized burst rate using DMEM/serum was more variable day to day. Fig 3B shows the burst rate of all experiments at each plating density recorded on the final day (which varied from

34 to 38 DIV). There was significant variation in the burst rates of the 250–1000 cells/mm^2 culture in the cultures using Neuron Culture Medium (left in Fig. 3B). It is noteworthy that two of the seven cultures plated at 250 cells/mm^2 did not exhibit burst activity. In addition, no burst was observed in any of the 100 cells/mm^2 cultures. In the cultures using DMEM/serum, one of the three cultures plated at 500 cells/mm^2 did not show synchronized bursts (right in Fig. 3B). Moreover, no burst was measured in any of the 250 cells/mm^2 or 100 cells/mm^2 cultures. These results indicate that synchronized burst activity requires an initial plating density of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum on MEAs in our culturing condition. When the initial density was lower than these densities, only a few spikes at a few electrodes were recorded and no synchronized burst was recorded.

Immunocytochemistry and image analysis

To investigate the morphological distribution of the cortical networks in different densities on MEAs, immunocytochemistry of MAP2 and NF200 was performed immediately after the final recording of electrical activity. Fig 4 shows immunofluorescence micrographs for typical cultures at five different densities using two different media. Fig 4A shows the cultures using Neuron Culture Medium and Fig 4B shows the cultures using DMEM/serum. Neuronal networks grown on MEAs were clearly recognized by this procedure. In the cultures using Neuron Culture Medium, the neurons that were initially plated at more than 500 cells/mm^2 formed complicated networks with many cell bodies and neurites (Fig. 4A(a–c)). The morphological distributions of the cultures initially plated at these densities did not differ significantly from each other. The cultures plated at 250 cells/mm^2 showed sparse cell bodies and neurites, and networks were more discernible than in denser cultures (Fig. 4A(d)). Two of the seven 250 cells/mm^2 cultures revealed sparser cell bodies and neurites than the other 250 cells/mm^2 cultures (Fig. 4A(e)). In these 250 cells/mm^2 cultures, cultures of Fig. 4A(d) showed synchronized bursts, whereas the cultures of Fig. 4A(e) did not. In the 100 cells/mm^2 cultures, the neurons were drastically decreased in number and did not form a sufficient network (Fig. 4A(f)). In the cultures with the plating densities of more than 1000 cells/mm^2, the cultures formed complicated networks, as shown in Fig. 4B(a) (an example of a 2500 cells/mm^2 culture). At the initial density, two of the three 500 cells/mm^2 cultures showed sparse and discernible networks (Fig. 4B(b)). These two 500 cells/mm^2 cultures exhibited synchronized burst activity. However, the other 500 cells/mm^2 culture did not form sufficient networks and showed a significant decrease in neurites (Fig. 4B(c)). In addition, this 500 cells/mm^2 culture did not show synchronized bursts. The cultures with initial plating densities below 500 cells/mm^2 showed no networks, as shown in Fig. 4B(d) (an example of a 100 cells/mm^2 culture). These fluorescence observations indicated that the cortical cultures required initial plating densities of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum to construct neuronal networks on MEAs. In addition, these minimum densities corresponded to the densities required for exhibiting synchronized bursts. When the initial plating densities were lower than these minimum densities, the cultures were not able to form sufficient networks or to exhibit synchronized bursts in these culturing conditions.

To determine the densities of neurons that survived at 5 weeks, we counted the number of somata of MAP2-positive neurons using the immunofluorescence images. Fig 5A shows the MAP2-positive final neuron density at 5 weeks for each plating density using two different culture media together as mean values, whereas Fig. 5B shows all individual data on MAP2-positive neuron density with respect to each medium. The MAP2-positive neuron densities using Neuron Culture Medium showed more than two-fold higher final densities at any initial plating density than when DMEM/serum was used. When cortical cells were cultured at a plating density of 100 cells/mm^2 using Neuron Culture Medium, few neurons survived at 5 weeks (left in Fig. 5B). As the plating density increased, the density of surviving neurons increased moderately. However, the MAP2-positive neuron density tended to become saturated in denser cultures, and did not increase linearly with plating density. The cultures using DMEM/serum showed similar tendencies in terms of the saturation of neuron density (right in Fig. 5B). Fig 5C shows the MAP2-positive neuron density at the final date as a percentage of each of the initial plating densities. Few neurons remained viable in the 100 cells/mm^2 cultures in Neuron Culture Medium (left in Fig. 5C). In the 250 cells/mm^2 cultures, the percentage of MAP2-positive neurons shows a large deviation, but indicated a higher survival rate than the 100 cells/mm^2 cultures. As the plating density increased, the percentage of surviving neurons decreased gradually. In the cultures using DMEM/serum, the cultures showed a similar tendency, whereas the percentage of MAP2-positive neurons decreased significantly (right in Fig. 5C). These results indicate that neurons did not decrease equally during l month of culture. Thus, in these culture conditions, the number of neurons decreased and the final density of surviving neurons became saturated as the initial plating density increased.

To more clearly determine the sizes of the networks formed at different densities, the percentage of the area of MEAs occupied by NF200-positive neurites at 5 weeks was also quantified in both media. When cortical cells were plated at 100 cells/mm^2, a small percentage of MEAs consisted of neurites in the culture using Neuron Culture Medium (left in Fig. 6A). At the plating density of 250 cells/mm^2, the NF200-positive area increased drastically up, but one of the seven cultures showed a smallpercentage of neurites. These differences have already been shown in Fig. 4A(d, e). As the plating of cells at the start of the culture became more dense, the area occupied by neurites did not change significantly. This saturation is similar to the results of the MAP2-positive neuron density. In the culture using DMEM/serum, the 100 cells/mm^2 and 250 cells/mm^2 cultures showed small percentages of NF200-positive neurite coverage on MEAs (right in Fig. 6A). At the plating density of 500 cells/mm^2, this coverage

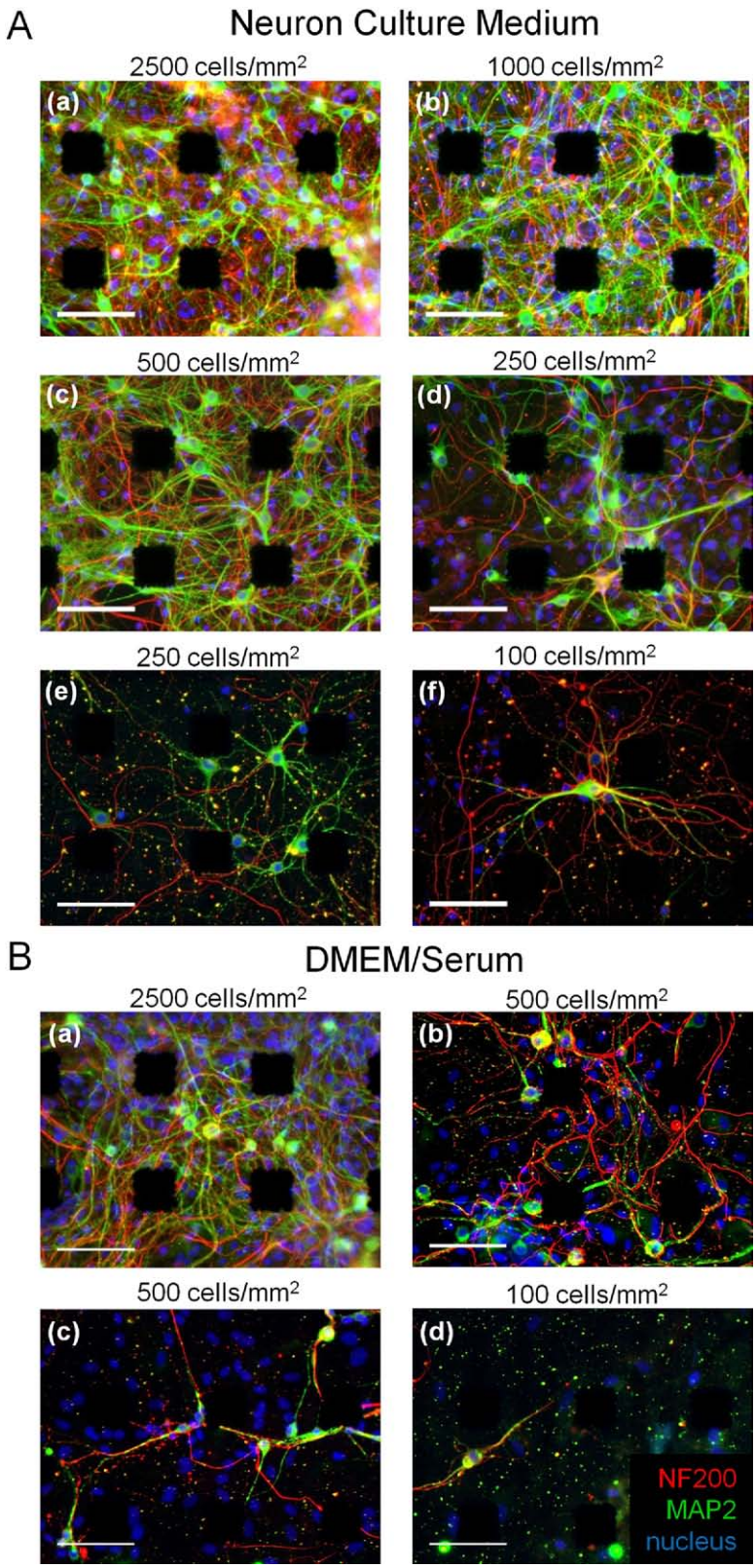

Fig. 4. Immunofluorescence micrographs of cortical cultures on MEAs at the five plating densities. All photographs show the merging of three fluorescence images of MAP2 (green), NF200 (red) and cell nuclei (blue). (A) Cultures using Neuron Culture Medium ((a) cultures of 2500 cells/mm^2, (b) 1000 cells/mm^2, (c) 500 cells/mm^2, (d) 250 cells/mm^2, (e) also 250 cells/mm^2, (f) 100 cells/mm^2). (B) Cultures using DMEM/serum ((a) cultures of 2500 cells/mm^2, (b) 500 cells/mm^2, (c) also 500 cells/mm^2, (d) 100 cells/mm^2). The scale bar represents 100 μm. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

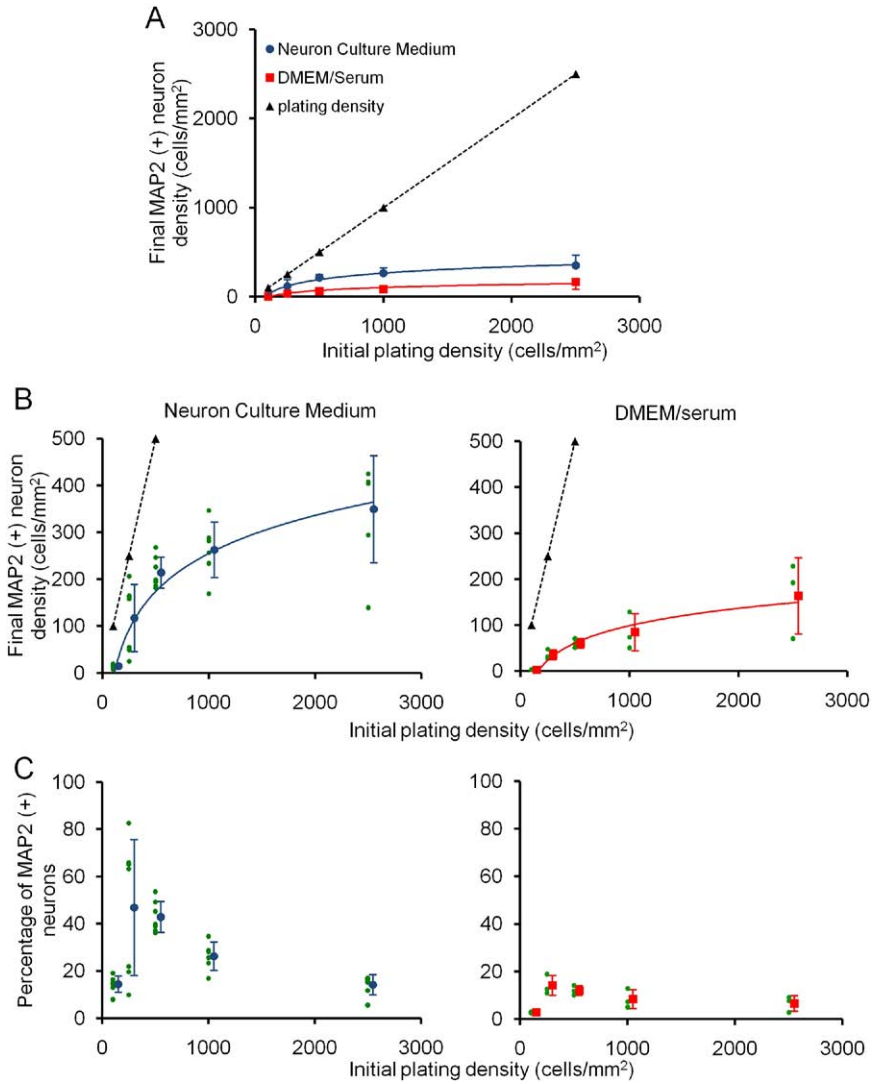

Fig. 5. Quantitative analysis of MAP2 immunofluorescence images. (A) The graph shows the MAP2-positive neuron density in the culture using Neuron Culture Medium (filled blue circles), that in the culture using DMEM/serum (filled red square) and the initial plating density (filled black triangles) at each of the five plating densities. Data are presented as mean±SD for Neuron Culture Medium and mean – SD for DMEM/serum. (B) The graphs provide an enlarged view below 500 cells/mm^2 in the y-axis area of graph (A). At left are the data obtained using Neuron Culture Medium and at right are those obtained using DMEM/serum. Filled green circles represent each culture. Filled blue circles and filled red squares represent the mean of each density (horizontal jittering was performed for visual clarity). The data are presented as the mean±SD value of neurons/mm^2. (C) The graphs show the MAP2-positive neuron density (as a percentage) for each plating density. Data are presented as the mean±SD percentage. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

area increased but showed a large deviation. This large deviation was derived from the differences between Fig. 4B(b) and Fig. 4B(c). The 1000 cells/mm^2 and 2500 cells/mm^2 cultures showed relatively higher values and seemed to become saturated in their percentages of neurites on MEAs. In addition, the percentages of saturated neurites showed little differences between the two media. Fig 6B shows the correlation plots between MAP2-positive neuron density and the percentage of the NF200-positive neurite area using both media. As the MAP2-positive final neuron density increased, the percentage of NF200-positive neurites also increased gradually at lower densities in both media. At the higher neuron density, the percentage of the area with neurites did not increase but reach saturation. These results also indicate that the minimum plating density needed to elongate a sufficient number of neurites and form complicated networks was 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum in these culture conditions. Moreover, the area occupied by neurites also became saturated as neuronal density increased.

Staining of cell nuclei indicated that the non-neuronal cells proliferated significantly in both media (Fig. 4). To quantify the proliferation of these non-neuronal cells at different densities, we counted the densities of cell nuclei and compared them with the neuronal densities. Fig 7 shows the Hoechst 33342-positive nuclear density for each plating density at 5 weeks using both media. Compared with the initial plating density (dashed line), the

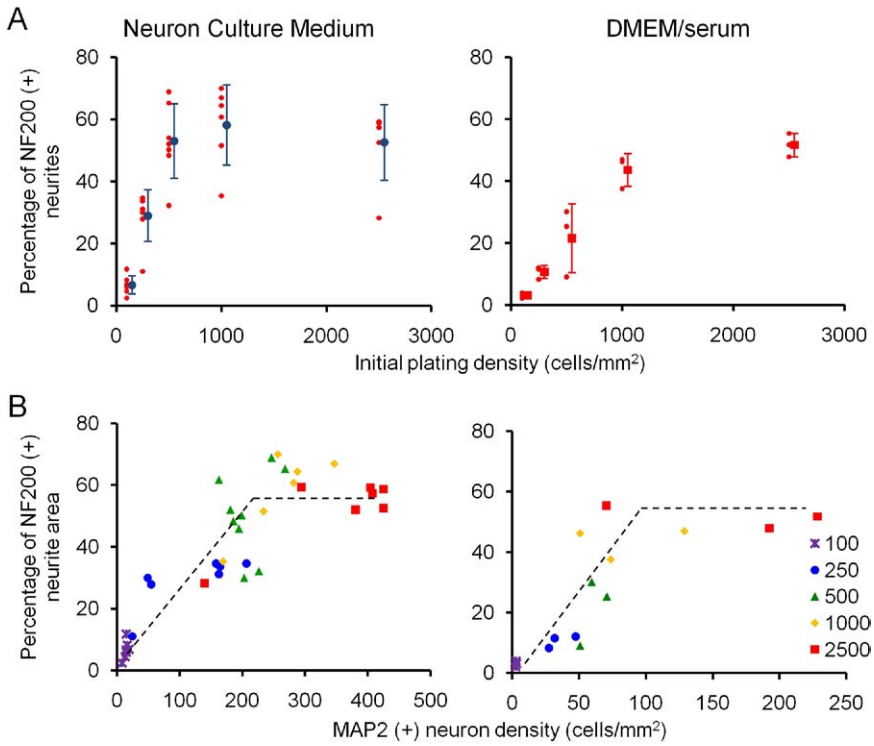

Fig. 6. Quantitative analysis of NF200 immunofluorescence images in the cultures using Neuron Culture Medium (left) and DMEM/serum (right). (A) The graphs show the percentage of the total surface area of MEAs occupied by NF200-positive neurites in each culture. Filled blue circles and filled red squares represent the mean of each density. Data are presented as the mean±SD percentage. (B) The graphs show correlation plots of the MAP2-positive neuron density in neurons/mm^2 against the NF200-positive neurite area as a percentage. The symbols represent each culture. Red squares represent the cultures of 2500 cells/mm^2, yellow rhombuses those of 1000 cells/mm^2, green triangles those of 500 cells/mm^2, blue circles those of 250 cells/mm^2, and purple asterisks those of 100 cells/mm^2. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

whole cell density increased significantly except for the 2500 cells/mm^2 cultures that used Neuron Culture Medium (left in Fig. 7). The density of cell nuclei became saturated (confluence) in most cultures of more than 250 cells/mm^2 at 2000–2500 cells/mm^2, with the exception that two of the seven cultures plated at 250 cells/mm^2 did not show such an increase in nuclear density. In the 100 cells/mm^2 cultures, cells did not show an increase up to confluence. The density at which the cells proliferated up to confluence corresponded to the minimum required for construction of sufficient networks (250 cells/mm^2). Similar tendencies were observed in the cultures using DMEM/serum, but the cultures required the initial plating density of 1000 cells/mm^2 to reach confluence (right in Fig. 7). These results show that non-neuronal cells proliferated significantly and became confluent in denser cultures, and that proliferation

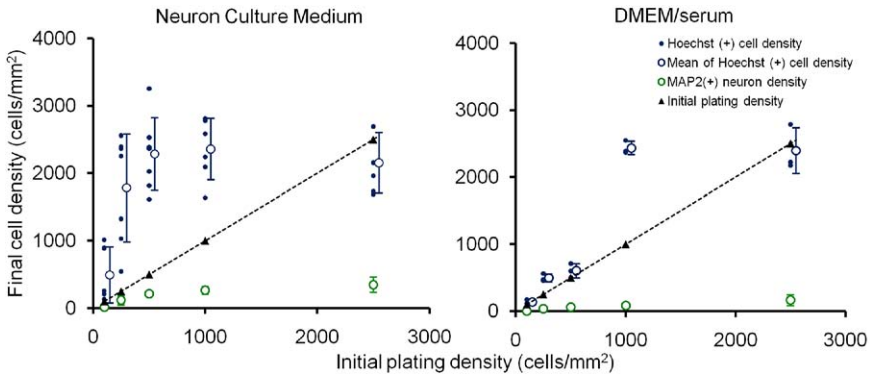

Fig. 7. Quantitative analysis of Hoechst 33342-stained images in the cultures using Neuron Culture Medium (left) and DMEM/serum (right). The graphs show the Hoechst 33342-positive cell nuclear density in each culture on MEAs at each of the five plating densities. Filled blue circles represent each culture, whereas open blue circles represent the mean of each density. Open green circles represent the mean MAP2-positive neuron density as shown in Fig. 5, and filled black triangles represent each initial plating density. Data are presented as the mean±SD cells/mm^2. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

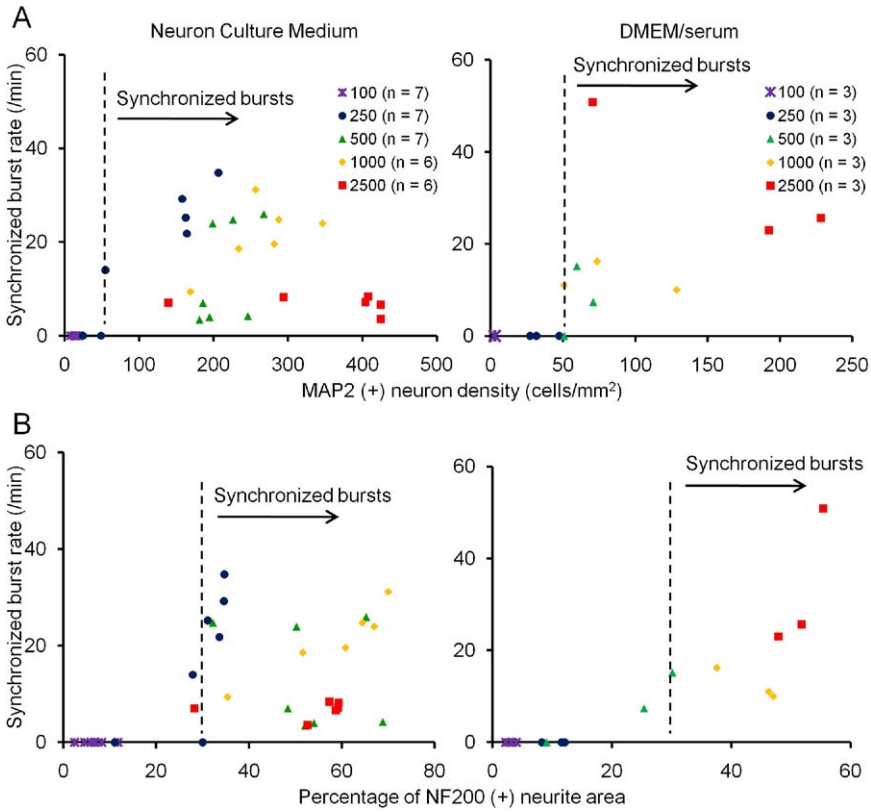

Fig. 8. Comparing the densities of surviving neurons and the neurite area with the synchronized burst rates in the culture using Neuron Culture Medium (left) and DMEM/serum (right). (A) The graphs show the synchronized burst rate of each culture in relation to the MAP2-positive neuron density. The symbols represent the rates recorded just before the immunocytochemical procedure (n=33 for Neuron Culture Medium and n=15 for DMEM/serum). Red squares represent the cultures of 2500 cells/mm^2, yellow rhombuses those of 1000 cells/mm^2, green triangles those of 500 cells/mm^2, blue circles those of 250 cells/mm^2, and purple asterisks those of 100 cells/mm^2. Dashed lines indicate the minimum survived neuron density required for synchronized bursts (approximately 50 neurons/mm^2 in both media). (B) The graphs show the synchronized burst rate of each culture in relation to the NF200-positive neurite area. The symbols indicate the rates for the same MAP2-positive neuron densities described in (A). Dashed lines indicate the minimum percentage of neurite area covering the surface required for synchronized bursts (approximately 30% in both media). For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.

of non-neuronal cells also required the minimum initial plating density. In addition, the results also showed a drastic decrease in surviving neuronal densities (MAP2-positive neuron densities), again compared with the initial plating densities in these culture conditions.

Analysis of neuron and neurite densities required for synchronized bursts

To determine the actual minimum network size required for synchronized bursts, we compared the densities of surviving neurons and the neurite area with the synchronized burst rates. For this purpose, we used the electrical activity recorded just before the cell fixation procedure. Fig 8A shows the synchronized burst rate of each culture in relation to the MAP2-positive neuron density. At a density of up to approximately 50 neurons/mm^2, no synchronized burst was recorded in the cultures using Neuron Culture Medium (left in Fig. 8A). The cultures containing more than approximately 50 neurons/mm^2 exhibited synchronized bursts. As the density of surviving neurons increased, the burst rate varied widely and independently of the density of surviving neurons. In the cultures using DMEM/serum, synchronized bursts were not observed up to a density of approximately 50 neurons/mm^2, the same as in the culture using Neuron Culture Medium (right in Fig. 8A). The cultures containing more than this density also showed synchronized bursts, and the burst rate was independent of the density of surviving neurons in DMEM/serum. Interestingly, the final neuron density at which the cultures began to show synchronized bursts in Neuron Culture Medium was consistent approximately with the final density in DMEM/serum. Fig 8B shows the synchronized burst rate of each culture in relation to the percentage of NF200-positive neurite area in both media. In both culture media, synchronized bursts were observed in the cultures in which neurites accounted for approximately 30% of the total area, whereas the neurites area accounted for only approximately 10% of the cultures that showed no synchronized bursts. These results indicate that a synchronized burst arises when the culture contains at least a minimum number of neurons, and each cortical culture requires more than approximately 50 neurons/mm^2 survival for synchronized bursts activity. This minimum density of neurons that survived did not vary in different media in this culture condition. The cultures that

exhibited synchronized bursts showed an increased percentage of the NF200-positive neurite area more than approximately 30% of the culture surface.

DISCUSSION

To investigate how many neurons a culture needs for synchronized burst activity, we cultured cortical cells at five different densities on MEAs for more than 1 month in two different culture media. We performed recording of spontaneous electrical activity using MEAs and subsequent observation of the network morphologies on MEAs using immunocytochemistry to evaluate the minimum neuronal density required for synchronized bursts. The main findings of our study were as follows: (1) Cortical cultures required initial plating densities of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum in order to exhibit synchronized bursts on MEAs. (2) Cortical cultures required initial plating densities of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum to form sufficient neuronal networks on MEAs. These densities corresponded to the minimum initial density required for synchronized bursts. (3) The number of neurons decreased during 1 month culture, and the density of survived neuron became saturated as the initial plating density increased. (4) The densities of the neurite covering the surface area and all cells also became saturated at higher initial plating densities. (5) Synchronized bursts were observed under conditions where a minimal density of neurons survived and sufficient numbers of neurites existed. These densities were consistent in both media under our culture conditions.

Minimum cortical density for synchronized bursts, and variation of burst rate

Since MEAs were first developed (Gross, 1979; Pine, 1980), many researchers have investigated the properties of dense neuronal networks *in vitro* (Kamioka et al., 1996; van Pelt et al., 2004; Chiappalone et al., 2006). However, few studies have used sparse cultures on MEAs. This is because there will be fewer cells in the vicinity of the electrode in sparser cultures, and thus spontaneous electrical activity (e.g., spikes and synchronized bursts) will be hardly recorded. In this study, we focused on sparser networks and assessed the presence or absence of the synchronized bursts. The results based on MEA recordings showed that the cultures required initial plating densities of at least 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum to exhibit synchronized bursts (Fig. 3A, B). However, it has to be noted that the culturing area (central area of MEAs, see *Cell culture on MEAs at five different densities*) in this study was smaller than it was under the conditions using cell culture dishes (e.g., Bi and Poo, 1998, 1999). Although we could not conclude that these densities are “universal” under any conditions, our results clarified, at least experimentally, that there was a minimum initial plating density of the cortical cultures below which no synchronized bursts would occur under these culturing conditions.

With the use of Neuron Culture Medium, most 250 cells/mm^2 cultures showed higher firing and burst rates than the denser cultures under our culturing conditions. The bursts of these cultures were characterized by high frequency, short duration and short inter-burst intervals. Conversely, most dense cultures exhibited surperburst-like activities—that is, sequences of global bursts, which were separated by intervals of several minutes (Wagenaar et al., 2005). These activities affected the decreases in firing and burst rates in denser cultures. In addition, the decreases in these rates might be related to the different densities of the inhibitory sub-networks. It is possible that the networks of the densest cultures contained higher densities of inhibitory sub-networks and showed subsequent reductions in firing and burst rates. However, this inverse correlation was not observed in the cultures using DMEM/serum, and densest cultures showed variable bursting patterns. These confusing results might be due to the culture condition that the medium was not changed frequently enough for the higher densities in the cultures using Neuron Culture Medium. One of more plausible possibilities is the different ratio of inhibitory neurons or inhibitory synapses in each culture. The ratio will be clarified using immunocytochemistry (Wagenaar et al., 2005; Brewer et al., 2009; Ito et al., 2010).

Minimum cortical density required for construction of networks and network saturation

We performed immunocytochemistry of neuronal components directly on MEAs and analyzed the spatial distribution of the network quantitatively (Figs. 4–7). The results revealed that the cultures required a density of 250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum at initial plating in order to establish networks. This minimum plating density corresponded to that of synchronized bursts. As described above (section *Cell culture on MEAs at five different densities*), Neuron Culture Medium is serum-free glial-conditioned medium based on DMEM/F-12 with N2 supplement (Banno et al., 2005; Takeuchi et al., 2005). Our results, which are shown in Figs. 4 and 5, suggest that Neuron Culture Medium improved the survival rate of neurons compared with DMEM/serum. When the initial plating density was lower than these densities (250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum), the cultures did not form sufficient networks and showed decreased numbers of neurons. It has been reported that low-density cultures were not viable, especially in a medium supplemented with serum (Brewer and Cotman, 1989; Brewer et al., 1993). Therefore, these densities (250 cells/mm^2 for Neuron Culture Medium and 500 cells/mm^2 for DMEM/serum) are thought to be the minimum initial plating densities in order to remain viable in our culturing spaces (center of the MEAs, see sections *Cell culture on MEAs at five different densities* and *Minimum cortical density for synchronized bursts, and variation of burst rate*).

Fig 5 shows drastic decreases in neuron numbers, and also saturation of survived neuronal densities in denser cultures. This kind of decrease in the neurons in dense cultures has also been shown in previous study (Voigt et

al., 1997). In contrast to this decrease of neurons, non-neuronal cells proliferated significantly compared with initial plating densities (Fig. 7). Therefore, it is possible that the proliferation of non-neuronal cells affects the neuronal reduction. Moreover, such a decrease in neurons might take part in the elimination of abundant neurons such as are found *in vivo* (Oppenheim, 1991; Ferrer et al., 1992). The saturation in denser cultures was observed not only in surviving neuronal densities but also in the portion of neurites covering the culture surface area (Fig. 6A). These results suggest that the cultures became "network-confluent" in denser cultures. However, it has to be noted that the neurons formed three-dimensional networks on planar MEAs. We obtained only the projected images, in which the neurites overlapped each other, as two-dimensional images using an epifluorescence microscope. Therefore, further investigation of the morphological distribution using confocal laser scanning microscopy will be needed to precisely quantify the amount of neurites.

Minimum neuron and neurite densities required for synchronized bursts

Comparing the results of the electrophysiological recordings with the immunocytochemical observation, we quantified the actual neuron and neurite densities required for synchronized bursts (Fig. 8). The results indicated that burst activity occurred suddenly under conditions allowing final neuron density of more than approximately 50 neurons/mm^2. The networks that contained more than 50 neurons/mm^2 showed little dependence on the synchronized burst rate. Interestingly, the final MAP2-positive neuron density at which the cultures began to show synchronized bursts in Neuron Culture Medium was consistent approximately with the final density in DMEM/serum. Therefore, it is possible that the neuronal density required for synchronized bursts will not vary in any culture media. However, it has been reported that patterned neuronal networks that were cultured at lower density compared with conventional dissociated cultures showed synchronized activity (Suzuki and Yasuda, 2007). Thus, it is necessary to verify the minimum neuron density under other culturing conditions, such as by using other media (Neurobasal/B27 and NbActiv4; Brewer et al., 1993, 2008) and by changing the culture dish to determine the "universal" minimum neuron density for synchronized bursts.

Our results also indicate that neurites accounted for approximately 30% of the total surface area of the MEAs in the cultures exhibiting synchronized bursts (Fig. 8B). Under the conditions that the culture did not show synchronized bursts, however, this percentage reached only 10%. This result suggests that whether the cultures showed synchronized bursts or not depends heavily on the extent to which neurites cover the surface area. Furthermore, it is possible that survival of the networks might be related in part with the presence of synchronized bursts. These functional significances of synchronized bursts remain to be resolved in future studies.

In summary, it was clarified experimentally that there was a minimum surviving neuron density required in order synchronized bursts to occur in cortical culture. The networks that showed synchronized burst activity contained more than a certain amounts of neurites.

Direct observation of the neuronal distribution on MEAs

These direct observations of cortical cultures on MEAs using fluorescence microscopy will be a useful tool with which to analyze spatial factors that affect neuronal dynamics. For example, they could be applied to computational modeling studies using a predetermined number of neurons (Izhikevich et al., 2004; Nomura et al., 2009). Moreover, it is thought to be easier to identify neuronal circuits *in vitro* than *in vivo* using electron microscopic techniques and transgenic strategies (Briggman and Denk, 2006; Livet et al., 2007). Further investigation of morphological distribution and statistical analysis using MEAs would clarify the process of spike transmission throughout neuronal circuits reconstructed *in vitro*.

CONCLUSION

This study clarified that cortical culture requires a minimum initial plating density in order to exhibit synchronized bursts, and that this minimum density corresponded to the formation of sufficient neuronal networks. Moreover, the number of neurons decreased in denser cultures during 1 month of culture, and the final density of surviving neurons became saturated as the initial plating density increased. The amount of neurites also became saturated in denser cultures. Finally, we revealed experimentally that there was a minimum density of surviving neurons that caused synchronized bursts.

Acknowledgments—This work was supported in part by Grants-in-Aid for Scientific Research from the Japan Society for the Promotion of Science (Nos. 20240023 and 21650049) to K. Gohara. We are grateful to S. Honma (Hokkaido University School of Medicine, Japan) for advice regarding the MEA-based recordings.

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(Accepted 18 August 2010)
(Available online 26 August 2010)