Effect of Mg$^{2+}$ on neural activity of rat cortical and hippocampal neurons

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Abstract. Mg$^{2+}$ plays an important role in biological functions, similar to that of Ca$^{2+}$. In terms of neural activity, it is well known that Mg$^{2+}$ blocks the NMDA receptor. However, the relationship between Mg$^{2+}$ and neural function has not been well understood. We have investigated the effect of low extracellular Mg$^{2+}$ concentration ([Mg$^{2+}$]$_o$) on neural activity in rat cortical and hippocampal neurons by using microelectrode array (MEA) measurements and glutamate measurements, with an enzyme modified MEA-based multi-array sensor. In this study, we investigated the effects of low [Mg$^{2+}$]$_o$ on intracellular Ca$^{2+}$ concentration ([Ca$^{2+}$]$_i$) using a confocal laser microscope and a flow cytometer with a fluorescence probe. The results indicate that low [Mg$^{2+}$]$_o$ has an effect on neural activity. The responses of cortical and hippocampal neurons to low [Mg$^{2+}$]$_o$ differed in the developmental period. The results suggest that hippocampal neurons are more sensitive to [Mg$^{2+}$]$_o$ than cortical neurons. The glutamate receptor distributions in the cortex and hippocampus may be different. Further investigation is required to understand the mechanisms of the Mg$^{2+}$ effect on neural activity.

Key words: Mg$^{2+}$, Ca$^{2+}$, MEA, flow cytometry, cortex, hippocampus

It is well known that Mg$^{2+}$ plays an important role in the inhibitory function to glutamate release [1], the Ca$^{2+}$ channel [2], and the NMDA receptor [3, 4]. However, few reports have investigated the effect of Mg$^{2+}$ on neural activity. Recent studies indicate that an Mg$^{2+}$ deficient diet has significant health effects since Mg$^{2+}$ is an important ion in relation to biological function.

[Ca$^{2+}$]$_i$ increases when extracellular Mg$^{2+}$ is removed from the experimental solution ([Mg$^{2+}$]$_o$ removal) [5]. The amount of [Ca$^{2+}$]$_i$ is 10 000 times less than that of [Ca$^{2+}$]$_o$. However, when cells are stimulated, [Ca$^{2+}$]$_i$ exhibits a transient increase as a result of the Ca$^{2+}$ influx from outside the cell and the Ca$^{2+}$ released from inside organelia, such as the endoplasmic reticulum (ER). The change in [Ca$^{2+}$]$_i$ caused by stimulation is mainly divided into two types. One is the Ca$^{2+}$ spike that indicates a rapid change in [Ca$^{2+}$]$_i$, and the other is Ca$^{2+}$ oscillation that indicates slow periodic changes. Ca$^{2+}$ oscillation is known as a signal induced by fertilization that initiates the differentiation and development of an egg, namely the beginning of life. It is also involved in various physiological phenomena, such as hormone and digestive enzyme release, immunity and gene expression [6-8], and neural circuits.

Here we investigated the effect of [Mg$^{2+}$]$_o$ on neural activity and its mechanism using a 64-channel microelectrode array (MEA) [9] and glutamate measurements performed with an enzyme-modified-MEA multi-array sensor [10-12]. We also investigated the difference in the effects of [Mg$^{2+}$]$_o$ on cortical and hippocampal neurons based on [Ca$^{2+}$]$_i$ measurements with a fluorescence probe.
Materials and methods

Neuron culture
This study used primary dissociated culture cells and slice cultures grown in a serum-free medium [13, 14]. An 18-day Wistar rat embryo (E18) was used for the culture. Cortex or hippocampus was removed from the rat brain, and then treated with 2.5 mg/mL trypsin (Invitrogen Corporation, Carisbad, CA, USA) for 10 min at 37°C. Then cells were centrifuged at 1,000 rpm for 5 min and triturated with a pipette. The culture was carried with a neurobasal medium (Invitrogen) that consisted of 0.074 mg/mL L-glutamine, 25 μM glutamate, 50 μg/mL gentamycin (Invitrogen) and 2% B27 supplement (Invitrogen). The cells were filtered with a lens paper, and then the cell density was adjusted to 1-5 x 10^6 cells/mL. The cells were plated on glass bottom culture dishes (35 mmø, uncoated, No. 0, (Matsunami Glass. Ind., Ltd, Osaka, Japan) pre-coated with 20 μg/mL laminin (Sigma-aldrich Corporation, St. Louis, MO, USA) and 100 μg/mL poly-D-lysine (M.W. 70 000-150 000, Sigma), and cultured for 1-4 weeks at 37°C under conditions of 5% CO2 and saturated humidity.

For a slice culture, we used postnatal 8-11th (P8-11) Wistar rat hippocampus. The hippocampus was sliced to a thickness of 300 μm using a Vibramat (Leica, Heidelberg, Germany). The slices were cultured on a 64ch microelectrode array (MEA) for 5-7 days at 34°C, in 5% CO2 and with saturated humidity after a one-hour recovery period. A medium (DMEM:HEPES = 2:1) including penicillin and streptomycin, 20 ng/mL BDNF and NGF was used for the culture.

Electrical activity measurement
A 64-channel MEA was used for the neural electric activity measurement. The array was fabricated by conventional photolithography, using quartz substrates with a sputtered layer of 150 nm indium tin oxide (ITO). After the ITO layer had been wet etched to form the electrode patterns, the surface was passivated with a silicon-based positive photoresist, except for the electrodes. The array was composed of 64 electrodes arranged in an 8 x 8 square grid. The recording electrodes were 30 x 30 μm in size and were 100-150 μm apart. The electrode impedance was around 100 kΩ at 1 kHz. The extracellular signals detected by the 64 electrodes were amplified using a specially designed 64-channel amplifier (bandwidth 0.1-10 kHz, NF Corp.) and stored at a rate of 3 kHz or more.

Flow cytometric membrane potential measurement
The membrane potential change was measured with flow cytometry (cell analyzer, EPICS XL ADC, Beckman Coulter Inc. Fullerton, CA, USA). Cells were stained with 2.5 μM DiBAC4(3) (Dojindo, λex = 495 nm, λem= 517 nm) for 10 minutes after being stained for one hour with 5 μM propidium iodide (PI, Sigma) for dead cell screening [15, 16]. Suspended cells (1-5 x 10^6 cells/mL) were then measured at 10 000 cells/time. Two major characteristics can be measured with this method; one is the forward angle light scatter (FS) and the other is fluorescence (FL). Since the FS value changes with cell size, we could use the former to estimate the latter. On the other hand, the dye we used – DiBAC4(3) – can accumulate inside the cell depending on the membrane potential. Since depolarization of the membrane potential causes an increase in fluorescence, we could estimate the membrane potential by measuring the fluorescence intensity change.

Spatial and temporal distribution measurement of glutamate release
The glutamate concentration induced by [Mg^2+]o removal was measured in real time for a rat hippocampal slice by using the enzyme-modified-MEA multi-array sensor. Glutamate oxidase was used to detect glutamate released from the cells. Consequently, the hydrogen peroxide generated was finally detected as an electrical current through horseradish peroxidase and osmium polymer (Os) mediator. This enzyme reaction results in highly selective and sensitive glutamate measurement. We measured the spatial distribution and the release of glutamate at various regions of the hippocampus in real time using this multi-array sensor.

Effect of low [Mg^2+]o on [Ca^2+]i
The primary dissociated neurons from the rat cortex were grown on glass dishes with a serum-free culture medium. The [Ca^2+]i responses were measured using 10 μg/mL fluo4-AM (Invitrogen, λex = 494 nm, λem= 516 nm) after one hour of staining, and the [Ca^2+]i change was measured as a fluorescence intensity change by using a confocal laser microscope (MRC1024MP, [Bio-Rad laboratories, Inc., Tokyo, Japan] equipped with a Zeiss Axiovert 135 [Carl Zeiss Inc., Jena, Germany], Kr/Ar laser). The stained cells were washed with HEPES buffer (Mg 2.0 mM) before use. Low Mg^2+ HEPES
buffer (Mg 1.9-0 mM) was used to measure the effect of a low Mg\(^{2+}\) concentration.

In this study, we investigated the [Mg\(^{2+}\)]\(_o\) response based on a [Ca\(^{2+}\)]\(_i\) oscillation measurement. The measurement was carried out on different culture days in vitro (DIV) to provide an understanding of the developmental change. The oscillation frequency is defined as the number of oscillations in 100 seconds, and a large numerical value indicates that the oscillation number is large.

Results and discussions

Change in membrane potential caused by low [Mg\(^{2+}\)]\(_o\)

The change in the electrical activity of rat cortical neurons was measured by removing Mg\(^{2+}\) from an external solution using the MEA (figure 1A). After the Mg\(^{2+}\) had been removed, the spontaneous electrical activity increased (figure 1B) and the evoked electrical activity indicated highly condensed activity (figure 1C).

Figure 2 shows the membrane potential change caused by a low Mg\(^{2+}\) concentration obtained with flow cytometry. Figure 2A shows the fluorescent distribution of FL1 (at 525 nm) and FL3 (at 620 nm) for rat cortical neurons. Region [a] (figure 2A) shows cells stained with 2.5 μM DiBAC\(_4\)(3), and region [b] (figure 2A) shows cells stained with 5 μM PI for dead cell screening. Figure 2B shows the change in the fluorescence distribution induced by different Mg\(^{2+}\) concentrations. [a] (figure 2B) shows density plots at 2.0 mM [Mg\(^{2+}\)]\(_o\) and [b] (figure 2B) shows density plots at 0.5 mM [Mg\(^{2+}\)]\(_o\). Figure 2C shows a histogram of total cell number vs fluorescence intensity for [a] (figure 2A). A typical fluorescence change for the membrane potential in cortical neurons is shown. The red line is the fluorescence intensity for 2.0 mM [Mg\(^{2+}\)]\(_o\) and the blue line is the fluorescence intensity for 0.5 mM [Mg\(^{2+}\)]\(_o\). The peak shifted to the right indicates an increase in fluorescence intensity caused by membrane depolarization. Figure 2D indicates the correlation between [Mg\(^{2+}\)]\(_o\) and membrane potential. [a] and [b] (figure 2D) show the changes in cortical and hippocampal neurons, respectively. The membrane potential depolarized when [Mg\(^{2+}\)]\(_o\) was decreased in both the cortical and hippocampal neurons. But the tendency of the membrane potential increase in the cortical neurons was different from that in the hippocampal neurons. The tendency for the hippocampal neurons was slightly more gradual than that for the cortical neurons.

Figure 1. [Mg\(^{2+}\)]\(_o\) removal induced electrical activity change. A) shows a 64-channel MEA. Each electrode is 10-30 μm square, and the electroconductive ITO film was patterned with a conventional photolithographic technique. Each electrode was connected to an amplifier. The neuronal electrical activity was measured at a rate of 3 kHz (or higher). B) shows spontaneous activity and (C) shows activity evoked by [Mg\(^{2+}\)]\(_o\) removal.
Spatial and temporal distribution measurement of glutamate release by \([\text{Mg}^{2+}]_o\) removal

**Figure 3** shows real-time glutamate release measurements at various regions of the hippocampus obtained using the multi-array sensor. As the \(\text{Mg}^{2+}\) blockade of the NMDA receptor is removed by \([\text{Mg}^{2+}]_o\) removal, the membrane potential is depolarized. This induces the glutamate release. The release was observed on dentate gyrus (DG), CA1 and CA3, but the response was different in each region. The effects of 500 \(\mu\)M MK801 (TOCRIS, Bristol UK), NMDA receptor antagonist, and 500 \(\mu\)M CNQX (TOCRIS), non-NMDA receptor antagonist on glutamate release were measured. The results indicate that MK801 inhibited the response at CA1 but did not affect DG or CA3. However, CNQX inhibited the response at DG and CA3 but did not affect CA1. This suggested that the receptor distribution was different in each region of the hippocampus. Neural activity in various regions of the hippocampus might be controlled/modulated differently by \(\text{Mg}^{2+}\).

**Effect of low \([\text{Mg}^{2+}]_o\) on \([\text{Ca}^{2+}]_i\)**

Although hardly any neural activities are detected immediately after the culture has started, the neu-
rons start to generate spontaneous activity after 3-4 days of culture in vitro (DIV) according to the MEA measurement [9, 17]. Then, the neural activities of the individual channels of the MEA begin to synchronize, depending on the increment of the synapse numbers of the input and output of the cell. As we reported previously, periodic \([\text{Ca}^{2+}]_i\) changes were observed at this culture stage [5]. With this as a basis, we measured the \([\text{Ca}^{2+}]_i\) responses from 5 DIV.

**Figure 4** shows \([\text{Mg}^{2+}]_o\) vs \([\text{Ca}^{2+}]_i\) responses depending on the culture day. The \([\text{Ca}^{2+}]_i\) responses were measured using a confocal laser microscope with a fluorescent probe. We investigated the sequential change in the \([\text{Ca}^{2+}]_i\) responses induced by low \([\text{Mg}^{2+}]_o\) (Mg 1.9-0 mM). A contour line graph showing culture period vs \([\text{Mg}^{2+}]_o\) and the ratio of the number of responsive cells to the total number of cells is shown in **figure 4A**. The \([\text{Ca}^{2+}]_i\) responses, which depended on \([\text{Mg}^{2+}]_o\), were detected from 5 DIV in both cortical and hippocampal neurons, but the responses for the two regions were different. The \([\text{Ca}^{2+}]_i\) oscillations exhibit a peak at 12 DIV in higher \([\text{Mg}^{2+}]_o\) of 1.2 mM in cortical neurons. But no response was detected when the culture period was long. The oscillations were observed only in low \([\text{Mg}^{2+}]_o\) of almost 0 mM. On the other hand, the \([\text{Ca}^{2+}]_i\) oscillations in the hippocampus were the most intense for the 14 DIV

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**Figure 3.** Change in glutamate concentration caused by \([\text{Mg}^{2+}]_o\) removal in hippocampal slice. The glutamate concentration change was measured by using an enzyme-modified-MEA multi-array sensor. The change in glutamate release was investigated by the addition of glutamate receptor antagonist (MK801 or CNQX). A) shows the response in DG, (B) shows the response in CA1 and (C) shows the response in CA3. The black, red, and blue lines show the responses before stimulation, after MK801 addition and after CNQX addition, respectively.
when $[\text{Mg}^{2+}]_o$ was 1.8 mM. The oscillations were observed in comparatively high $[\text{Mg}^{2+}]_o$ even if the culture period was long. Figure 4B shows the oscillation frequency (times/100 seconds) with a bar graph. The difference between cortical neurons and hippocampal neurons was also noticeable in terms of oscillation frequency. The frequency was high when $[\text{Mg}^{2+}]_o$ was low (0 mM), and it did not depend on the culture period in the cortical neurons. The frequency was high even if the culture period was long and $[\text{Mg}^{2+}]_o$ was high, and the frequency was high for 12-16 DIV particularly in the hippocampal neurons. Overall, the oscillation frequency of the cortical neurons was low compared with that of the hippocampal neurons. As the oscillation frequency indicates the response intensity, it is suggested that hippocampal neurons are much more sensitive to $[\text{Mg}^{2+}]_o$ than cortical neurons.

The $[\text{Ca}^{2+}]_i$ responses induced by $[\text{Mg}^{2+}]_o$ removal were measured when the glutamate receptor was inhibited by the addition of 500 $\mu$M MK801 (TOCRIS) and 500 $\mu$M CNQX (TOCRIS) for cortical and hippocampal neurons (figure 5). The $[\text{Ca}^{2+}]_i$ oscillation was induced because the Mg$^{2+}$ blockade of the NMDA receptor is removed. The glutamate release was inhibited by MK801 or CNQX (figure 3).
The \([\text{Ca}^{2+}]_i\) responses induced by \([\text{Mg}^{2+}]_o\) removal were inhibited with the addition of MK801, but no noticeable inhibition was observed in CNQX in cortical neurons. On the other hand, the \([\text{Ca}^{2+}]_i\) responses induced by \([\text{Mg}^{2+}]_o\) removal were inhibited by MK801, and greatly inhibited by CNQX in hippocampal neurons. These different responses to \([\text{Mg}^{2+}]_o\) in cortical and hippocampal neurons indicate a good correlation with the receptor distribution. This suggests that the \([\text{Mg}^{2+}]_o\) response might be caused by the glutamate receptor response. However, \(\text{Ca}^{2+}\) channels, such as a voltage gated \(\text{Ca}^{2+}\) channel (VGCC), might be involved in these different responses. Further investigation of the difference in \([\text{Mg}^{2+}]_o\) response between cortical and hippocampal neurons is required.

**Conclusion**

We investigated the effect of low \([\text{Mg}^{2+}]_o\) on neural activity using MEA-based neural activity measurement, real-time glutamate measurement using a multi-array sensor and optical measurement with a fluorescence probe, using a confocal laser microscope and a flow cytometer. The spontaneous and evoked electrical activity was increased and the glutamate release was also increased by \([\text{Mg}^{2+}]_o\) removal. The glutamate release was different in different regions of the hippocampus. The membrane potential was depolarized by \([\text{Mg}^{2+}]_o\) removal, and the change in the \([\text{Mg}^{2+}]_o\) dependent membrane potential differed for cortical and hippocampal neurons. The effect of low \([\text{Mg}^{2+}]_o\) on \([\text{Ca}^{2+}]_i\) also differed for cortical and hippocampal neurons. The \([\text{Ca}^{2+}]_i\) responses were noticeably different with respect to culture period and \(\text{Mg}^{2+}\) concentration. In cortical neurons, the \([\text{Ca}^{2+}]_i\) oscillations were the most noticeable at 12 DIV. The responses were measured throughout the entire culture period at low \([\text{Mg}^{2+}]_o\), namely 0 mM, in cortical neurons. On the other hand, in hippocampal neurons, the \([\text{Ca}^{2+}]_i\) oscillations were the most intense at 14 DIV. The responses were observed at high \([\text{Mg}^{2+}]_o\), namely 1.8 mM, and for a long culture period in hippocampal neurons. The results suggested that hippocam-
pal neurons are more sensitive to [Mg<sup>2+</sup>]<sub>o</sub> than cortical neurons. The glutamate receptor distributions in the cortex and hippocampus may be different, because the [Ca<sup>2+</sup>]<sub>i</sub> responses caused by removing [Mg<sup>2+</sup>]<sub>o</sub> with the addition of MK801 or CNQX were different in cortical and hippocampal neurons. Further investigation is required for an understanding of the mechanism of the effect of [Mg<sup>2+</sup>]<sub>o</sub> on neural function.

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References


