Abstract—Investigation of hypersensitivity caused by peripheral sensitization progression is important for developing novel pain treatments. Existing methods cannot record plastic changes in neuronal activity because they occur over a few days. We aimed to establish an efficient method to evaluate neuronal activity alterations caused by peripheral sensitization on high-density microelectrode arrays (HD-MEAs) which can record neuronal activity for a long time. Rat dorsal root ganglion (DRG) neurons were dissected from rat embryos and cultured on HD-MEAs. DRG neurons were labeled with NeuO, live staining dye. Neurons were detected with the fluorescence signal and electrodes were selected with the fluorescence images. The number of DRG neurons, whose activity were recorded, detected based on fluorescence observation was five times greater than that based on neuronal activity. Analysis of changes in neuronal activity observed in pharmacological stimulation experiments suggested that substance P induced peripheral sensitization and enhanced capsaicin sensitivity. In addition, results of immunofluorescence staining suggested that peripheral sensitization occurred mostly in neurons that co-expressed transient receptor potential vanilloid 1 (TRPV1) and neurokinin 1 receptor (NK1R). In conclusion, we established an efficient method for assessing the effects of peripheral sensitization on DRG neurons cultured on HD-MEAs.

I. INTRODUCTION

Enhanced pain sensation caused by substances secreted from damaged tissues increases the likelihood of survival by prompting individuals to escape from the stimuli[2]. However, excessive secretion can cause peripheral sensitization, a phenomenon where the sensitivity of nociceptors becomes higher than normal[2]. This sensitized state leads to allodynia, the sensation of pain from non-nociceptive stimuli, and hyperalgesia, an abnormally strong perception of pain. Furthermore, if sensitivity enhancement persists chronically, amplified neuronal activity from peripheral neurons can change the properties of central nervous system and cause the development of chronic pain, reducing quality of life[3].

Researchers have revealed mechanisms of peripheral sensitization[4]. In culture systems, substance P, a neuropeptide, is used to enhance capsaicin sensitivity as a model of peripheral sensitization[5]. Substance P binds to the neurokinin 1 receptor (NK1R) and activates protein kinase C pathways, which phosphorylate transient receptor potential vanilloid 1 (TRPV1), the capsaicin receptor, resulting in enhancement of capsaicin sensitivity[6]. Although acute hypersensitivity, one of the symptoms of peripheral sensitization, has been investigated, the mechanisms for developing chronic hypersensitivity after the transient enhancement of sensitivity are less clear[7]. The patch-clamp technique[5], a traditional method of recording electrical activity, is invasive, making it difficult to assess time-dependent elevation in neuronal activity developing over a few days. Evaluation of the alterations contributes to clarifying the mechanisms of the chronicity of peripheral sensitization in neurons and to developing therapeutic methods.

Recently, high-density microelectrode arrays (HD-MEAs) have been used to record neuronal activity[8][9]. Although the signal-to-noise ratio is small, HD-MEAs can noninvasively record electrical activity over time[9]. Additionally, the high electrode density enables recording with high spatial resolution[10]. Thus, HD-MEAs are useful for detailed research on the progression of peripheral sensitization.

When conducting activity recording with HD-MEAs, selecting the appropriate electrodes under the neurons for recording is crucial. Typically, recording electrodes are selected by scanning activity across all electrodes, which necessitates consistent neuronal activity for over 30 minutes[8]. However, sensory neurons exhibit minimal spontaneous activity and short-lasting pharmacological responses[3][11], presenting a challenge when it comes to selecting electrodes for recording on HD-MEAs.

Here, we aimed to establish an efficient method to evaluate the effects of peripheral sensitization on sensory neurons cultured on HD-MEAs. We cultured rat dorsal root ganglion (DRG) neurons on HD-MEAs and pharmacologically induced acute hypersensitivity to capsaicin. The recording electrodes were selected by detection of neurons using a live cell staining method. The reproduction of peripheral sensitization was examined by the results of pharmacological stimulation experiments and immunofluorescent staining.

II. METHODS

A. Cell culture

Fig. 1 shows primary sensory neurons cultured on HD-MEAs (Maxwell Biosystems AG, Zurich, Switzerland). HD-MEAs were pretreated and coated according to Maxwell...
B. Pharmacological Stimulation Experiments

The sensitization state was evaluated with the number of sensitivity of sensory neurons was reproduced using substance sensitization, we compared responses to capsaicin with and without aprepitant, an antagonist of NK1R.

NGF/ml nerve growth factor (FUJIFILM Wako Pure Chemical Corp.), 100 μg/ml streptomycin (Thermo Fisher Scientific), and 50 ng/ml nerve growth factor (FUJIFILM Wako Pure Chemical Corp.). Samples were added to the electrodes and left at 4°C overnight. After washing with sterilized water, 0.1% polyethyleneimine in 70% ethanol for 30 minutes after hydrophilic treatment. Biosystems protocol. Briefly, chips were sterilized by soaking in 70% ethanol for 30 minutes after hydrophilic treatment. After washing with sterilized water, 0.1% polyethyleneimine (Sigma-Aldrich Co., Ltd., St. Louis, MO, United States) was added to the electrodes and left at 4°C overnight. After washing four times with sterilized water, 20 μg/ml laminin (Thermo Fisher Scientific, Waltham, MA, United States) was added and incubated at 37°C for at least one hour.

DRG neurons were dissected from the day-15 Wistar rat embryos (Charles River Laboratory Japan Inc., Kanagawa, Japan). The ganglia were treated with 0.25% Trypsin solution diluted in Hanks’ balanced salts solution (FUJIFILM Wako Pure Chemical Corp., Osaka, Japan) at 37°C for 17 minutes. DRG neurons were isolated by pipetting and plated on HD-MEAs at a concentration of 3000 cells/mm². All procedures were performed in accordance with the University of Tokyo Animal Experiment Manual with the approval of the University of Tokyo Animal Experiment Committee (KA19-14). Culture medium was Neurobasal Plus medium (Thermo Fisher Scientific) containing the following supplements: B-27 Plus supplement (Thermo Fisher Scientific), 0.5 mM GlutaMAX (Thermo Fisher Scientific), 100 units/ml penicillin - 100 μg/ml streptomycin (Thermo Fisher Scientific) and 50 ng/ml nerve growth factor (FUJIFILM Wako Pure Chemical Corp.). Samples were kept in a humidified 5% CO₂ incubator at 37°C except during operation, and half of the medium was replaced every 3 days.

B. Pharmacological Stimulation Experiments

The peripheral sensitization that enhances capsaicin sensitivity of sensory neurons was reproduced using substance P. The sensitization state was evaluated with the number of spikes. To examine the contribution of NK1R on the sensitization, we compared responses to capsaicin with and without aprepitant, an antagonist of NK1R[12].

In pharmacological stimulation experiments, the initial neuronal activity and pharmacological responses were recorded with 5 stimulation conditions: “Control” with culture medium only, “C” with capsaicin only, “C S” with capsaicin and substance P, “C A” with capsaicin and aprepitant, and “C S A” with capsaicin, substance P and aprepitant. We set the stimulation time for each reagent from preliminary experiments. As shown in Fig. 2, capsaicin was added in the second and fourth recordings except for in “Control.” In “C S” and “C S A,” substance P was added in the third recording and remained until the end. Similarly, in “C A” and “C S A,” aprepitant was added in the second recording and remained until the end. Samples were washed out between each recording. We used 200 nM capsaicin (FUJIFILM Wako Pure Chemical Corp.);[13], 2 μM substance P (FUJIFILM Wako Pure Chemical Corp.;[13] and 2 μM aprepitant (Sigma-Aldrich Co.).

C. Activity Recording

The MaxOne recording system (Maxwell Biosystems) was used to record the activity of sensory neurons. 26,400 electrodes exist on the HD-MEA, and the circuit can be switched to enable simultaneous recordings of extracellular potentials from 1,024 electrodes[10]. The recording electrodes were selected with the fluorescence images. Normally, “activity scan” is used to select the recording electrodes based on activity recorded with all electrodes in advance[8]. However, sensory neurons have little spontaneous activity and it’s difficult to select the electrodes with “activity scan.” Here, we used 0.25 μM NeuO (Stemcell Technologies Inc., Vancouver, British Columbia, Canada) and incubated for one hour to label neurons for selecting the recording electrodes. The NeuO is a membrane permeable fluorescent probe that can label live neurons[14]. To verify the effectivity of our selection method, we compared the number of detected neurons that responded to pharmacological stimulations. We detected 60 neurons by “activity scan” or fluorescence images and select 16 electrodes under each neuron. Stimulation condition was “C S,” and 4 and 6 samples were used for “activity scan” and fluorescence observation, respectively. The samples on 21 - 28 days in culture were used.

After selecting recording electrodes based on fluorescence images, we recorded responses of neurons to pharmacological stimulations in the order of “Control,” “C,” “C S,” “C A” and “C S A” with an interval of at least one hour between each condition. We used 6 samples on 21 - 28 days in culture.

D. Immunocytochemistry

Neurons cultured on HD-MEAs were fixed with 4% paraformaldehyde (PFA, FUJIFILM Wako Pure Chemical Corp.) for 30 minutes at room temperature. After washing the samples with phosphate buffered saline (PBS, Thermo Fisher Scientific), 4% Block ace (Sumitomo Dainippon Pharma, Osaka, Japan) and 0.25% Triton X-100 (Merck, Darmstadt, Germany) diluted in PBS were added to the samples and left for 2 hours at room temperature. 0.4% Block ace and 0.25% Triton X-100 were diluted in PBS, and the primary antibody solution prepared with the dilution was added to the samples and left overnight at 4°C. After washing with PBS for 5 minutes three times, the secondary antibody solution was added to the samples and left at room temperature for 4 hours. After washing three times with PBS, the samples were observed. The following primary and secondary antibodies were used: Anti-TRPV1 antibody (mouse, 1:500; Abcam, Cambridge, UK), Anti-substance P receptor antibody (rabbit, 1:500; Sigma-Aldrich Co.), Anti-NeuN antibody (chicken, 1:2,000; Sigma-Aldrich Co.), Alexa Fluor 488 anti-mouse IgG antibody (goat, 1:500; Thermo Fisher Scientific), Alexa Fluor 568 anti-rabbit IgG...
647 anti-rabbit IgG antibody (goat, 1:500; Thermo Fisher Scientific) and Alexa Fluor 555 anti-chicken IgY antibody (goat, 1:500; Thermo Fisher Scientific).

E. Data Analysis and Evaluation

For analysis, 60 seconds of data before and after the addition of ligands or culture medium were used. The data of the initial condition were used for 120 seconds from 1 minute after starting the recording. First, the data from all electrodes were filtered using a bandpass filter from 100 to 3000 Hz. The negative peaks with absolute values five times greater than the standard deviation of the signal at each electrode were selected as spikes. If multiple spikes were detected within 50 ms, only the first spike was used in the analysis. For each neuron, out of the 16 electrodes, only data from the electrode with the highest number of spikes were used for analysis.

In the comparison of electrode selection methods, the ratio of pharmacologically responsive neurons was evaluated. The ratio of capsaicin-sensitive neurons, increased in the number of spikes with the addition of capsaicin, was calculated. Similarly, the ratio of capsaicin- and substance P-sensitive neurons was calculated. Both ratios were compared between the “activity scan” and the fluorescence observation.

To examine the reproduction of peripheral sensitization induced by pharmacological stimuli, the enhancement of the capsaicin sensitivity with the addition of other reagents was evaluated. In each recorded neuron, the difference in the number of spikes between the second and fourth recordings were calculated. The changes in the number of spikes were used to compare the hypersensitivity to capsaicin between the following conditions: “C” and “C S,” “C” and “C A,” and “C S” and “C S A.”

The consistency of the results of immunofluorescence staining, a live cell labeling technique and pharmacological responses was evaluated. The overlapping ratio of neurons labeled by live cell staining and neuronal nuclei detected by immunofluorescence staining was examined. The proportion of the expression of TRPV1 in capsaicin-sensitive neurons and that of NK1R in substance P-sensitive neurons was evaluated. Finally, the type of receptors expressed on neurons that increased capsaicin sensitivity in the pharmacological stimulation experiments was investigated.

III. RESULTS

A. Comparison of “activity scan” and Live Cell Staining

The mean ratio of capsaicin-sensitive neurons was 8.9% with the “activity scan” and 47.6% with the live cell staining method, which was significantly higher than that using “activity scan”(p = 4.0 × 10⁻³, Welch’s t-test). Similarly, the mean ratio of capsaicin- and substance P-sensitive neurons was 6.0% with the “activity scan” and 31.9% with the live cell staining method, which was significantly higher than that using “activity scan”(p = 0.030, Welch's t-test). These results indicate that the live cell staining method is effective in selecting the recording electrodes.

B. Reproduction of Peripheral Sensitization by Substance P

In “Control,” addition of culture medium did not clearly increase the activity. Fig. 3A shows the spike frequencies of the second and fourth recordings in “C S.” From the results, the addition of substance P increased the capsaicin response by 28%. Similarly, Fig. 3B shows the results in “C S A.” From the results, the addition of aprepitant reduced the capsaicin response by 18%.

The median increase in the number of spikes at the fourth recording compared to those at the second were 1.0 (from -20 to 22.5) (n=99) in “C,” 16.5 (from 1 to 42.8) (n=126) in “C S,” 5.0 (from -10.8 to 25.3) (n=70) in “C A,” and -11.0 (from -23 to 8) (n=59) in “C S A.” The change in “C S” was significantly larger than that in “C” (p = 2.7 × 10⁻⁴, Mann-Whitney's U test) and “C S A” (p = 1.7 × 10⁻⁷, Mann-Whitney's U test). There was no significant difference between the changes in “C” and “C A” (p = 0.34, Mann-Whitney's U test).

In summary, capsaicin sensitivity was enhanced by substance P, but was not by aprepitant. Also, the enhancement of capsaicin sensitivity was inhibited by aprepitant.

C. Characterization of Neurons by Immunostaining

The ratios of TRPV1+ and/or NK1R- neurons detected by the live cell staining method was examined. Fig. 5A shows the superimposition fluorescent image of live neurons (red) and the immunofluorescence staining image of neuronal nuclei (green). The merged image indicates overlapping positions of live neurons and neuronal nuclei. Fig. 5B shows TRPV1+ neurons (red) and NK1R+ neurons (green). The merged image indicates that the type of receptor expressed in each neuron can be detected. These results showed that it’s possible to detect the neurons using the live cell imaging method and to evaluate

![Image](https://example.com/image333x82)

**Figure 3.** Comparing neuronal activity in 2nd (black) and 4th (red) recordings. (A) Substance P increased the capsaicin response in “C S.” (B) Aprepitant reduced the capsaicin response in “C S A.”

![Image](https://example.com/image350x269)

**Figure 4.** Results of live cell images and immunofluorescence images. (A) Red and green colors show live neurons and neuronal nuclei. (B) Red and green colors show TRPV1 and NK1R expressed in neurons.
the relationship between pharmacological responses and neuron types.

The consistency of the expression of receptors and responsiveness of capsaicin and substance P was examined. The proportion of TRPV1+ neurons that responded to capsaicin was significantly higher than that of TRPV1- neurons ("C S", p = 1.1 × 10^{-2}; "C S A", p = 2.0 × 10^{-2}; "C A", p = 3.0 × 10^{-2}; "C S A", p = 1.9 × 10^{-2}: Welch's t-test). Similarly, in "C S," the proportion of NK1R+ neurons that responded to substance P was significantly higher than that of NK1R- neurons (p = 2.9 × 10^{-2}, Welch's t-test). On the other hand, there was no significant difference in the proportion of neurons that responded to substance P between the presence and absence of NK1R expression in "C S A" (p = 0.37, Welch's t-test). These results indicate that pharmacological responses are consistent with the type of expressed receptors.

The proportion of neurons with increased capsaicin response in the fourth recording compared to the second was calculated by each neuron type. The proportion of TRPV1+ NK1R- neurons was approximately 4% regardless of the stimulus conditions, and there was no significant difference between the results in "C S" and in "C S A" (p > 0.50, Welch's t-test with Bonferroni correction). In contrast, the proportions of TRPV1+ NK1R+ neurons were 27.6% in "C S" and 4.02% in "C S A," and there was a significant difference (p = 2.4 × 10^{-2}, Welch's t-test with Bonferroni correction). These results indicate that the NK1R was functionally expressed and that substance P and aprepitant bound with the receptor.

IV. DISCUSSION

To reproduce peripheral sensitization and evaluate changes in neuronal activity in a culture system on HD-MEAs, it is necessary to satisfy the following two requirements: 1) detection of neurons to select recording electrodes and 2) reproduction of peripheral sensitization which is consistent with expressed receptors and neuronal responses.

Firstly, we selected recording electrodes using a live cell staining method to detect sensory neurons. This method resulted in recording activity from five times more neurons than the “activity scan.” The immunofluorescent staining results showed that cells detected with live cell staining were neurons expressing TRPV1 and/or NK1R, suggesting that sensory neuron activity can be recorded using the live cell staining method.

Secondly, we reproduced peripheral sensitization using substance P to enhance capsaicin sensitivity. The capsaicin response with substance P was significantly increased compared to that without substance P or with aprepitant. Immunofluorescent staining results showed that the hypersensitivity to capsaicin was caused by neurons that co-expressed TRPV1 and NK1R, indicating that we can induce peripheral sensitization and evaluate its effects on HD-MEAs.

V. CONCLUSION

We aimed to develop a method for assessing the impact of peripheral sensitization on sensory neuron activity using HD-MEAs. We detected neurons using a live cell staining technique with NeuO, selected recording electrodes, and evaluated changes in activity produced by pharmacologically induced hypersensitivity. After recording, we identified the receptors expressed in each neuron. Our analysis of neural activity changes suggested that substance P induced peripheral sensitization. Furthermore, results of immunofluorescence staining were consistent with the relationship between neuronal responses to pharmacological stimuli and neuron types. We conclude that we have established a method for recording and evaluating the effects of peripheral sensitization on sensory neurons cultured on HD-MEAs.

REFERENCES