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Change in network dynamics over time by administering Notch response inhibitor DAPT to hippocampal culture

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***Abstract*— Although previous researches have investigated the relationship between learning and memory function in the hippocampus and continuously produced newborn neurons, the detailed role of newly generated neurons remains unclear. Here, we investigated the correlation between immature neurons and the electrical activity of the hippocampus at the network level *in vitro*. We showed that administrating the Notch response inhibitor DAPT to the hippocampal network enhances the neuronal differentiation of newborn cells and decreases the ratio of immature neurons in hippocampal culture. Unlike the hippocampal network without DAPT, the network with DAPT decreased the burst duration and the coefficient of variation of interburst intervals over culturing time and showed a higher synchronization level of the network over time. Moreover, the number of neurons playing a receiver or sender neuron was lower in the network with DAPT than without DAPT. Our results indicate that immature neurons may contribute to assigning neurons specific nodes as the receiver of the sender and to the diversity of the network activity while altering connections among neurons in the network.**

***Clinical Relevance*— Our research demonstrated the effect of DAPT on the ratio of immature neurons. Furthermore, our study showed the role of immature neurons in the hippocampus at the network level.**

I. Introduction

In the hippocampus of the adult brain, newborn neurons are produced continuously throughout life. Thus, immature neurons continue to exist in the hippocampal network. Previous *in vivo* researches reported relationships between learning and memory functions and newly generated neurons in the hippocampus. For example, several reports demonstrated that decreasing the number of newborn neurons attenuated learning and memory function [1][2][3]. However, the detailed role of newborn neurons remains unclear. Here, we investigated the correlation between hippocampal activity and the newborn neurons at the network level *in vitro*. First, we assessed the effect of DAPT, a γ-secretase inhibitor of Notch signaling[4], on proliferation and differentiation of neural stem cells in hippocampal culture. Next, we compared the spontaneous activity of the hippocampal network using a high-density microelectrode array (HD-MEA) with and without DAPT. We analyzed the change in electrical activity over culturing time dependent on the number of immature neurons.

II. Methods

A. Cell culture

All animal experiments were performed with the approval of the Animal Experiment Ethics Committee of the University of Tokyo and following the University of Tokyo Guidelines for the Care and Use of Laboratory Animals. Hippocampal cultures were prepared from Wistar rat brains at embryonic day 19. Hippocampal tissue was separated and dissected using HBSS. The isolated hippocampal cells were digested with a solution containing 0.5% trypsin (Life Technologies) for 15 min at 37 °C. The addition of FBS-containing medium stopped digestion. Cells were plated on MEA substrates treated with 0.1% polyethyleneimine (Wako) overnight, followed by 20 μg/mL of laminin (Life Technologies) for 1 hour. The initial cell densities were 1000 cell/mm^2 on glass-based dishes for immunocytochemical analysis and 5000 cell/mm^2 on MEA substrates for recording neural activity. Cells were cultured in primary culture medium containing Neurobasal Medium (Thermo Fisher Scientific), 2% B27 supplement (Thermo Fisher Scientific), 2 mM GlutaMAX (Thermo Fisher Scientific), and 1% penicillin/streptomycin (Thermo Fisher Scientific). Half of the culture medium was changed twice per week. Cultures were maintained in a CO_2 incubator under 5% CO_2 at 37 °C in a water-saturated atmosphere. One μM DAPT was added during 12-15 days *in vitro* (DIV) after seeding and then washed away.

B. Immunocytochemistry

The immunocytochemical analysis was performed as a previously described method [5]. Newborn cells were detected with 5- ethynyl-2'-deoxyuridine (EdU, Invitrogen), incorporated into DNA during the S phase of cell division. Briefly, 1 μM EdU was applied to cultures fixed at 19 DIV

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from 15–19 DIV and to cultures fixed at 29 DIV from 25-29 DIV. EdU-positive nuclei were detected with a click reaction. Cells were fixed in 4% paraformaldehyde (FUJIFILM Wako Pure Chemical), permeabilized with 0.25% Triton X-100 (Merck) in phosphate buffer, and blocked with 4% BlockAce (KAC Co., Ltd.) and 0.25% Triton X-100 in phosphate-buffered saline (PBS; Thermo Fisher Scientific). The cells were then incubated with the primary antibodies for 24 h. After 3 washes with PBS, the cells were incubated with the secondary antibodies for 20 h. The following primary and secondary antibodies were used: anti-DCX (rabbit, 1:1000; Abcam), anti-NeuN (chicken, 1:1000; Abcam), Alexa Fluor 488 anti-rabbit IgG (goat, 1:500; Thermo Fisher Scientific), and Alexa Fluor 647 anti-chicken IgG (goat, 1:500; Thermo Fisher Scientific). In addition, cell nuclei were identified by counter-staining with 300 nM 4'-6-diamidino-2-phenylindole (DAPI, Thermo Fisher Scientific). The number of cells at 19 DIV and 29 DIV were counted using ImageJ software. Immature neurons were detected as both positive cells for DCX, an immature neuronal marker, and negative cells for NeuN, a mature neuronal marker.

C. *Recording electrical activity*

Spontaneous activity of hippocampal cell culture was recorded at 19-21 and 28-30 DIV using HD-MEA (MaxWell Biosystems), where 26,400 electrodes were aligned with a pitch of 17.5 µm. First, spontaneous activity was recorded for 20 min, and approximately 1000 electrodes with firing rates > 0.1 spikes/s at 19-21 DIV were selected. Second, spontaneous activity of the selected electrodes was recorded for 20 min at both 19-21 and 28-30 DIV. Extracellularly recorded spikes were detected based on threshold calculation of the signal median

$$T = 5 \times median\left(\frac{|x|}{0.6745}\right) \quad (1)$$

where x is the 6-raw Butterworth bandpass-filtered (0.1–3 kHz) signal [6]. The synchronization level was assessed based on cross-correlations of the number of spikes between pairs of electrodes.

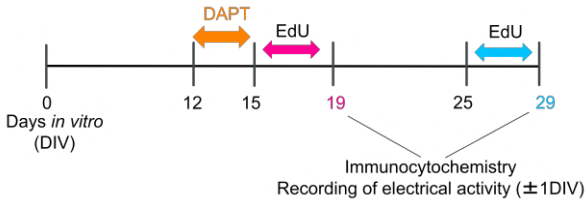

Figure 1. Scheme of experiment. For +DAPT group, 1 µM DAPT was administrated during 12-15 days *in vitro* (DIV). At 19-21 and 28-30 DIV, immunocytochemical analysis was performed to assess the effect of DAPT on the number of immature neurons and the electrical activity was recorded using high-density microelectrode array.

D. *Analysis of spontaneous activity*

The recorded spike train data were binned into 100 ms overlapping windows sliding at 10 ms for spontaneous activity. The number of active electrodes and the total number of spikes at these electrodes were calculated for each bin. The threshold for burst detection was defined as the 93rd percentile of the product for all bins. The start and end times of a network burst event were identified if the product was larger than the 0.1 thresholds. Consecutive bursts with inter-burst intervals of < 200 ms were combined.

Burst duration (BD), inter-burst intervals (IBI), and coefficient of variation (CV) of BD and IBI were calculated to assess changes in characteristics of network bursts. To evaluate connections among neurons, cross-correlations between pairs of electrodes were calculated as described in [7]. The synchronized level was evaluated as the peak time of cross-correlation fitted by the Gaussian function. The median value of the peak time of each sample was compared between 19-21 DIV and 28-30 DIV. In addition, to understand the structure of the network, the percentages of receiver and sender nodes were calculated. Briefly, each connection between pairs of channels was categorized into incoming or outgoing connections according to the sign of the peak time (positive or negative). Then, the channel with the ratio of

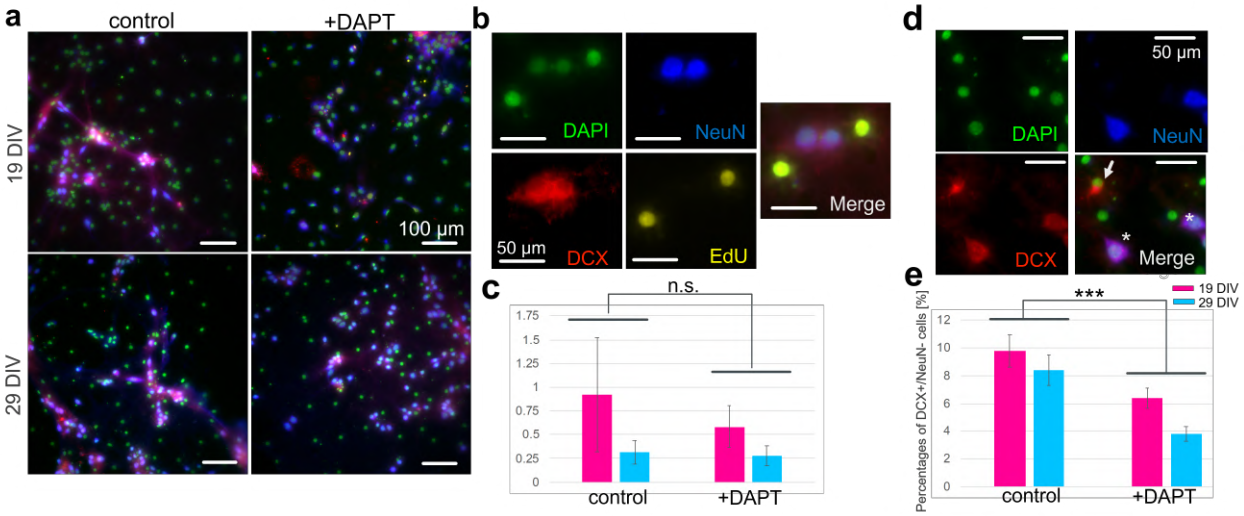

Figure 2. (a) Immature and mature neurons in a hippocampal culture with and without DAPT (b) Newborn cells which is positive for EdU. (c) The percentage of EdU+ cells. (mean±S.E.M; n.s., $p > 0.5$; ANOVA followed by Tukey's HSD test, N = 12) (d) Immature neurons which is positive for DCX. (arrow, DCX+/NeuN- cells;asterisk, DCX+/NeuN+ cells) (e) The percentage of DCX+/NeuN- cells. (mean±S.E.M; *, $p < 0.05$; ANOVA followed by Tukey's HSD test, N = 12)

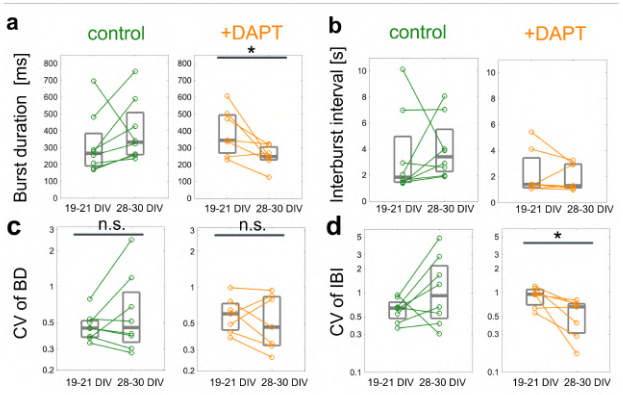

Figure 3. Comparison of characteristics of network bursts between 19-21 DIV and 28-29 DIV. (a) Burst duration (BD). (b) Inter-burst interval (IBI) (c) Coefficient of variation (CV) of BD. (d) CV of IBI. (*, $p < 0.05$; n.s., $p > 0.5$; Wilcoxon signed rank test; $N_{control} = 8$; $N_{+DAPT} = 7$; box, quantile)

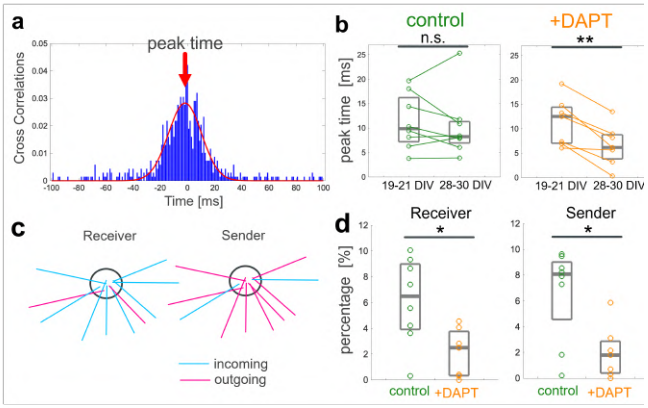

Figure 4. (a) Cross-correlation. Synchronized level was calculated as the peak time of cross-correlation fitted by gaussian function (red). (b) Difference in the synchronized level between 19-21 DIV and 28-30DIV. (*, $p < 0.05$; n.s., $p > 0.5$; Wilcoxon signed rank test; $N_{control} = 8$; $N_{+DAPT} = 7$; box, quantile) (c) Illustrations of receiver and sender neurons. (d) Difference in the percentages of receiver and sender neurons at 19-21 DIV. (*, $p < 0.05$; Rank sum test; $N_{control} = 8$; $N_{+DAPT} = 7$; box, quantile)

incoming (outgoing) connections exceeding 80 % of the total connections is identified as a receiver (sender) node.

E. Statistical analysis

All analyzed parameters were first tested for normality according to the Shapiro-Wilk test. If the test for normality indicated that the data were not normally distributed, non-parametric tests were used. The number of immature neurons is compared by analysis of variance (ANOVA) test followed by Turkey's test. The burst parameters and the synchronization level were compared using the Wilcoxon signed-rank test. Percentages of receiver and sender nodes were compared by the Wilcoxon rank-sum test. All analyses were performed using MATLAB software.

III. Results

A. The effect of DAPT on the newborn cells and the immature neurons

The percentage of newborn cells detected by EdU+ cells and immature neurons ¥ detected by DCX+/NeuN- cells were compared between the control and +DAPT group at 19 DIV and 29 DIV (Fig. 2(a), (c), (e)). The ratio of newborn cells was not significantly different among all groups. The Control group showed that the percentage of DCX+/NeuN- cells were showed 10.0 ± 2.1 % at 19 DIV and 10.7 ± 1.8 % at 29 DIV. On the other hand, the +DAPT group showed fewer percentages of DCX+/NeuN- cells, 6.3 ± 1.5 % at 19 DIV and 3.1 ± 1.1 % at 29 DIV ($p < 0.05$, ANOVA followed by Tukey's HSD test). These results suggest that hippocampal culture contains stable percentages of newborn cells and immature neurons over time, and the administration of DAPT during 12-15 DIV was effective in decreasing the number of immature neurons while did not affect the proliferation of newborn cells in a hippocampal culture *in vitro*.

B. Characteristics of electrical activity

As shown in Fig. 3, unlike the control group, the +DAPT group showed shorter BD and lower CV of IBI at 28-30 DIV than at 19-21 DIV ($p < 0.05$, Wilcoxon signed-rank test). As for CV of BD, both group no significant difference between 19-21 DIV and 28-30 DIV ($p > 0.5$, Wilcoxon signed-rank test).

Synchronization level was evaluated as the peak time of cross-correlation fitted by Gaussian function (Fig. 4(a)). The closer to zero the peak time is, the higher the synchronization level is. As shown in Fig.4(b), the synchronization level across the whole network was not significantly different between 19-21 DIV and 28-30 DIV in the control group ($p > 0.5$, Wilcoxon signed-rank test). On the other hand, the +DAPT group showed a higher synchronicity level over culturing time ($p < 0.05$, Wilcoxon signed-rank test).

A receiver or sender nodes were identified as channels with more than 80 % of incoming or outgoing connections (Fig. 4(c)). At 19-21 DIV, percentages of both receiver and sender nodes were lower in the +DAPT group than in the control group (Fig. 4(d), $p < 0.05$, Wilcoxon rank-sum test).

IV. Discussion

A. The effect of DAPT on the newborn neurons and the immature neurons

DAPT-mediated inhibition of the Notch response resulted in enhanced differentiation of neural stem cells [4][8]. Also, DAPT significantly depressed cell viability [9]. We demonstrated that administering 1 μM DAPT during 12-15 DIV into a hippocampal culture resulted in lower percentages of immature neurons while did not affect the proliferation of newborn cells. Our results indicate that the concentration of DAPT might be high enough for enhancing neuronal differentiation but too low to inhibit the proliferation of neural stem cells in hippocampal culture. Remaining newborn cells after being administered DAPT during 12-15 DIV would keep proliferating over time.

B. Characteristics of electrical activity

The network with DAPT showed shorter BD and lower . CV of IBI. Higher values of a CV of IBI reflect a lack of temporal structure to activity, and values closer to zero indicates temporal organization [10]. Considering the result of immunocytochemistry, the network with DAPT contains lower percentages of immature neurons. Altogether, the results

that hippocampal network with lower portions of immature neurons showed a decrease in the CV of IBI over culturing time, indicating that immature neurons contribute to the randomness of a temporal network burst pattern and resulted in a diversity of network dynamics. Immature neurons are more prone to fire because of their higher input resistance and lower threshold voltage [11][12]. Thus, immature neurons in hippocampal cultures might recruit more network bursts or lead to propagating bursts, resulting in longer BD and diversity of network bursts. Overall, our results that the network with a decrease in those immature neurons showed shorter BD and higher temporal organization, suggesting the role of immature neurons as a contributor to the diversity of the network activity.

An increase in the synchronized level over culturing time in the hippocampal network with DAPT indicates that constructing networks with lower percentages of immature neurons improved synchronicity over time. A network with only immature synapses fire less synchronously[13], and the propagation velocity of network bursts becomes faster as neurons mature[14]. Our results show that a decrease in the ratio of immature neurons with more delayed and less synchronous firing might enhance the synchronization level across the entire network. Furthermore, we found that lower percentages of both receiver and sender nodes in the +DAPT group compared to the control group. This result suggests that immature neurons may contribute to the diversity of the structure of the network by assigning several neurons the specific role, such as receiver and sender nodes. To study the detailed function of immature neurons in the synchronized activity, the relationship between the synchronicity and the distance of pairs of electrodes should be investigated further.

V. Conclusion

We investigated the relationship between immature neurons and electrical activity of the hippocampus at the network level. Administrating the Notch response inhibitor DAPT decreased the ratio of immature neurons while did not affect the percentages of newborn cells. Also, we demonstrated that the network with DAPT showed a change in characteristics of network bursts, improved the degree of synchronization level over culturing time, and reduced the specific roles in the network. These results indicate that differences in the several percentages of immature neurons in hippocampus culture alter the entire network activity over time and would reveal the contribution of immature neurons in the diversity of the network.

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