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Modulation of dynamics in a pre-existing hippocampal network by neural stem cells on a microelectrode array

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Abstract

Objective. Neural stem cells (NSCs) are continuously produced throughout life in the hippocampus, which is a vital structure for learning and memory. NSCs in the brain incorporate into the functional hippocampal circuits and contribute to processing information. However, little is known about the mechanisms of NSCs’ activity in a pre-existing neuronal network. Here, we investigate the role of NSCs in the neuronal activity of a pre-existing hippocampal in vitro network grown on microelectrode arrays. Approach. We assessed the change in internal dynamics of the network by additional NSCs based on spontaneous activity. We also evaluated the networks’ ability to discriminate between different input patterns by measuring evoked activity in response to external inputs. Main results. Analysis of spontaneous activity revealed that additional NSCs prolonged network bursts with longer intervals, generated a lower number of initiating patterns, and decreased synchronization among neurons. Moreover, the network with NSCs showed higher synchronicity in close connections among neurons responding to external inputs and a larger difference in spike counts and cross-correlations during evoked response between two different inputs. Taken together, our results suggested that NSCs alter the internal dynamics of the pre-existing hippocampal network and produce more specific responses to external inputs, thus enhancing the ability of the network to differentiate two different inputs. Significance. We demonstrated that NSCs improve the ability to distinguish external inputs by modulating the internal dynamics of a pre-existing network in a hippocampal culture. Our results provide novel insights into the relationship between NSCs and learning and memory.

1. Introduction

Adult neurogenesis, whereby neural stem cells (NSCs) give rise to neurons and glia continuously throughout life, occurs in specific regions of the mammalian brain [1]. One of these areas is the dentate gyrus of the hippocampus, which plays an essential role in learning and memory functions [2]. Although a number of in vivo studies have reported positive correlations between neurogenesis and learning and memory [3–6], several reports have identified no such correlation [7, 8], or even a negative one [9]. Thus, the relationship between neurogenesis and learning and memory remains controversial. This discrepancy may be due to several factors such as differences in tasks evaluating learning and memory functions, or environmental stressors known to affect neurogenesis in animals [10, 11]. Therefore, assessing clear correlations at the network level of the brain is required.

At the level of the neuronal network, NSCs are integrated into pre-existing neuronal circuits and contribute to the plasticity of the network [12]. Electrophysiological features differ between mature neurons and the early developmental stages of NSCs. For example, newborn neurons are more prone to fire and have high plasticity due to their higher input...
resistance and lower threshold voltage [1, 13]. Computational models studying the role of neurogenesis in hippocampal function have demonstrated that NSCs likely affect the function of the entire dentate gyrus and hippocampal circuits [14]. However, the distinct role of NSCs in pre-existing hippocampal circuits remains unclear.

The in vitro hippocampal network shows well-organized neuronal activity by culturing and generating events called bursts, where multiple neurons fire in a synchronized manner [15–17]. These spontaneous activities are similar to those observed in vivo [18] and are strongly related to the plasticity and dynamics of the network state. The biophysical mechanisms of network bursts are complex, ranging from ion channel dynamics in single cells via dendritically generated bursts to dynamics of whole networks [19]. Persistent sodium currents govern burst onset, and synaptic functionality and excitatory connection are fundamental for the network-wide bursting [20, 21]. Particularly, blocking AMPA, NMDA, or GABA receptors results in changes in the characteristics of network bursts, such as distribution of burst duration (BD) and interburst interval (IBI) [22]. Microelectrode arrays (MEAs) allow long-lasting and noninvasive extracellular recordings from a large number of neurons and are useful for studying long-term processes in neuronal networks [23, 24]. They also allow tracking of spatiotemporal neuronal activities in the network [25] and evaluation of information processing in the network by application of electrical stimulations [26]. In particular, the ability of a network to differentiate external inputs can be measured from evoked activity in response to stimuli on MEAs [27]. A previous study showed that a higher number of NSCs improved learning a specific spatial pattern using two different types of stimulations with MEAs [28]. However, how NSCs affect a pre-existing network, and in particular, how NSCs change the internal dynamics and the responses to novel different external input patterns of the pre-existing network still remain unclear.

To better understand the role of NSCs in pre-existing neuronal circuits, we investigated changes in the electrical activity of the hippocampal network by adding NSCs using MEAs. To reveal the dynamics of the network, we first analyzed spontaneous activities. Next, we measured evoked activities to evaluate the information processing of different external spatial input patterns. In particular, we quantified the ability to discriminate between two different external inputs by analyzing the spike count code (using mutual information between the number of spikes and input patterns) and spike timing code (using cross-correlation among neurons).

2. Materials and methods

2.1. Primary cell cultures

All animal experiments were performed with the approval of the Animal Experiment Ethics Committee of the University of Tokyo, in accordance with the University of Tokyo Guidelines for the Care and Use of Laboratory Animals. Hippocampal cultures (n = 5) 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 (Thermo Fisher Scientific, Waltham, MA, USA) for 15 min at 37 °C. Digestion was stopped by the addition of FBS-containing medium. Cells were plated on MEA substrates treated with 0.1% polyethylenimine (Sigma–Aldrich, St Louis, MI, USA) overnight followed by 20 µg ml⁻¹ of laminin (Thermo Fisher Scientific) for 1 h. The initial cell densities were 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 CO₂ incubator under 5% CO₂ at 37 °C in a water-saturated atmosphere.

2.2. Preparation of NSCs and additional seeding

NSCs were prepared using the neurosphere method [29–31]. Dissociated hippocampal cells were plated at a density of 1.0 × 10⁵ cells ml⁻¹ in 5 ml complete NSC medium containing 1 × N-2 supplement (Thermo Fisher Scientific), 1% v/v non-essential amino acid (Sigma–Aldrich), 0.1 mM 2-mercapto ethanol (Sigma–Aldrich), 5 µg ml⁻¹ Insulin (Sigma–Aldrich), 20 ng ml⁻¹ basic fibroblast growth factor (bFGF, ReproCELL, Kanagawa, Japan), 20 ng ml⁻¹ epidermal growth factor (FUJIFILM Wako Pure Chemical), and DMEM/F12 with GlutaMax (Thermo Fisher Scientific). 5 ml of medium was used in a 60 mm ultra-low attachment dish for suspension culture. Neurospheres formed after 4–7 d of incubation at 37 °C in a humidified incubator with 5% CO₂. When the neurospheres were 150–200 µm in diameter, they were mechanically dissociated and passaged into a new complete NSC medium. Here, we used NSCs from neurospheres passed more than three times to improve the proliferation potential of NSCs.

At 39 d after seeding a primary culture of hippocampal neurons, the medium was removed from the MEA and NSCs were plated at 1000 cells mm⁻² on MEA substrates. Once the NSCs adhered to the primary culture, neural differentiation medium
containing neurobasal medium, 20 ng ml\(^{-1}\) bFGF, 20 ng ml\(^{-1}\) BDNF (ReproTech), 2% B27 supplement, 2 mM GlutaMax, and 1% penicillin/streptomycin.

2.3. Immunocytochemistry
The immunocytochemical analysis was performed as previously described [32]. Cells were fixed in 4% paraformaldehyde (FUJIFILM Wako Pure Chemical, Osaka, Japan), permeabilized with 0.25% triton X-100 (Merck, Darmstadt, Germany) in phosphate-buffered saline (PBS; Thermo Fisher Scientific), and blocked with 4% BlockAce (KAC Co. Ltd, Kyoto, Japan) and 0.25% triton X-100 in PBS. The cells were then incubated with the primary antibodies overnight. The cells were then washed three times with PBS, before being incubated with the secondary antibodies overnight. The following primary and secondary antibodies were used: anti-PSA-NCAM (mouse, 1:400; Merck) anti-DCX (rabbit, 1:1000; Abcam, Cambridge, UK), anti-MAP2 (chicken, 1:1000; Abcam), Alexa Fluor 488 anti-mouse IgG (goat, 1:500; Thermo Fisher Scientific), Alexa Fluor 546 anti-rabbit IgG (goat, 1:500; Thermo Fisher Scientific), and Alexa Fluor 647 anti-chicken IgG (goat, 1:500; Thermo Fisher Scientific). Additionally, cell nuclei were identified by counterstaining with NucBlues Fixed Cell Ready Probes Reagent (a DAPI-based preparation, Thermo Fisher Scientific). To confirm that NSCs obtained from the neurosphere assay differentiated into neurons, NSCs were immunostained after being cultured with neurobasal medium with bFGF and BDNF for 10 d. Furthermore, to check that the additional NSCs differentiated into neurons to integrate into a pre-existing neural network, we compared the primary culture at 30 d after seeding (pre-existing) and 10 d after adding NSCs to primary culture (+NSCs).

2.4. MEA recording and stimulation
Electrical stimulations and recordings were performed at a pitch of 250 \(\mu\)m using a custom-made MEA system (NF Corporation, Japan [33]) and handmade MEA dishes with 8 \(\times\) 8 distributed micro-electrodes (50 \(\times\) 50 \(\mu\)m). Signals were amplified by a factor of 20, filtered with a 100–2000 Hz band-pass filter, and converted to 12-bit signals via an A/D converter (National Instruments). Signals were then amplified by a factor of 250 and sampled at a frequency of 25 kHz. The LabVIEW software (National Instruments) was used to record neuronal activity.

After being placed into the recording chamber, the cultures were allowed to equilibrate for 10 min at 37 °C under 5% CO\(_2\) before recording was initiated. Spontaneous activity was recorded for 20 min and stimulation-evoked activity was recorded for 15 min. We used two spatial pattern stimulations provided by eight electrodes arranged in the left and right columns. Stimulation consisted of a biphasic pulse (1 V, 200 \(\mu\)s), and two spatial patterns of stimulations were alternately applied 150 times at 3 s intervals.

Spontaneous and stimulation-evoked activities were recorded 30–32 d after seeding of the primary culture and at 10 d after additional seeding of NSCs (i.e. 49 d after seeding of the primary culture).

2.5. Spike detection
For spontaneous activity, the standard deviation (\(\sigma\)) of the amplitude of the extracellular potential over the recorded time of 20 min was calculated for each recording electrode. Spikes were detected as negative peaks crossing a \(-5\sigma\) threshold. For electrodes with \(\sigma > 10 \mu V\) and for firing rates < 0.5 spikes s\(^{-1}\) were excluded from the analysis.

For stimulation-evoked activity, we corrected signals for the baseline and noise. The recorded signals were filtered with a Mexican-hat type wavelet band-pass filter around 2 kHz [34] and the amplitude of the extracellular potential was calculated for a 20 s period of recording before the initiation of stimulations. Spikes were detected as negative peaks crossing a \(-5\sigma\) threshold. For electrodes with \(\sigma > 10 \mu V\) and for firing rates < 0.5 during 10 s before initiation, were excluded from further analyses.

2.6. Data analysis
To quantify the changes in the internal dynamics of the network, the regime of synchronized bursting events (network bursts), initiating patterns of network bursts, and cross-correlation among spikes were assessed for spontaneous activity. To evaluate the ability to differentiate between different two external inputs, we utilized two frameworks for analyzing information processing by the network, namely the spike count code and spike timing code for stimulation-evoked activity. The former proposes that the network can process information from external inputs through the number of spikes evoked by stimuli [35], and the latter proposes that information is processed through the timestamps of spike trains in response to input stimuli [36]. Therefore, we used spike counts and cross-correlation of the evoked response to two different spatial input stimuli to evaluate the ability to process input information.

2.7. Burst detection and analysis
For spontaneous activity, the recorded spike train data were binned into 100 ms overlapping windows sliding at 10 ms. The product of the number of active electrodes and the total number of spikes at these electrodes was 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 threshold. Consecutive bursts with IBIs of < 200 ms were combined.
To assess changes in network bursts, the following parameters were analyzed: BD, IBI, and burst percentage (BP) [37].

BP was used as an indicator of burstiness and calculated as follows:

$$\text{BP(\%)} = \frac{\text{total number of spikes within bursts}}{\text{total number of spikes}} \times 100.$$  

(1)

2.8. Motif analysis

The network bursts showed a similar spatiotemporal propagation profile (a repeating motif). These motifs reflect the stability of the global dynamics of the network and the functional correlation among neurons in cultures. We identified repeating motifs using a previously described method [38] which strictly depended on the delays between initiations of network bursts. First, the similarity of accuracies in the time delay between where \(p\)th and \(q\)th network bursts (\(S\)), was calculated as follows:

$$S(A_p, A_q) = \frac{1}{n(n-1)} \sum_{i \neq j} H(th - |A_p(i,j) - A_q(i,j)|)$$  

(2)

where \(A_p(i,j)\) represents the delay in milliseconds between the 1st spike of electrode \(i\) and the 1st spike of electrode \(j\) in the \(p\)th network bursts, \(H\) is the Heaviside step function, and \(th\) is the threshold parameter. Here, we calculated the similarity \(S\) for increasing \(th\) with a time step of 1 ms and chose the \(th\) where the mean similarity exceeded 0.3.

The number of activation motifs (\(M\)) was determined using a two-stage method. In the 1st stage, the rows and columns of a similarity matrix (\(S\)) were re-ordered by a standard agglomerative dendrogram method (MATLAB) so that network bursts sharing the most similar activation patterns were placed next to each other. In the 2nd stage, a single mean activation matrix (\(A_{set}\)) was calculated using equation (3):

$$A_{set}(i,j) = \frac{1}{N} \sum_{k=1}^{N_{tot}-N+1} A_k(i,j).$$  

(3)

The mean similarity within the set was calculated using equation (4)

$$S_{set} = \frac{1}{N} \sum_{k \in set} S(A_k, A_{set}).$$  

(4)

where \(N_{tot}\) is the total number of network bursts and \(N\) is the number of network bursts per motif.

The first selected motif was the set with the highest \(S_{set}\). Next, all optional sets that included network bursts satisfying either of the following two conditions were removed: (a) previously detected motifs, and (b) those showing similarity with previously detected motifs to a higher degree than the lowest similarity value of the network bursts in the motifs. The next identified motif would be the set with the highest \(S_{set}\) of the remaining sets. This procedure was repeated until no optional sets remained. Finally, we obtained \(M\) different motifs, each consisting of \(N\) network bursts. \(M\) depends on the number of network bursts \(N_{tot}\) and the set size \(N\). Here, we used \(N_{tot} = 313\), which was the minimum number of network bursts of all cultures. Setting \(N \sim \sqrt{N_{tot}}\) resulted in the best detection of motifs [38]. Thus, we used \(N = 17\) in this study.

2.8.1. Cross-correlation

Cross-correlation among pairs of electrodes was quantified based on the number of spikes as follows [39]

$$C_{xy}(\tau) = \frac{1}{\sqrt{N_xN_y}} \sum_{t=t_0}^{t_0+\Delta\tau} X(t) Y(t_0 - t).$$  

(5)

where \(N_x\) and \(N_y\) are the total number of spikes during the recorded time at \(X\) and \(Y\) electrodes, respectively, \(t_0\) is the time stamp in the spike train at the \(X\) electrode, and \(\Delta\tau\) is the bin size (2 ms). To get information on how an electrode \(x\) is correlated to all the other electrodes \(y\) (with \(y \neq x\)), the mean correlogram was defined and calculated as:

$$C_x(\tau) = \frac{1}{n-1} \sum_{y \neq x} C_{xy}(\tau)$$  

(6)

where \(n\) is the total number of electrodes available. The coincidence index (CI) was calculated to quantify the functional connectivity between pairs of electrodes. CI is the ratio of the integral of cross-correlation in a specified area around zero to the integral of the total area and was calculated as follows [40]

$$CI = \frac{\sum_{\tau=\Delta\tau}^T C_{xy}(\tau)}{\sum_{\tau=-T}^{-\Delta\tau} C_{xy}(\tau)}$$  

(7)

where \(k\) is the time number of bins around zero and was set at 2.

For spontaneous activity, spike trains during the recorded time of 1200 s were used to calculate cross-correlation, and \(T\) was set at 150 ms. For the evoked activity, spike trains during 5-200 ms after each stimulation were used, and \(T\) was set at 50 ms. To analyze the relationship between CI and the distance between pairs of electrodes, we divided the electrodes into six groups in distance windows of 400 \(\mu\)m ranging from 100 \(\mu\)m to 2500 \(\mu\)m.

2.9. Mutual information

Mutual information was estimated by the number of spikes evoked during 5–50 ms after each stimulation for each electrode as follows [41]:

$$I_{plug-in} = \sum_{\tau} P(s) \sum_{r} P(r|s) \log_2 \frac{P(r|s)}{P(r)}$$  

(8)
where $P(s)$ is the probability of the presentation of stimulus $s$, i.e. $P(s) = 0.5$; $P(r)$ is the probability of observing response $r$ across all trials to any stimulus, and $P(r|s)$ is the probability of observing response $r$ given a presentation of a stimulus $s$. In our dataset, the maximum number of responses to a stimulus ($\bar{R}$) was 35 for the single electrodes. Because the number of trials per pattern ($N_s$) was 150, the ratio of the trials to responses satisfied $N_s/\bar{R} > 4$. Therefore, we used the quadratic extrapolation correction for the bias as shown in equation (9) [42, 43]:

$$I_{\text{plug-in}}(S; R) = I_{\text{true}}(S; R) + \frac{a}{kN} + \frac{b}{(kN)^2}$$ (9)

where $a$ and $b$ are free parameters and estimated by re-computing the MI from fractions $1/k$ ($k = 2, 3, 4, 5$) of the trials as follows: the dataset was broken into random partitions, and MI quantities were computed for each sub-partition individually. The average of all values obtained from all partitions provided an estimate of the MI corresponding to the fraction of the trials. We repeated this procedure ten times for each $k$ and the average of $I_{\text{plug-in}}(S; R)$ was plotted against $k$. Following this, $a$ and $b$ were extrapolated as parameters of the parabolic function and the actual MI was taken to be the zero-crossing value.

2.10. 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 burst parameters were compared using the Student’s paired $t$-test. CI among all different distance groups and conditions for both spontaneous and evoked responses were compared using the Kruskal–Wallis
3. Results

The role of NSCs in pre-existing neuronal circuits remains elusive. Here, we investigated the changes in the electrical activity of the hippocampal network by adding NSCs using MEAs (figures 1(a) and (b)). By comparing electrical activities before (30–32 d after seeding primary culture) and after adding NSCs (10 d after additional seeding), we investigated the effect of NSCs on the pre-existing hippocampal network (figure 1(c)). Before measuring electrical activity, we confirmed that NSCs differentiated into neurons (supplementary figure 1 (available online at stacks.iop.org/JNE/18/0460e2/mmedia)), and that immature neurons, which were positive for PSA-NCAM, existed after additional seeding of NSCs (figure 1(d)). First, the internal dynamics of the network state were analyzed based on spontaneous activity. Next, the ability to process external input information and discriminate two different spatial input patterns was assessed from the evoked activity in response to electrical stimulation.

3.1. Spontaneous activity

Hippocampal cultures started to show synchronized activity over the entire network at 2 and 3 weeks following seeding (figure 2(a)). To characterize the regime of synchronized bursting events (network bursts), we quantified IBI, BD, the number of bursts, and BP. The hippocampal network generated a bimodal distribution of IBIs and BDs (figures 2(b) and (c)). However, the shape of the histogram clearly changed while maintaining the locations of the two modes after the introduction of additional NSCs. There were two modes in the distribution of IBIs;
Figure 3. Motif analysis. (a) Similarity matrix between bursts of a representative sample. (b) Sorted similarity matrix of (a). (*) symbols correspond to motifs shown in (c)). (c) Extracted motif from (b). (d) The number of motifs ($p = 0.0625$; Wilcoxon signed-rank test; $n = 5$) ‡, $p < 0.1$.

Network bursts have a limited number of spatiotemporal patterns called motifs that reflect the stability of the global dynamics of the network. To
determine reproducibility, the network bursts were sorted by the similarity in delays in the 1st spike of the network bursts at the recording electrodes. The mature hippocampal cultures showed a rich repertoire of network bursts before NSCs were added (figures 3(a) and (b)). Based on the similarity matrix of the time delay, repeating motifs were detected (figure 3(c)). We found that the number of motifs decreased in all cultures with additional NSCs (figure 3(d), $p = 0.0625$, Wilcoxon signed-rank test), indicating that NSCs led the network to a more stable state globally.

To investigate the synchronization level in a neuronal network, we calculated the cross-correlation function of spike trains between all pairs of available electrodes. Figure 4(a) represents the cross-correlation of the recording electrodes averaged over all possible pairs and the mean cross-correlation over the network. The shape of the cross-correlation function changed less steeply with additional NSCs, and the peak value of the mean cross-correlation of the network with NSCs was lower than that of the pre-existing network. To evaluate the degree of synchronization, we calculated the ratio of the peak around zero to the cross-correlations of all considered time windows, i.e. the CI. Figure 4(b) plots the CI of all possible pairs, and their distribution was clearly changed by the addition of NSCs. The median value of CI in each culture was compared, and the CI decreased from $0.083 \pm 0.07$ to $0.073 \pm 0.06$ by the introduction of additional NSCs in all cultures (figure 4(c), $p = 0.0417$, Student’s paired $t$-test). The cross-correlation function depends on the distance between neurons and reveals the strength of the connections among them. Therefore, we measured CI according to the distance between electrodes to investigate whether the effect of NSCs is related to the proximity of connection among neurons. Separating all pairs of electrodes into six groups based on their distance, we compared the CI before and after adding NSCs. CI significantly decreased in all additional NSC groups (figure 4(d), $p < 0.0001$, Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm’s stepwise Bonferroni correction), suggesting that NSCs reduced the synchronization level as a whole, independent of the proximity of connection.

3.2. Evoked activity

Recent studies have demonstrated that in vitro networks respond to external stimuli and transfer and...
process information [27, 43]. Based on two frameworks, namely the spike count code and spike timing code that evaluate the ability to process input information, we used spike counts and cross-correlation of the evoked response to two different spatial input stimuli.

To analyze the change in neuronal activities in response to the external inputs, two different spatial patterned stimulations were applied to the network: pattern 1, where eight electrodes on the left column were stimulated, and pattern 2, where eight electrodes on the right column were stimulated (figure 5(a)), patterns 1 and 2 were given to the network alternatively a total of 150 times for each, and these stimulations evoked neuronal activity. Figure 5(a) shows the evoked responses to stimuli averaged over all trials for each pattern. To quantify the amount of information that a neuronal population conveys about the two different stimulations, we calculated the mutual information between the responses and the stimuli (pattern 1 and pattern 2). Here, we utilized the concept of spike count coding and considered evoked responses during 5–50 ms after stimuli. Compared to spike counts in the pre-existing network, differences in the distribution of spike counts between the two input patterns appeared in the network with additional NSCs (figure 5(b)). The mutual information at recording electrodes was compared, and we found that the network with NSCs showed a higher value of mutual information, suggesting that NSCs improved the ability to discriminate between two different input patterns (figure 5(c), $p = 0.00094$, Mann–Whitney U test).

Since spike timing is more accurate at the beginning of spike trains in evoked responses to electrical stimulations [44], we calculated the cross-correlation functions of evoked spike trains during 5–200 ms after application of stimuli among the recording electrodes. The synchronization level of spikes evoked by external inputs was measured by CI in the same way as the analysis of spontaneous activity. As mentioned above, we found that additional NSCs similarly decreased the CI for all groups depending on the distance between the electrodes during spontaneous
Figure 6. Cross-correlations in evoked responses to two different spatial input patterns. (a) CI of evoked response to external inputs (center line, median; shadow area, 95% confidence intervals; box, interquartile ranges; $p = 0.0905$, $p = 0.5251$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, respectively; Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm's stepwise Bonferroni correction; pre-existing, $n = 1343, 1944, 1752, 772, 164$; +NSCs, $n = 583, 817, 1039, 658, 250$, 34 pairs of electrodes from five samples in ascending order of distance). (b) The top 40 pairs of highest CIs for each external input of a representative sample. Circles indicate 64 electrodes on MEA. (c) The difference in CI between two spatial input patterns (center line, median; shadow area, 95% confidence intervals; box, interquartile ranges; $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p < 0.0001$, $p = 0.00011$, respectively; Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm's stepwise Bonferroni correction; pre-existing, $n = 1343, 1944, 2794, 1752, 772, 164$; +NSCs, $n = 583, 817, 1039, 658, 250$, 34 pairs of electrodes from five samples in ascending order of distance) $\ddagger$, $p < 0.1$, $\ast$, $p < 0.05$; $\ast\ast\ast$, $p < 0.001$; $\ast\ast\ast\ast$, $p < 0.0001$; n.s., $p > 0.5$.

On the other hand, we found that the network with NSCs showed a higher value of CI for close connections (distance between electrodes $< 500 \mu m$) and a lower CI value for remote connections (distance $> 900 \mu m$) than the pre-existing network in the case of evoked activity (figure 6(a), $p = 0.0905$, $p < 0.0001$, Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm's stepwise Bonferroni correction). The groups were not significantly different between close and remote distances of connection (figure 6(a), $p = 0.5251$, Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm's stepwise Bonferroni correction). These results suggest that NSCs raised the degree of synchronization with external inputs in close connections among neurons. Furthermore, we analyzed the differences in CI between evoked responses to the two patterns to evaluate the ability to discriminate different spatial inputs. The top 40 pairs of the highest CI values in the pre-existing network mostly overlapped between the two external input patterns (figure 6(b)). In contrast, the network with additional NSCs demonstrated specific connections with a high CI value for different input patterns. The network with NSCs generated a larger difference in CI for all groups with the same distances (figure 6(c); $p < 0.0001$ for the 1st five groups, $p = 0.0011$ for the last group in ascending order of distance; Kruskal–Wallis test followed by Mann–Whitney $U$ post-hoc tests with Holm's stepwise Bonferroni correction). These results suggested that the pre-existing network distinguished two different inputs more clearly after the addition of NSCs.

In summary, the analysis of network bursts of spontaneous activity revealed that additional NSCs changed the diverse internal dynamics of the network state to a more stable and organized one globally. In light of the cross-correlation function among spike trains, NSCs decreased the synchronicity of the pre-existing circuits in the neuronal state without any external inputs. In contrast, NSCs increased the synchronization level only for short-distance connections in response to external inputs. Moreover, NSCs led to a larger difference in the degree of synchronization of evoked responses between two different spatial input patterns and a higher value of mutual information, indicating that additional NSCs showed more specific responses to different external inputs, thus improving the ability to discriminate different input patterns.

4. Discussion

In the present study, we investigated the role of NSCs in a pre-existing hippocampal network to compare...
the electrical activities of the network before and after adding NSCs using MEAs. The characteristics of network bursts were altered, and synchronicity among neurons in the spontaneous activity decreased, suggesting that additional NSCs modulated the internal dynamics of the network state. Moreover, a higher synchronization level to novel external inputs in short connections and a more specific response to two spatial input patterns indicated that additional NSCs improved the ability to distinguish different external inputs.

We observed two modes in the BD and IBI histograms, which is in line with previous research that reported that a dense neuronal network shows a bimodal distribution of BDs and IBIs [16, 45]. The network with additional NSCs demonstrated the characteristics of network bursts and generated longer bursts with longer intervals. Network bursts are related to excitation and inhibition balance. When disrupting the balance to increase the excitation level, the network has been reported to show longer BDs and IBIs [18, 46]. NSCs are more excitable in the early course of maturation because they have higher resistance, a lower threshold for firing, and conversion of GABA<sub>A</sub> receptor-mediated inhibition of excitation [47, 48]. Thus, additional NSCs would increase the excitation level in the network, and as a result, the network with NSCs generated longer BDs with longer IBIs. Although the network with NSCs showed more spikes within bursts than the pre-existing network, the synchronization level of spike trains was reduced. Taken together, our results indicate that NSCs may drive the pre-existing network to produce network bursts with slightly loosened connections among neurons so that bursts have a tendency to prolong instead of fire in a concentrated manner.

Motif analysis demonstrated that the reproducible patterns of initiating network bursts were limited by additional NSCs. A previous study discussing the mechanism of the beginning of bursts showed that the diversity of spatiotemporal patterns of bursts is regulated by AMPA receptors [22]. Other studies have claimed that several highly active neuronal populations (pacemaker neurons) lead to the emergence of bursts [49], and that competition among these neurons contributes to the richness of burst patterns [50]. It has been reported that transplanted NSCs could trigger the bursting behavior of hippocampal slices, when using optical stimulations and whole cell patch clamp technique [51]. Considering the high ratio of NMDA receptors to AMPA receptors [32] in the early development of NSCs and in conditions of high excitability, our results showing that adding NSCs further limited the initiating burst patterns of the pre-existing network are consistent with the results of other studies. A decrease in the number of motifs indicated that the dynamics of the network state became more stable with additional NSCs. Network bursts are globally synchronized over the entire network. In contrast, the CI between pairs of electrodes in close connections indicated locally synchronized activity. Our results suggest that additional NSCs modify the internal dynamics of the pre-existing network to be more globally stable and locally variable.

Since neuronal responsiveness to external inputs depends on the state of the network [53], we applied a mutual information and cross-correlation analysis to the evoked activity to quantify the sensitivity to external inputs. NSCs are incorporated into the network to make connections with neighboring neurons [54], and they respond to a broader range of inputs [55]. A study using calcium imaging and electrophysiology demonstrated that immature neurons respond at higher rates to a given stimulus [56]. Our finding that synchronization levels increased in close connections between neuronal populations and decreased in remote connections to external inputs suggested that the effect of NSCs—which easily respond to novel stimuli—could appear in only short connections.

An increase in mutual information and a larger difference in the synchronicity between two input patterns were observed across the entire network after the addition of NSCs, indicating that the network has a trend to discriminate two patterns more clearly with regard to both spike counts and spike timing. This <i>in vitro</i> result is consistent with <i>in vivo</i> research, which reported that newborn neurons actively participate in encoding novel information [57]. Previous research has shown that the activity produced by external inputs must propagate throughout the network in order for them to be sensitive to those inputs [58]. Since the balance between local processing and global integration is essential for transferring information [59, 60], NSCs would lead the pre-existing network to spread evoked activity more effectively by increasing synchronicity among short connections and decreasing it among long connections. A study has reported that the newly generated neurons contribute to creating sparse synaptic connectivity, resulting in distinct activation profiles to external inputs [61]. Thus, this change in connections among neurons is a critical aspect of information processing. Correlations among neuronal responses are stimulus-dependent, and changes in correlations are strongly related to the functionality and regulation of the flow of neural information [62]. Stimulus-specific response synchronization has been observed both <i>in vivo</i> [63] and <i>in vitro</i> [64]. Interestingly, <i>in vivo</i> research has demonstrated that stimulus-specific synchronization of neuronal responses is facilitated when spontaneous activity is desynchronized rather than synchronized [65]. This is consistent with our results that NSCs decreased synchronization level in the internal state while enhancing differences in the
synchronicity of evoked responses between two different inputs. Further investigation will reveal the clear correlation between external responses and the internal state, which is globally stable and locally variable.

Our work is the first to explore the potential mechanisms underlying network level changes caused by the introduction of NSCs into a hippocampal culture with MEA recordings and the idea of spike count and timing code. Our study remains limited in that we did not identify locations of additional NSCs on MEAs. For example, combining visualization of NSCs by fluorescent proteins [66] with MEA recordings will uncover the relationship between positions of NSCs and spatial changes in the electrical activity of the pre-existing network. However, it is noteworthy that changes in internal dynamics and the ability to process information occurred over the entire network by additional NSCs. Several computational studies regarding hippocampal adult neurogenesis speculate that NSCs in the dentate gyrus contribute to encoding novel inputs [67] and reducing interference among new information [68, 69]. Since these studies simulated information processing using either a trisynaptic circuit or feedforward structure of the hippocampus, we will combine these results with a simulated network similar to our experimental conditions, to investigate in more detail the role of NSCs in future work. In the present study, we showed that the introduction of additional NSCs modified the internal dynamics of the network to be more stable globally, all the while maintaining loosened connections among neurons. This change in a steady-state might drive the pre-existing network to acquire higher sensitivity to novel external inputs, thus improving the ability to discriminate between two different spatial inputs. Distinguishing two different inputs efficiently at the network level is vital for learning and memory in the hippocampus. Therefore, our results lay forth new insights with regard to the relationship between NSCs and learning and memory at the network level.

5. Conclusion

NSCs in the hippocampus are hypothesized to contribute to learning and memory. Here, we demonstrated that NSCs alter the internal dynamics of a pre-existing hippocampal in vitro network and enhance the ability to discriminate different external input patterns. The addition of NSCs stabilized global synchronized activities (network bursts) and decreased local synchronicity in the internal state of the pre-existing network. These changes led to more specific responses to different external inputs, suggesting that NSCs improve the ability to distinguish external inputs by modulating the internal dynamics of a pre-existing network in hippocampal culture.

Data availability statement

All data that support the findings of this study are included within the article (and any supplementary files).

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