

A neurospheroid-based microrobot for targeted neural connections in a hippocampal slice

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Abstract

Functional restoration by the re-establishment of cellular or neural connections remains a major challenge in targeted cell therapy and regenerative medicine. Recent advances in magnetically powered microrobots have shown potential for use in controlled and targeted cell therapy. In this study, a magnetic neurospheroid (Mag-Neurobot) that could form both structural and functional connections with an organotypic hippocampal slice (OHS) was assessed using an ex vivo model as a bridge toward in vivo application. The Mag-Neurobot consists of hippocampal neurons and superparamagnetic nanoparticles (SPIONs); it is precisely and skillfully manipulated by an external magnetic field. Furthermore, the results of patch-clamp recordings of hippocampal neurons indicated that neither the neuronal excitabilities nor the synaptic functions of SPION-loaded cells were significantly affected. Analysis of neural activity propagation using high-density multi-electrode arrays showed that the delivered Mag-Neurobot was functionally connected with the OHS. The applications of this study include functional verification for targeted cell delivery through the characterization of novel synaptic connections and the functionalities of transported and transplanted cells. The success of the Mag-Neurobot opens up new avenues of research and application; it

offers a test platform for functional neural connections and neural regenerative processes through cell transplantation.

1. Introduction

Cell transportation, transplantation, and tissue engineering have important roles in regenerative therapies that aim to achieve regeneration and functional recovery after disease or other forms of tissue damage.^[1-4] In the central nervous system, these strategies have focused on facilitating the regeneration of native tissues and directly restoring lost functions based on structural integration.^[5-7] Direct surgical transplantation of therapeutic cells into the central nervous system is the most commonly used form of cell therapy. However, direct injection into the central nervous system is invasive, risky if repeated, and may cause side effects.^[8-9] As alternatives to conventional direct injection into the brain, several efforts have been made to employ magnetically powered microrobots.^[10-13] Such microrobots precisely deliver therapeutic agents to target lesions in a non-invasive or minimally invasive manner.^[14-16] Of the various energy sources used to power microrobots, a magnetic field penetrates the body wirelessly, harmlessly, and effectively; the body permeability is similar to that of air.^[13, 17-18] Recently, magnetically powered microrobots for targeted delivery of therapeutic agents to the central nervous

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system have exploited the advantages of magnetic actuation.^[8, 19-22] Multiple studies have demonstrated the potential uses of magnetically powered microrobots in controlled and targeted cell therapy.^[11, 20, 23-25] For example, three-dimensional (3D) microrobots have been used for 3D cell culture and transplantation in various microfluidic environments, including a body-on-a-chip.^[8, 25-27] Initially, the magnetic spheroids used to generate 3D microrobots were constructed using magnetic monosized polymer microspheres,^[28] streptavidin paramagnetic particles,^[29] or magnetic polystyrene microparticles,^[30] none of these products were approved by the Food and Drug Administration (FDA). However, superparamagnetic nanoparticles (SPIONs) have already received FDA approval for use in human brain studies; thus, they have potential for use in microrobot studies with medical applications.^[31] The cationic transfection agent poly-L-lysine (PLL) is widely used to enhance the cellular uptake efficiency of nanoparticles. Positively charged amino acid chains of PLL-modified nanoparticles interact with negatively charged cell membranes.^[8, 32-34] Surface modification of SPIONs with PLL reduces cytotoxicity and improves both biocompatibility and stability.^[35-37] Previous studies found that PLL-functionalized nanosized particulate materials of different sizes^[8, 34, 38] and morphologies^[39-40] electrostatically interacted with negatively charged cell membranes and were then

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internalized. PLL-modified nanoparticles could be internalized by cells and were not cytotoxic for 3–4 weeks *in vivo*.^[41–42] Jeon et al. reported that stem cells combined with PLL-SPIONs proliferated for 2 days and differentiated into neuronal cells over 21 days of cultivation; this is evidence of high-level SPION biocompatibility.^[8] The use of a permanent magnet that enables magnetic spheroids to make multiple connections has been demonstrated,^[28, 30, 43–44] and cell-carrying microrobots have been developed to deliver cells to *in vitro* and *in vivo* environments.^[8] Although these studies have demonstrated the feasibility of targeted cell delivery using microrobots, to the best of our knowledge, our group has performed electrophysiological analyses to investigate whether the delivered cells can contribute to the morphological and functional connections with the surrounding cells.^[20]

Unlike other organs, healthy function in the brain depends on accurate information transfer between regions through synaptic connections.^[20, 24–25] The specificity of connections among brain regions and subregions, as well as among neurons within a brain region, has been extensively characterized.^[45–48] Aberrant neuronal connectivity and imbalances in excitability control often lead to neurological or psychiatric brain disorders.^[49–51] Transplanted engineered neurons must therefore be integrated into the

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neural circuit, then gain the structure and function needed to reconstruct neural circuits.

Despite the importance of neural connectivity, most attention in the field of tissue engineering has focused on the use of biomimetic 3D scaffolds to promote the development of complex 3D neural synaptic networks.^[52-55] However, studies concerning the structure and function of the bioengineered neuronal constructs were performed using in vitro culture systems, in which brain regions are not reproduced and connectivity is largely random. The organotypic brain slice system offers an alternative because it has many of the critical attributes needed to assess whether transplanted neurons have assumed a normal structure and can function in a neural circuit.^[56-60]

Additionally, neuronal connectivities are better preserved and thus provide a more natural 3D neural network with which to examine the transplanted neurons. Thus, cell-based 3D models with brain slices could be a useful platform for novel drug screening and diagnostic platforms;^[61] they may also be useful for cell therapy and regenerative medicine focused on restoring functional connectivity.^[1-3] Similar to in vivo systems, the organotypic slice system enables mechanistic studies of information processing and operations in the brain.^[56-60] The delivery of a 3D neuron spheroid-based microrobot to a

specific site in the brain can also be simulated using an organotypic slice; this approach facilitates morphological, electrophysiological, and molecular assessments of neurons.

In previous work, we developed a magnetically actuated microrobot with microgrooves for use in neuron delivery and the establishment of selective functional neural connectivity in vitro.^[14] In the present study, we describe the delivery and neural connections of a hippocampal spheroid microrobot, demonstrated through an ex vivo model that consists of an organotypic hippocampal slice (OHS) culture. Through its SPIONs, the magnetic neurospheroid microrobot (hereafter regarded as the "Mag-Neurobot") could be actively and precisely driven by an external magnetic field, thereby forming connections with the OHS; this process resulted in the ex vivo reconstruction of neural circuitry. The fabricated Mag-Neurobot is morphologically similar to in vivo neurites, and its cellular extensions are electrically active. The morphological and functional connections of the delivered Mag-Neurobot with the OHS were confirmed using high-density multi-electrode arrays, which also revealed the propagation of neural activity. To our knowledge, this is the first description and functional analysis of a targeted ex vivo delivery system for hippocampal neural spheroids. The proposed platform could be used to obtain ex vivo functional neural connections and neural

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regenerative processes by precise cell transplantation. It could also lead to expanded applications, including drug and toxicity tests of cell-delivery-based functional systems that use neural, cardiac, and stem cells in ex vivo and in vivo models.

2. Results and Discussion

2.1. Magnetic neurospheroid microrobot (Mag-Neurobot)

The Mag-Neurobot was fabricated by seeding primary hippocampal neurons (PHNs) with PLL-coated SPIONs in U-shaped-bottom wells. The neurospheroid without SPIONs served as a control in comparisons with the Mag-Neurobot. As shown in **Figure 1a**, 3D spheres in control and Mag-Neurobot groups formed within 5 days. Based on our previous work, the target concentration of SPIONs was 0.1 mg mL^{-1} (100 pg per cell; 350 ng per 3,500 cells), a concentration that has no negative impacts on cell viability or proliferation rate.^[8, 62-63] PHNs with PLL-coated SPIONs, but not PHNs with bare (non-PLL-coated) SPIONs, formed 3D spheroids in 5 days (Figure S1a and S1b, Supporting Information). PLL plays a crucial role in inducing PHNs to form aggregates with SPIONs because the positively charged amino acid chains of PLL-modified SPIONs interact with the negatively charged cell membrane.^[64] It is difficult to closely observe how cells bind to PLL-coated SPIONs in

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a 3D environment, such as U-shaped-bottom wells, in which cells do not attach to the well surface but aggregate with each other. Therefore, PHNs were cultured in a cell culture dish with PLL-SPIONs and bare SPIONs to observe how PLL-coated SPIONs bind to PHNs in a two-dimensional environment (Figure S2, Supporting Information). PLL-SPIONs became attached to cells (red arrows in Figure S2), but bare SPIONs did not (red circles in Figure S2) 1 day after seeding. The lack of bare SPION binding may prevent neurospheroid formation by PHNs in U-shaped wells (Figure S1b, Supporting Information). Formation of 3D spheroids composed of PHNs required PLL on the surfaces of SPIONs. The control neurospheroid showed a generally bright core region, whereas the Mag-Neurobot showed a dark central region because of its SPIONs, as shown in Figure 1a and Figure S1a (Supporting Information). The size distributions of the control neurospheroid and the Mag-Neurobot are shown in Figure 1b; the mean diameters were $296.1 \pm 5.6 \mu\text{m}$ and $292.3 \pm 6.4 \mu\text{m}$, respectively (Table S1, Supporting Information). The size of the 3D spheroids can be controlled by changing the number of seeded PHNs per well. Spheroids $>500 \mu\text{m}$ in diameter decrease cell viability because they exhibit poor diffusion of oxygen and other necessary nutrients.^[65] Therefore, the target diameter of

the spheroid was set to 300 μm ; 3,500 PHNs were used to form each spheroid. To match the diameter of the Mag-Neurobot, the thickness of the OHS was also set to 300 μm .

The viability of neurons in the spheroid was then determined. In live cells, the nearly non-fluorescent (green) cell-permeant dye calcein AM is enzymatically converted to the intensely fluorescent dye calcein; in dead cells, BoBo-3 Iodide red dye enters through damaged membranes and fluoresces brightly when it binds to DNA. Live/dead staining indicated that cell viabilities in control and Mag-Neurobot groups were generally similar (Figure 1c). Most PHNs were alive (green), with only a few dead cells (red) in both control and Mag-Neurobot groups (250 ng and 350 ng SPIONs) (Figure 1c, Figure S3, Supporting Information). PHN viability was not affected by even high levels of SPIONs (Figure S4, Supporting Information). However, quantitative analysis of cell death using this method was restricted by the imaging depth required for reliable optical sectioning and staining. Furthermore, a direct comparison could not be made when SPIONs were present. SPIONs are opaque; the light required for confocal imaging is blocked. Rather, cell viabilities were quantitated using other methods. First, we measured cytoplasmic adenosine triphosphate (ATP) levels. The ATP level of PHNs with Mag-Neurobots did not change significantly, suggesting that cell viability was not affected by PLL-SPIONs up to

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day 14 (Figure 1d). The ATP results indicated a lack of necrosis within the Mag-Neurobot; the live cell numbers of the Mag-Neurobot were similar to those of the control. To explore apoptotic cell death status, we performed a terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP) nick-end labeling (TUNEL) assay (Figure S5, Supporting Information). Very few TUNEL-positive nuclei were seen in the control and Mag-Neurobot samples. Additionally, we measured reactive oxygen species (ROS) levels using the CellROX Green reagent (Figure S6, Supporting Information). The control and Mag-Neurobot groups stained similarly. These results confirmed the live/dead staining and ATP assay results; PLL-SPIONs did not trigger significant apoptotic cell death. Therefore, any cytotoxic effects of PLL-SPIONs in neurospheroids were negligible; the cell viabilities of Mag-Neurobots and the control were similar. The presence of SPIONs was validated using energy-dispersive X-ray spectroscopy and scanning electron microscopy (SEM). SPIONs inside the Mag-Neurobot were detected based on their Fe content, which was detectable in all examined Mag-Neurobots (Figure 1e and 1f). Bright-field microscopy revealed dark brown SPIONs inside the cryosectioned Mag-Neurobots (inset of Figure 1f). As shown in Figure S7a (Supporting Information), PLL-Fluorescein (FITC) labeled SPIONs within the neurospheroids were

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scattered peripherally but more concentrated in the core. Transmission electron microscopy (TEM) of cryosectioned Mag-Neurobots revealed SPIONs in the cytoplasm (Figure S7b, Supporting Information). The morphologies of PLL-coated and bare SPIONs were investigated via SEM and TEM (Figure S8a and S8b, Supporting Information). This confirmed the lack of any morphological change after PLL coating. PLL incorporation onto the surface of SPIONs was confirmed via dynamic light scattering (DLS) and the zeta potential test (Figure S8c, S8d, and Table S2, Supporting Information). The hydrodynamic sizes of bare and PLL-coated SPIONs were 147.65 ± 5.23 and 541.42 ± 78.79 nm ($n = 4$), respectively. The surface charge of bare SPIONs was negative; the zeta potential was -43.20 ± 1.36 mV ($n = 4$). For PLL-coated SPIONs, the PLL coating led to partial compensation by protonated amine groups of PLL, resulting in a zeta potential of -30.26 ± 0.25 mV ($n = 4$). According to the increase in the hydrodynamic sizes of PLL-coated SPIONs (Table S4, Supporting Information), particle aggregation during PLL coating may induce partial surface coverage of PLL on the surfaces of SPIONs. In agreement with previous studies,^[66-67] our results showed a negative zeta potential for PLL-coated iron oxide nanoparticles. The negatively charged surface-modified nanoparticles, which had a negative zeta potential, showed cellular uptake resulting in

endocytosis.^[67-68] Accordingly, despite the negative zeta potential of the functionalized SPIONs, the presence of PLL on the surface of SPIONs may facilitate cell attachment in this study. Furthermore, as shown in our previous research, the Fourier transform infrared spectra result showed that most SPIONs have formed amide bonds and have been functionalized with PLL.^[8] These results implied that SPIONs did not affect cell viability during spheroid culture. Based on these results, the control neurospheroid and the Mag-Neurobot were cultured for 5 days and used in the following experiments.

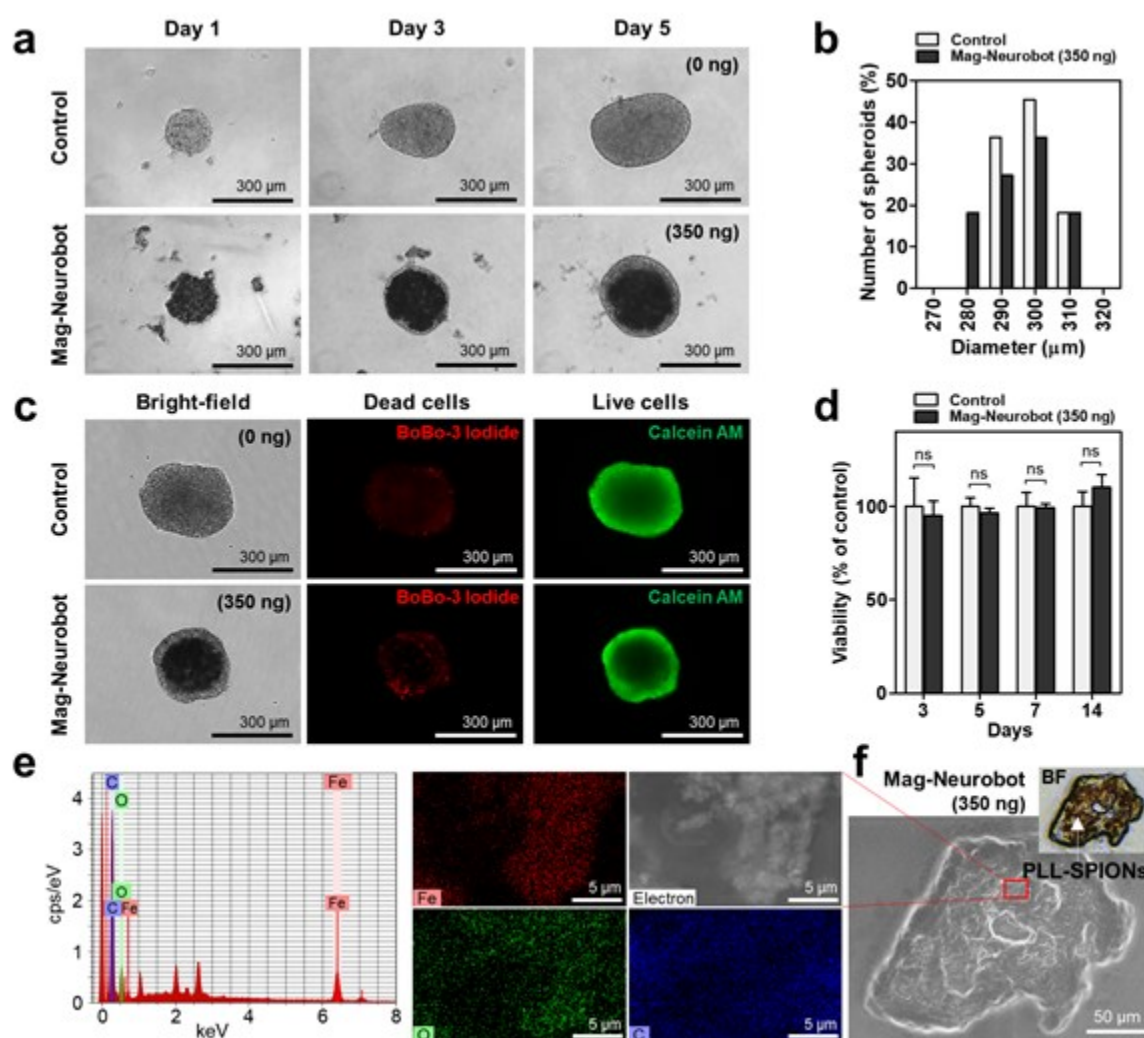


Figure 1. Size and viability of the control and magnetic neurospheroid microrobot (Mag-Neurobot). a) Microscopy images of the neurospheroid without (control) and with (Mag-Neurobot) 350 ng of superparamagnetic nanoparticles (SPIONs) after 5 days of incubation. b) Histograms of the mean diameter of the control neurospheroid and the Mag-Neurobot with 350 ng of SPIONs ($n = 11$). c) Bright-field and fluorescence images

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taken during the live/dead assay (green: live cells; red: dead cells) by the control and Mag-Neurobot. d) Adenosine triphosphate (ATP) assay of the control neurospheroid and the Mag-Neurobot with 350 ng of SPIONs. Error bars represent $\pm$ standard deviations (S.D.s) ($n = 5$) and statistical significance was assessed by unpaired Student's *t*-test. *ns*, not significant. e) Scanning electron microscopy-energy dispersive X-ray spectroscopy analyses of the Mag-Neurobot with 350 ng of SPIONs. f) Scanning electron microscopy (SEM) and microscopy images (inset of the scanning electron microscopy image on the right) of the Mag-Neurobot with 350 ng of SPIONs.

2.2. Morphological and functional characterization of the Mag-Neurobot

For the Mag-Neurobot to be useful in regenerative therapy, its PHNs must develop normal neuronal connectivity and activity. Therefore, we examined whether the PHNs in the Mag-Neurobot maintained the molecular features of healthy neurons. Immunostaining with an early neuronal marker (i.e., neuron-specific class III β -tubulin [Tuj1], in red) and a mature neuronal marker (i.e., neuron-specific nuclear protein [NeuN], in green)^[69] confirmed that both the control neurospheroid and the Mag-Neurobot showed the typical morphological and molecular characteristics of neurons (**Figure 2a**).

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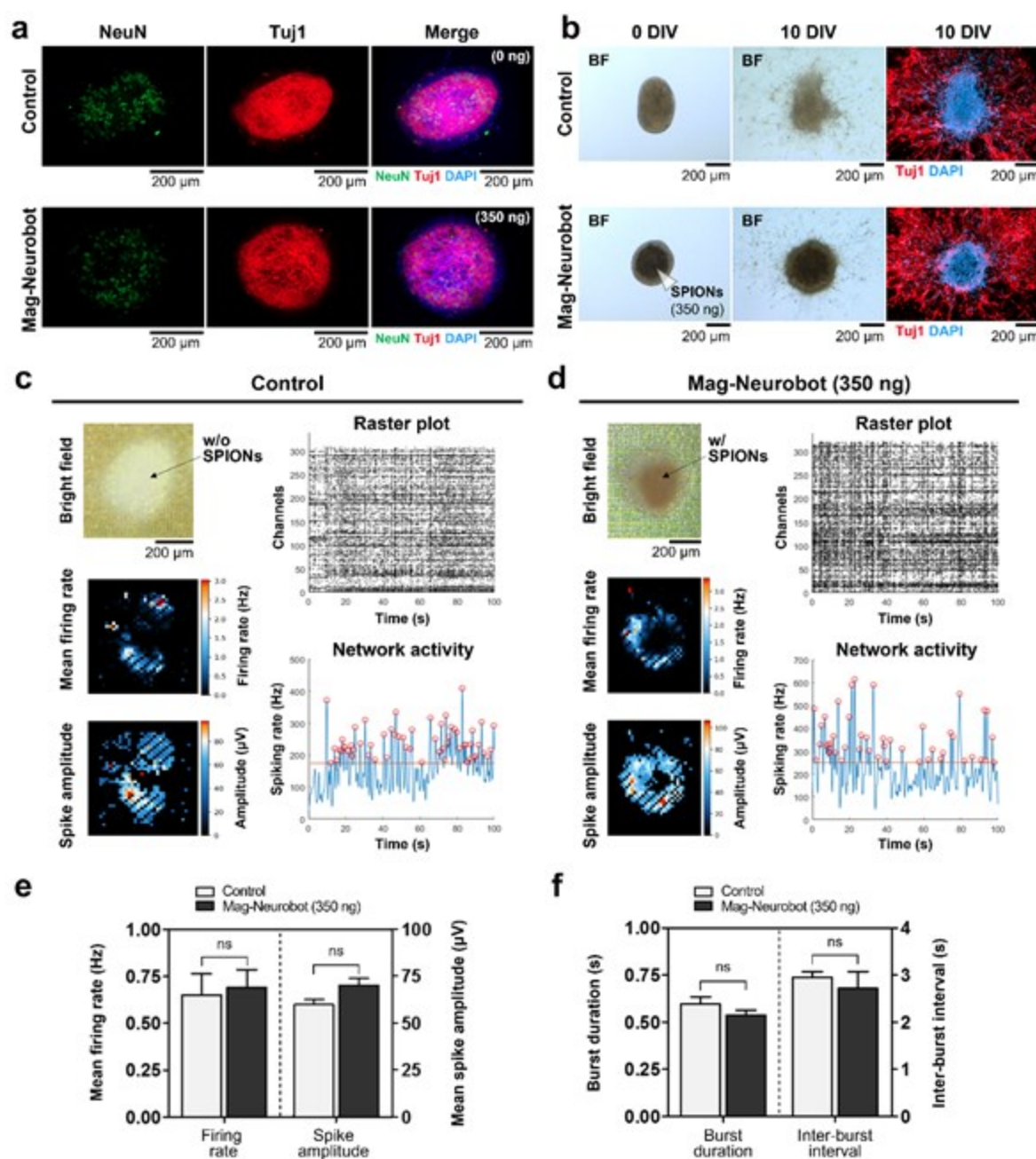
Although neurons in the Mag-Neurobot exhibited qualitative normality, quantitative validation was challenging because of their morphological diversity. Because Matrigel is widely used to mimic the 3D extracellular environment, neurite outgrowth into Matrigel from the Mag-Neurobot was measured before the Mag-Neurobot was applied to the OHS. In bright-field and immunofluorescence images taken at 10 days in vitro (DIV), thus about 16 days after cell isolation, the Mag-Neurobot evidenced neurite outgrowth levels comparable to those of control neurospheroids (Figure 2b, Figure S9a). The neurite outgrowth areas occupied by elongated neurites from PHNs in control and Mag-Neurobot samples did not differ significantly at 10 DIV (Figure S9b, Supporting Information). These results indicated that the Mag-Neurobot, with its magnetic nanoparticles, maintained cell viability and neurite growth at levels comparable with the levels in the control neurospheroid.

The control neurospheroid and the Mag-Neurobot were then functionally characterized with respect to their neural activity (Figure 2c–2f and Figure S10, Supporting Information). Extracellular action potentials (APs) from spontaneous activity at 8 DIV were measured using high-density multi-electrode arrays (HD-MEAs) to assess connectivity with the existing neural network. At this time, about 2 weeks had passed

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from the time of cell isolation from embryos (Figure S11, Supporting Information). Characteristic synchronous network activity, such as bursts, was clearly preserved in the presence of the Mag-Neurobot (Figure 2c and 2d, right panels). Synchronized oscillatory neuronal activities are common in hippocampal brain slices^[70-71] and cultured hippocampal neurons, especially when the neurons are densely cultured, as in neurospheroids^[72-74]. These activities are presumed to reflect the actions of inhibitory neurons. In cultivated brain slices and in vitro, when multiple single neurons begin to fire, when possibly random synaptic connections form, synchronized oscillatory activities develop at the cell boundaries. The firing rate and spike amplitude were comparable (0.65 ± 0.27 Hz and 60.21 ± 6.79 μ V for the control neurospheroid, and 0.69 ± 0.23 Hz and 70.57 ± 8.96 μ V for the Mag-Neurobot; Figure 2c–2e). The mean burst duration and mean inter-burst interval were not significantly different: 0.60 ± 0.1 s and 2.96 ± 0.28 s for the control neurospheroid, and 0.54 ± 0.06 s and 2.72 ± 0.88 s for the Mag-Neurobot (Figure 2f). As shown in Figure 2c–2f, it was found that the Mag-Neurobot showed statistically comparable neural activity with the control neurospheroid regardless of the presence or absence of PLL-SPIONs. It indicates that PLL-SPIONs can be used as a

cell delivery carrier under magnetic manipulation without any effect on neuronal function and activity.



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Figure 2. Morphological and functional characterization of the control neurospheroid and the Mag-Neurobot. a) Co-staining of a mature neuronal marker (NeuN, in green) and an early neuronal marker (neuron-specific class III β -tubulin [Tuj1], in red) to track neural differentiation and connections in the control neurospheroid and the Mag-Neurobot. Cellular nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI, blue). b) Immunostaining of the control neurospheroid and the Mag-Neurobot cultured in Matrigel for 10 DIV after spheroid formation (neuronal marker [Tuj1], red; nuclei, blue). c,d) Neural activity analysis of (c) the control neurospheroid and (d) the Mag-Neurobot. e) Mean firing rate and mean spike amplitude of spontaneous scan activity. f) Burst duration and inter-burst interval of the control neurospheroid and the Mag-Neurobot at 8 days in vitro on an HD-MEA chip. Error bars represent $\pm$ standard error of the mean ($n = 6$) and statistical significance was assessed by unpaired Student's t -test. *ns*, not significant.

2.3. Neuronal excitability and connectivity of PHNs with and without SPIONs

The extracellular AP data demonstrated that Mag-Neurobot neurons integrate synaptic inputs to generate APs and send neural activity downstream of the network. To confirm the normal integration of excitation and firing, the membrane properties of neurons with

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(w/) and without (control) SPIONs were measured using whole-cell patch-clamp recordings (**Figure 3a, b**). The whole-cell capacitance (i.e., neuron size) was indistinguishable (Figure 3c, left panel, control: 38.69 ± 2.86 pF vs. w/ SPIONs: 33.20 ± 3.80 pF; Table S3, Supporting Information). The resting membrane potentials (RMPs) and input resistance (R_{in}) of the PHNs without and with SPIONs also did not significantly differ (-66.72 ± 0.69 mV and 194.9 ± 38.62 M Ω vs. -65.33 ± 3.04 mV and 196.2 ± 31.11 M Ω) (Figure 3c). The membrane potential rebound in response to hyperpolarizing current injection (voltage sag) reflects the size of hyperpolarization-activated cyclic nucleotide-gated channels; it often determines rhythmic firing frequency.^[75-77] Voltage sag did not significantly differ between the two groups (control: 11.33 ± 2.14 mV vs. w/ SPIONs: 10.26 ± 2.42 mV by -200 -pA current injection, $p = 0.74$) (Figure 3d). According to these results, SPIONs did not significantly change the passive membrane properties of PHNs. The membrane properties that contributed to AP output were then tested. Similar to the above findings, a comparable frequency of APs was evoked in response to varying amplitudes of depolarizing current injection (Figure 3e); control PHNs and PHNs w/ SPIONs were indistinguishable in terms of their current-AP frequency (I-F) curves. Consistent with this result, there were no substantial changes in AP threshold (control:

– 38.93 ± 0.91 mV vs. w/ SPIONs: -38.92 ± 2.04 mV, $n = 7$), half-width of AP (control: 2.28 ± 0.19 ms vs. w/ SPIONs: 2.97 ± 0.57 ms), or amplitude of after-hyperpolarization (control: 11.83 ± 1.48 mV vs. w/ SPIONs: 10.52 ± 1.47 mV) (Figure 3f). These results indicated that SPIONs did not affect the integration of excitatory input or AP firing.

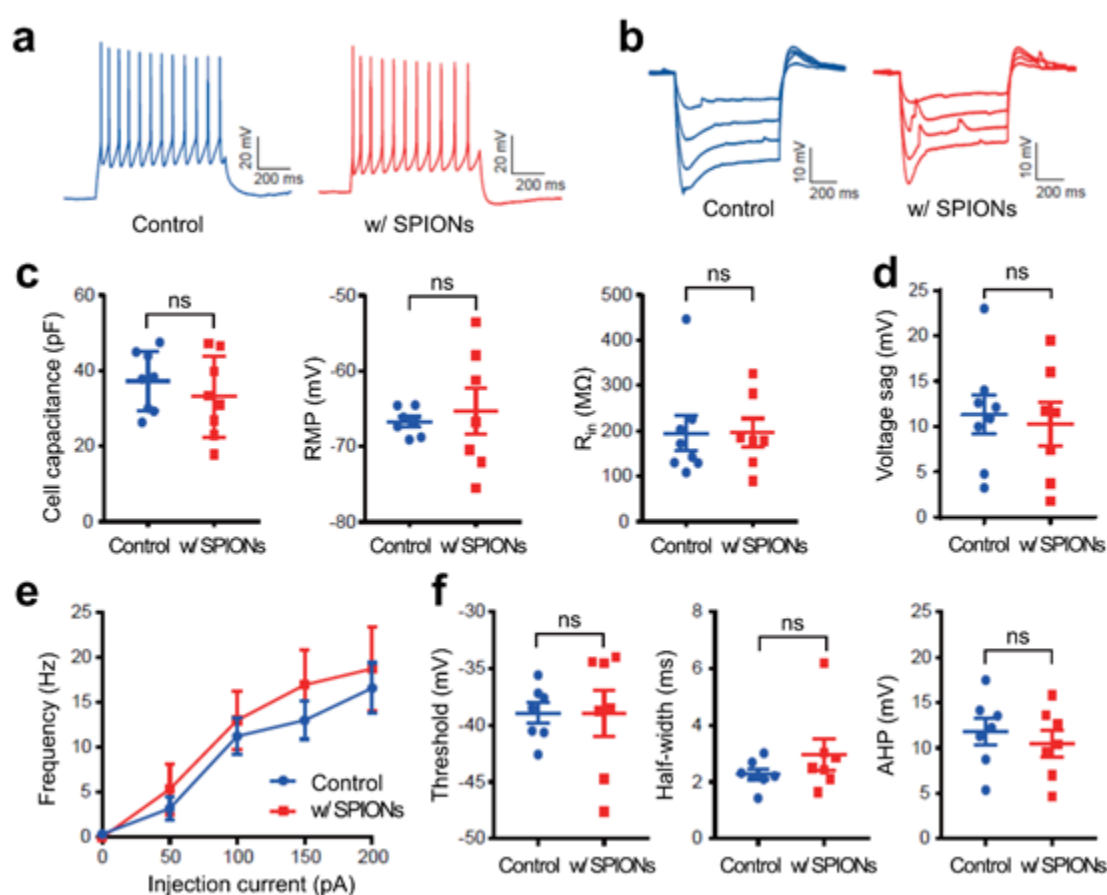


Figure 3. Effects of SPIONs on neuronal excitability in primary hippocampal neurons (PHNs). a) Representative traces of repetitive action potentials (APs) evoked by 100-pA current injection for 800 ms. Scale bars: 20 mV, 200 ms. b) Representative traces of

voltage sags generated through negative current pulses (-200 pA to -50 pA, in 50 -pA increments). Scale bars: 10 mV, 200 ms. c) Passive membrane properties. Averaged whole-cell capacitance (C_m), resting membrane potentials (RMPs), and input resistance (R_{in}). d) Voltage sag in response to -200 -pA current injection in PHNs without (control) and with (w/) SPIONs. e) Frequencies of APs fired in response to injected currents of 0 – 200 pA (in 50 -pA increments) for 800 ms. f) Firing properties of the first APs evoked by 100 -pA current injection. Averaged threshold, half-width, and after-hyperpolarization (AHP) of APs. Error bars represent $\pm$ S.D.s ($n = 7$) and statistical significance was assessed by unpaired Student's t -test. *ns*, not significant.

Because neuronal excitability was not altered by SPIONs, changes in the frequency of postsynaptic responses were considered indicative of the number of synapses, the release probability, or both. Therefore, the frequencies of spontaneously evoked excitatory and inhibitory synaptic currents (sEPSCs and sIPSCs, respectively) were measured. However, the frequencies of sEPSCs (**Figure 4a** and **4b**) and sIPSCs (**Figure 4c** and **4d**) remained consistent in the presence of SPIONs, indicating that the sEPSC/sIPSC frequency ratio did not differ between the two groups (control: 4.09 ± 1.26 , vs. w/

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SPIONs: 2.91 ± 0.49 , $p = 0.42$; Table S4, Supporting Information); moreover, the excitation/inhibition balance was not affected by SPIONs. Taken together, these results demonstrated that SPIONs did not alter the excitability or synaptic transmission of excitatory and inhibitory neurons.

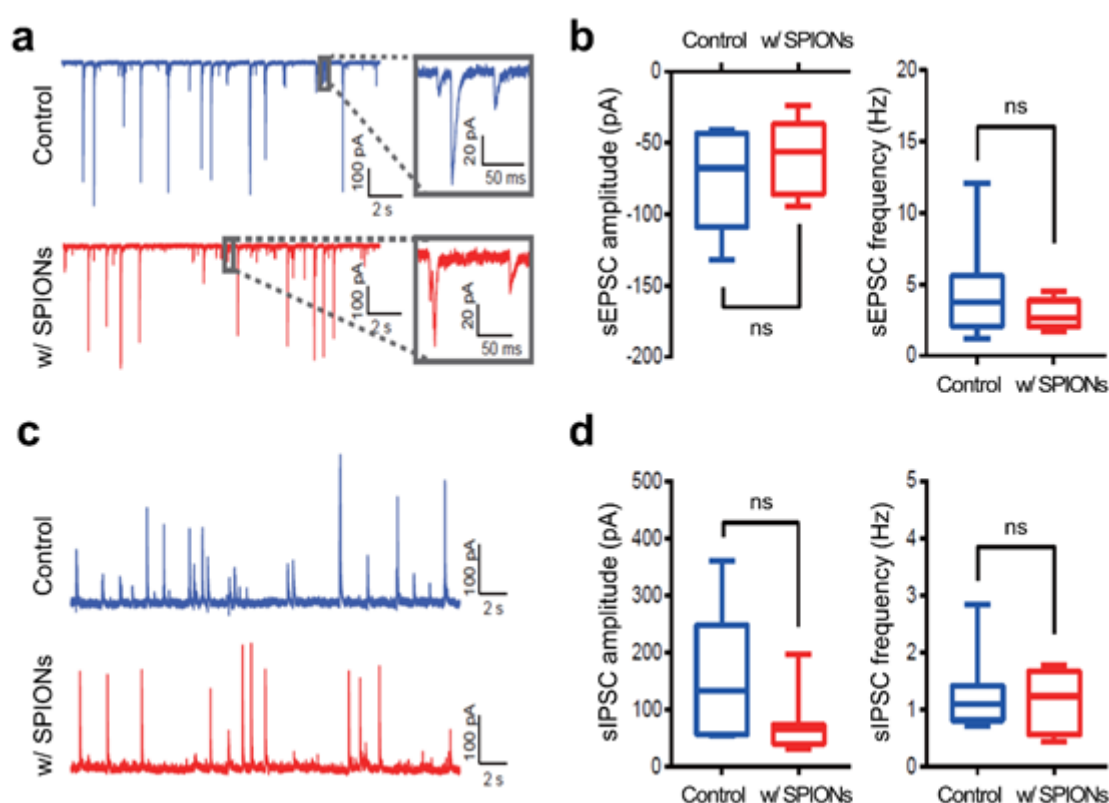


Figure 4. Characterization of synaptic inputs in primary hippocampal neurons (PHNs) with and without SPIONs. Spontaneously evoked excitatory and inhibitory synaptic currents (sEPSCs and sIPSCs) were recorded in the same PHNs at -70 mV and 0 mV,

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respectively. a) Representative traces of sEPSCs in control PHNs without SPIONs (top) and in PHNs with SPIONs (bottom). Scale bars of the left traces: 100 pA, 2 s. The right traces are expansions of the gray boxes in the left traces. Scale bars of the expanded traces: 20 pA, 50 ms. b) Summary of sEPSC amplitude (left) and frequency (right). c) Representative traces of sIPSCs in control PHNs without SPIONs (top) and PHNs with SPIONs (bottom). Scale bars: 100 pA, 2 s. d) Summary of sIPSC amplitude (left) and frequency (right). Error bars represent $\pm$ minimum and maximum values ($n = 7$). Statistical significance was assessed by unpaired Student's t -test. *ns*, not significant.

2.4. Manipulation of the Mag-Neurobot

Figure 5 shows the magnetic characterization and actuation of the Mag-Neurobot. Magnetic hysteresis loops (magnetization curves) of the control neurospheroid and the Mag-Neurobot were obtained using a vibrating sample magnetometer (Figure 5a). Compared with the control neurospheroid, the Mag-Neurobot clearly showed superparamagnetism, similar to SPIONs, with zero magnetism in the absence of an external magnetic field (Figure 5a, inset). The saturation magnetizations (M_s values) of SPIONs were 45.9 emu g⁻¹ and 1.84×10^{-11} A·m² ng⁻¹, thus within the experimental range for SPIONs (30–80 emu g⁻¹). The saturation magnetizations of the Mag-Neurobot with

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PLL-SPIONs and control neurospheroids were $4.46 \times 10^{-9} \text{ A} \cdot \text{m}^2$ and $2.57 \times 10^{-10} \text{ A} \cdot \text{m}^2$, respectively. When the Mag-Neurobot was loaded with 350 ng of SPIONs, the weight of PLL-SPIONs in the Mag-Neurobot was estimated to be ~243 ng from the measured saturation magnetization ratio (M_s of Mag-Neurobot divided by the M_s of SPIONs per nanogram [ng]) (Figure S12a, Supporting Information). The reduction presumably resulted from the vibrating sample magnetometer sampling procedure and the multiple washing steps during the fixation process.

The Mag-Neurobot was manipulated using an electromagnetic coil system (OctoMag), as shown in Figure 5b. The velocity of the Mag-Neurobot was $\sim 3.25 \text{ mm min}^{-1}$, measured using a 40-mT rotating magnetic frequency until 4 Hz, after which the velocity gradually decreased as the rotating magnetic frequency increased (Figure 5c). The decreased velocity at high frequencies may have been caused by exposure of the Mag-Neurobot to an unsynchronized rotating magnetic field.^[78] Therefore, 3 Hz was chosen as the rotating frequency for the following experiment. The Mag-Neurobot was used with 350 ng of SPIONs, rather than 100 or 250 ng of SPIONs, because of its higher-velocity performance in the velocity test using a permanent magnet (Figure S12b–S12d, Supporting Information). The maneuverability of the Mag-Neurobot was confirmed by its

control through a trajectory ("BMR") that was achieved by rotating the magnetic field at 3 Hz with an intensity of 40 mT, as shown in Figure 5d (Movie S1, Supporting Information). The Mag-Neurobot was also controlled with an OHS to demonstrate its targeted manipulation before the formation of ex vivo neuronal connections (Figure 5e, Movie S2, Supporting Information). The OHS is a widely used circuit model because of its good experimental accessibility, along with its simple circuit composition and connectivity. In particular, dentate gyrus (DG)-to-cornu ammonis 3 (CA3) synapses and CA3-to-CA1 synapses have been extensively characterized (DG-CA3-CA1).^[79-80] In this study, the hippocampal circuit was used to assess the ability of the Mag-Neurobot to form functionally relevant synaptic interactions with existing neural circuits. In the first step, the ability of the Mag-Neurobot to deliver healthy neurons to the targeted OHS area was examined. Magnetic manipulations were used to deliver the Mag-Neurobot in proximity to the CA3 region in the OHS. The Mag-Neurobot reached the vicinity of the CA3 within 3 min (Figure 5e (i)); it was then co-cultured with the OHS (Figure 5e (ii) and (iii)).

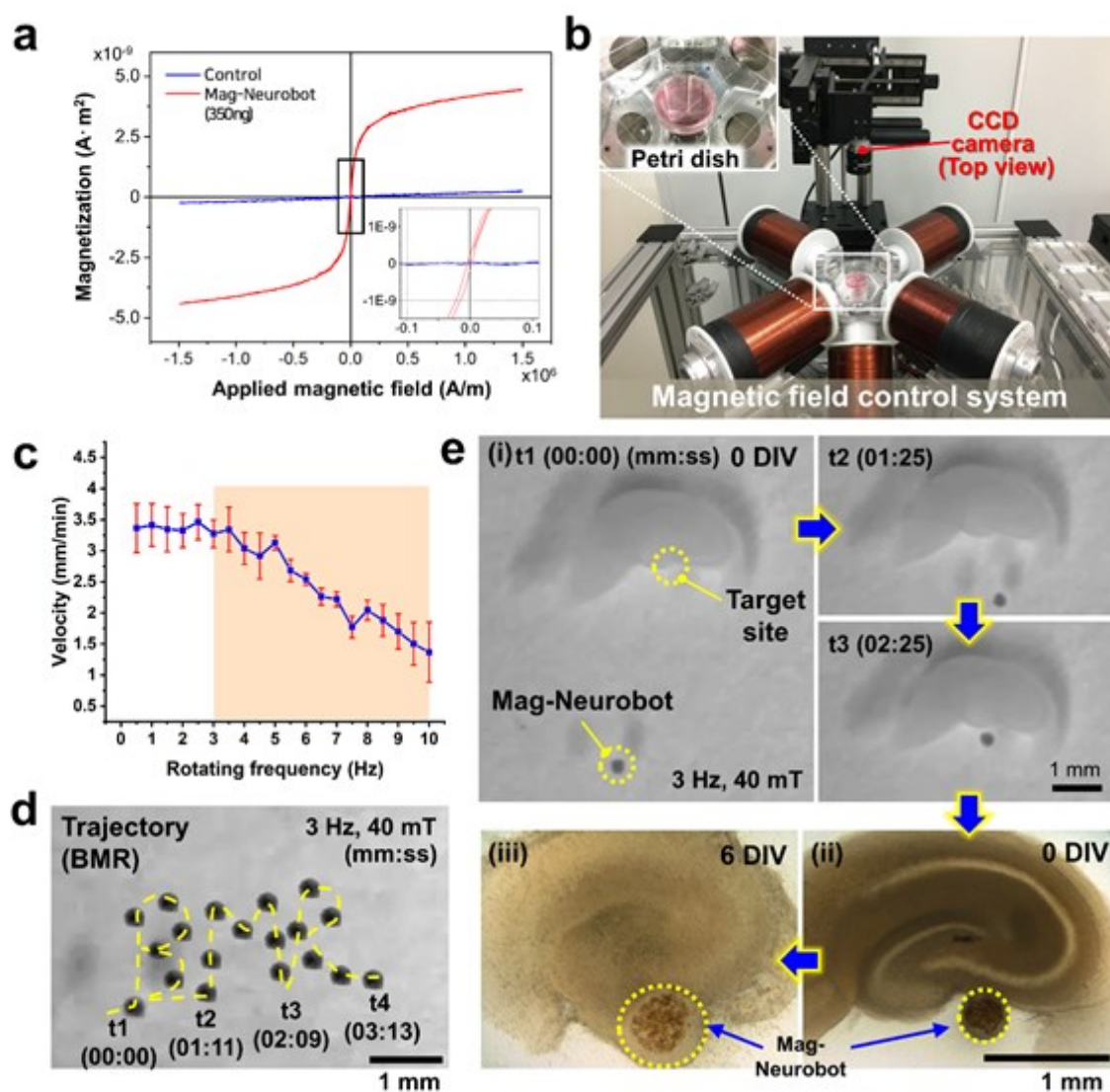


Figure 5. Magnetic characterization and manipulation of the Mag-Neurobot. a) Magnetization hysteresis loops of the control neurospheroid and the Mag-Neurobot. b) The electromagnetic coil system (OctoMag) controls the Mag-Neurobot with a rotating magnetic field. c) Velocity of the Mag-Neurobot under various frequencies of the rotating magnetic field. Error bars represent $\pm$ S.D.s ($n = 3$). d) Tracing the trajectory

("BMR"). e) The precisely targeted delivery of the Mag-Neurobot to the cornu ammonis 3 (CA3) region of the OHS after 6 days in vitro (DIV). CCD, charge-coupled device.

2.5. Morphological connections of the Mag-Neurobot and the OHS

The Mag-Neurobot transported by magnetic manipulation was cultured with the OHS to investigate their morphological connections (**Figure 6a**). In bright-field images of the OHS, the dense regions of the PHNs (including DG, CA3, and CA1) were visible at 1 DIV (Figure 6a, Figure S13a, Supporting Information). OHSs with and without the Mag-Neurobot were stabilized for 6 days to recover from sectioning damage.^[81-82] The neurons in both the control OHS and the OHS cultured with the Mag-Neurobot formed dense connections, as indicated by neuronal somata (NeuN) and neurite outgrowth (Tuj1) after 14 days (Figure 6b). Ortho view images of the CA3 region obtained to detect grafting between the OHS and the Mag-Neurobot showed 3D neuronal spreading, as well as the formation of connections between the pyramidal neurons in CA3 and the PHNs in the Mag-Neurobot (Figure 6c, Movie S3, Supporting Information). Although the origin of neuronal sprouting in the region between the Mag-Neurobot and the CA3 region could not be definitively determined, the presence of massive bundles of neuritic processes implied a strong morphological connection (Figure 6c, white arrows).

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Flattening of the OHS culture with preservation of its 3D organization was also observed (Figure 6c, Figure S13a, Supporting Information).^[83] These observations indicated that the Mag-Neurobot delivered by magnetic manipulation forms physical and neuronal connections with an ex vivo OHS. Furthermore, immunostaining with markers for mature neurons and neuronal processes showed dense neuronal networks and neuronal spreading between the Mag-Neurobot and the CA3 region; this finding implied that the magnetically driven Mag-Neurobot could deliver multiple healthy neurons to a target area, and thus potentially to damaged brain areas, where it could then form morphological connections.

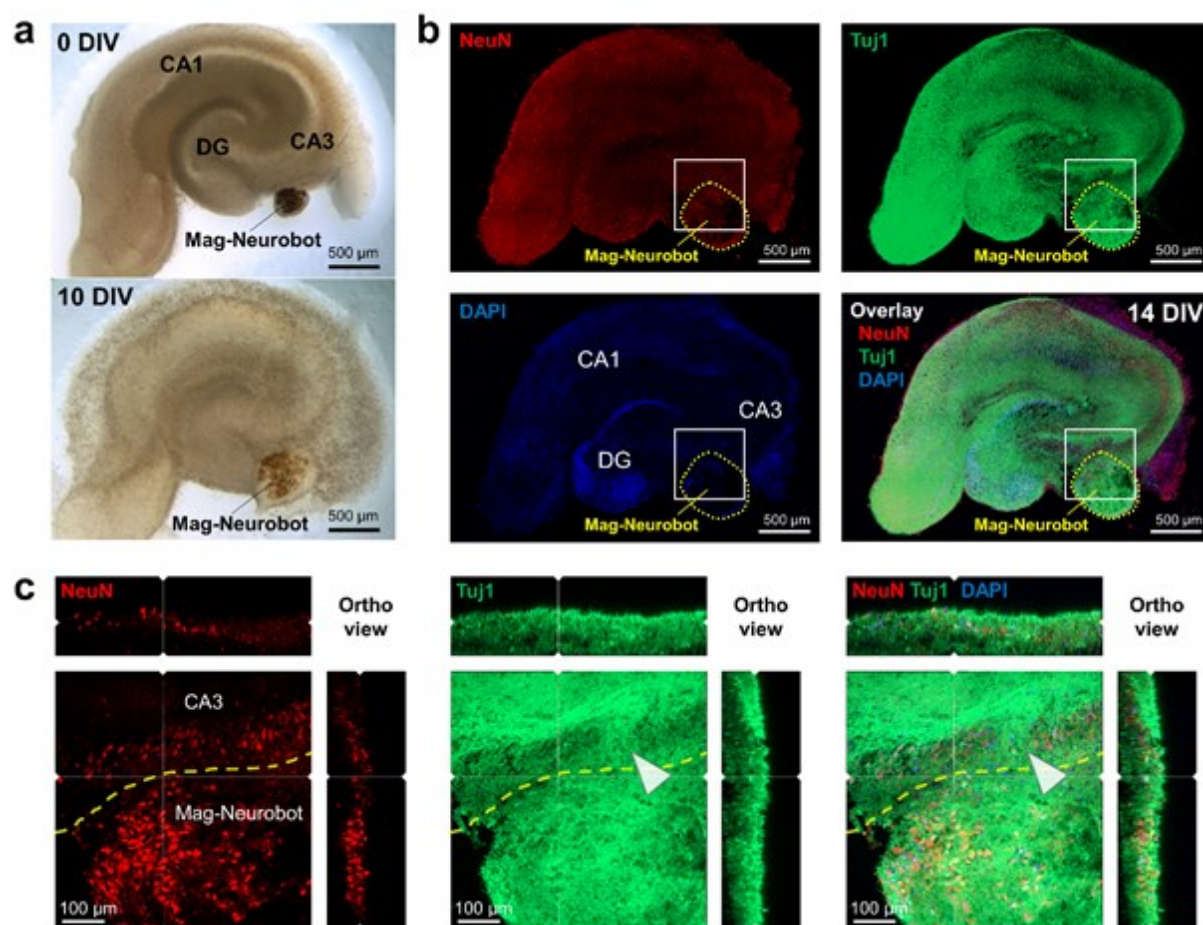


Figure 6. Morphological connections of the OHS with the Mag-Neurobot. a) Bright-field images of the OHS with the Mag-Neurobot on the cell culture insert membrane at 0 and 10 DIV. b) Immunostaining of the OHS cultured with the Mag-Neurobot (neuronal nuclei [NeuN], red; neuronal marker [Tuj1], green; nuclei, blue). c) Ortho view images of the white square box in (b). White arrows indicate neuronal connections formed between the OHS and the Mag-Neurobot.

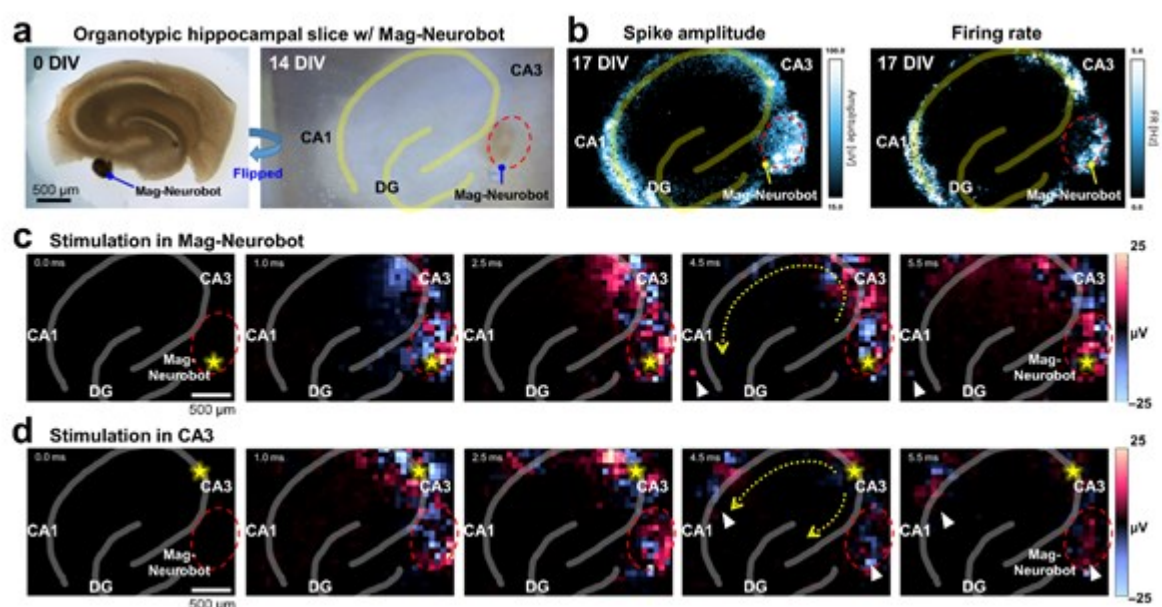
2.6. Functional connectivity between the OHS and the Mag-Neurobot

Finally, the functional connectivity of the Mag-Neurobot engrafted onto the OHS was investigated using HD-MEAs (**Figure 7**). The Mag-Neurobot was positioned in the CA3 region of the OHS (Figure 7a, left). After 10 DIV, the cell culture insert membranes on which OHSs had been cultured with and without the Mag-Neurobot were flipped, placed onto the HD-MEA chip (Figure S13b, Supporting Information), and incubated for an additional 3–7 days. At this time, about 3 weeks had passed since cells of the Mag-Neurobot had been obtained from embryos (Figure S11c, Supporting Information). The dark brown color of the Mag-Neurobot present in the bright-field images revealed its location in the OHS (Figure 7a, right panel). The spontaneous neural activity of the OHS connected with the Mag-Neurobot was identified by maps of the mean firing rate and spontaneous spike amplitude (Figure 7b, Figure S14 and Movie S4, Supporting Information). As shown in Figure S14c (Supporting Information), AP traces show that most neurons in CA3, CA1, and the Mag-Neurobot areas were bursting and thus were synchronously active, implying their connections.

The functional neuronal connectivity between the OHS and the Mag-Neurobot was examined by measuring propagating neuronal activation in selectively stimulated

neurons surrounding two different areas: the Mag-Neurobot and CA3. Because the Mag-Neurobot forms functional output connectivity, its stimulation showed signal propagation to CA3 (at ~2.5 ms) and CA1 (at ~4.5 ms) (Figure 7c, Figure S14d, and Movie S5, Supporting Information). Upon stimulation in the CA3 region, neural spikes propagated to the Mag-Neurobot (at ~1 ms) and to CA1 (at ~4.5 ms), indicating functional input connections from existing neural circuits to the Mag-Neurobot (Figure 7d, Figure S14e, and Movie S5, Supporting Information). As shown in Figures S14d and S14e (Supporting Information), stimulus-triggered average activities show that electrical stimulation evoked propagating APs between the Mag-Neurobot and the CA3 region. These stimulation-induced responses were consistent with functional connectivity between the Mag-Neurobot and cells in the CA3 region, after the transport of the Mag-Neurobot and its co-culture with the OHS.^[84-85] In the OHS without the Mag-Neurobot (control) (Figure S15a, Supporting Information), the spontaneous spike amplitude map showed activity mainly in the CA3 and CA1 regions (Figure S15b, Supporting Information). AP traces show that parts of neurons neighboring the CA3 region produced synchronous spike activities, implying their neuronal connections (Figure S15c, Supporting Information). Electrical stimulation in the CA3 region demonstrated propagating neural activity around

CA3 (at ~1.5 ms) and CA1 (at ~4.0 ms) (Figure S15d, Supporting Information). As shown in Figure S15e (Supporting Information), stimulus-triggered average activities also show evoked APs in neighboring neurons of the CA3 region. As in previous analyses of neural activity in an OHS,^[86-87] the OHS in this study showed functional neural activity in the CA3 region after its co-culture and integration with the transported Mag-Neurobot. These results demonstrate that the Mag-Neurobot delivered by magnetic manipulation can form functional neural connections with neighboring cells of the OHS in an ex vivo environment.



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Figure 7. Spontaneous neural activity and signal propagation after stimulation of the OHS co-cultured with the Mag-Neurobot. a) Microscopy images of the OHS with the Mag-Neurobot on the cell culture insert membrane and an HD-MEA chip. b) Spike amplitude and firing rate of the OHS with the Mag-Neurobot. c) Electrical stimulation of the Mag-Neurobot results in neural signal propagation to the CA3 and CA1 regions. d) Electrical stimulation in the CA3 region results in neural signal propagation to both the Mag-Neurobot and the CA1 region.

3. Conclusion

The reconstruction of damaged brain circuits requires the precise delivery of healthy and functional neurons to the target area, followed by their incorporation into existing brain tissue through stable synaptic connections. Our ex vivo study showed that the Mag-Neurobot, loaded with up to 350 ng of SPIONs, can meet these requirements. SPIONs had no effect on normal neuronal function, consistent with the viability and intact membrane properties of the cells; moreover, SPIONs did not impact neurite outgrowth or neural activity. The results of patch-clamp recordings of hippocampal neurons indicated that SPIONs did not alter neuronal excitability or synaptic function. The precise control of a magnetic field allowed the delivery of the Mag-Neurobot to the target brain

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region, where it strengthened existing synaptic connections by normal differentiation and formed new synaptic connections, thereby allowing regeneration of the neuronal circuit. The functional connectivity of the Mag-Neurobot with the hippocampal slice was also successfully demonstrated by recording electrical stimulation-evoked signals. Further studies are needed to determine whether neurons delivered by the Mag-Neurobot remain healthy and normal for an extended period in vivo and develop functional specificity, consistent with the phenotypes of other neurons in vivo (e.g., orientation selectivity in the primary visual cortex). Nonetheless, our study provides an essential bridge toward the reconstruction and circumvention of damaged neural circuits.

Moreover, the magnetic cell spheroid-organotypic slice connection based on an HD-MEA described in this study may be useful as a functional verification platform for targeted cell delivery, thus enabling characterization of the functional properties of transported and transplanted cells. The designated and targeted 3D neural networks can be engineered to allow studies of the mechanisms that underlie reconstructed neuronal connections. Mag-Neurobot-based approaches can therefore serve as valuable tools in the field of neuroengineering and neuroscience related to regenerative medicine.

4. Experimental Section

Surface modification of SPIONs with PLL: SPIONs (Fe_3O_4 ; 15–20 nm, 20 wt%) dispersed in deionized water were purchased from US Research Nanomaterials, Inc. (Houston, TX, USA). PLL (Sigma-Aldrich, St. Louis, MO, USA; cat.# P5899) was used to functionalize the SPION surface. Fifty microliters of SPIONs were added to a mixture of 0.45 mL of PLL (1 mg mL^{-1}), 1 mL of deionized water, and 1 mL of serum-free neurobasal medium (Thermo Fisher Scientific, Waltham, MA, USA; cat.# 21103049). The preparation was sonicated for 2 h, washed three times with serum-free neurobasal medium, and then resuspended in cell culture medium to a final SPIONs concentration of 1 mg mL^{-1} .

Isolation of PHNs and fabrication of magnetic spheroids: Pregnant Sprague Dawley rats (E18) were purchased from KOATECH (Gyeonggi-do, South Korea) and euthanized to obtain PHNs, in accordance with a previously described protocol.^[88] All procedures were conducted in accordance with the regulations of the Animal Experiment Ethics Committee of the DGIST (approval no. DGIST-IACUC-19050903-08). The rat embryos were decapitated; the entire hippocampus from each embryo was trypsinized and dissociated with 0.25% trypsin-ethylenediaminetetraacetic acid (Thermo Fisher Scientific; cat.# 25200056), then washed with phosphate-buffered saline (Thermo Fisher Scientific; cat.# 10010023) and subjected to mechanical trituration. The cell density was determined using an automated cell counter (Countess II FL, Thermo Fisher Scientific) and diluted to $3.5 \times 10^4 \text{ cells mL}^{-1}$. To generate

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3D neurospheroids without SPIONs (control), 3,500 cells per well were plated in a U-shaped-bottom 96-well microplate (Thermo Fisher Scientific; cat.# 174925). For Mag-Neurobot, spheroids were formed using 3.5 μ L of a stock solution of PLL-coated SPIONs (1 mg mL⁻¹) that contained ~350 ng of SPIONs. The control neurospheroid and the Mag-Neurobot were cultured in supplemented neurobasal medium that contained 1% L-glutamine (Thermo Fisher Scientific; cat.# 25030149), 2% B-27 supplement (Thermo Fisher Scientific; cat.# 17504044), and 1% penicillin/streptomycin (Thermo Fisher Scientific; cat.# 15140122). Both the control neurospheroid and the Mag-Neurobot were maintained in a humidified incubator (5% CO₂ and 37°C).

Spheroid cryosectioning and magnetic characterization. Before cryosectioning, each Mag-Neurobot was fixed with 4% paraformaldehyde (Sigma-Aldrich) in phosphate-buffered saline at 4°C overnight and stored in a 30% (wt vol⁻¹) sucrose solution (Sigma-Aldrich; cat.# S7903) at 4°C overnight, in accordance with a modified version of a previously described protocol.^[89] The Mag-Neurobot was then immersed in a warmed gelatin (Sigma-Aldrich; cat.# G1890-100G) sucrose solution at 37°C for 15 min, embedded in the Tissue-Tek optimal cutting temperature compound (Sakura Finetek USA Inc., Torrance, CA, USA; cat.# 4583), and frozen at – 80°C. Each frozen specimen was cut into 20- μ m-thick sections at – 20°C using a cryostat cryosectioning system (Leica CM3050S,

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Leica Microsystems, Nussloch, Germany) and mounted onto cover slips. The samples were examined by field emission scanning electron microscopy (S-4800, Hitachi, Japan). The magnetic properties of each fixed Mag-Neurobot were analyzed using a vibrating sample magnetometer (7400-S Series, Lake Shore Cryotronics Inc., Westerville, OH, USA).

Invasion study. To assess neurite outgrowth in the 3D environment, the control neurospheroid and the Mag-Neurobot were placed in a flat-bottom 96-well plate (Corning Inc., NY, USA; cat.# 3474), embedded in growth factor-reduced Matrigel (Corning; cat.# 354230), and cultured for 10 days.

Preparation of organotypic hippocampal slices. OHSs were prepared from 7-day-old postnatal rat pups from wild-type Sprague–Dawley rats, as previously described.^[56, 58, 90] Briefly, after decapitation of the pups, the extracted brains were gently removed and stored in ice-cold oxygenated (95% O₂ + 5% CO₂) Hanks' balanced salt solution (Thermo Fisher Scientific; cat.# 14175-095) mixed with D-glucose (45%, Sigma-Aldrich; cat.# G87691), kynurenic acid (1 mM, Tocris), and penicillin/streptomycin (5 U mL⁻¹, Gibco; cat.# 15140) under sterile conditions. The hippocampi were dissected under microscopic

guidance and embedded in 1% low melting temperature agarose (Research Products International Corp., Mount Prospect, IL, USA; cat.# A20070-250.0). Sagittal OHSs (300 μ m thickness) were prepared using a vibratome (Leica VT1200 S); slices with intact DG and CA regions were identified using a microscope. The OHSs were then placed on air-fluid interface-style Millicell culture inserts (Millipore, Bedford, MA, USA; cat.# PICM0RG50, 30 mm, hydrophilic polytetrafluoroethylene, 0.4 μ m) in 6-well culture plates (Thermo Fisher Scientific).^[91-92] OHSs with or without a Mag-Neurobot were maintained in culture medium containing L-glutamine-free Eagle's basal medium (Thermo Fisher Scientific; cat.# 21010-046), Hanks' balanced salt solution (Thermo Fisher Scientific), inactivated horse serum (Thermo Fisher Scientific; cat.# 26050-088), 45% D-glucose (Sigma-Aldrich), and GlutaMAX (Thermo Fisher Scientific; cat.# 35050-061), penicillin/streptomycin, and B-27 supplement. After 3 days the culture medium was replaced with B-27-free culture medium, and the medium was changed completely three times per week.

Cell viability assay, immunostaining, and optical imaging. The viabilities of the control neurospheroid and the Mag-Neurobot were examined using the Live/Dead™ cell imaging kit (Thermo Fisher Scientific; cat.# R37601). Briefly, the samples were incubated for 15

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min at room temperature in working solution that had been prepared in accordance with the manufacturer's instructions. For immunostaining, the control neurospheroid and the Mag-Neurobot were immunostained to visualize the neuronal cell marker β -III tubulin Tuj1 (red) and the neuronal nuclei marker NeuN (green). Cell nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI, blue). Cells that had been fixed with 4% paraformaldehyde at 4°C overnight were permeabilized in 0.25% Triton X-100 (Sigma-Aldrich) at room temperature for 1 h, then incubated in blocking buffer that consisted of phosphate-buffered saline supplemented with 1% bovine serum albumin (Sigma-Aldrich) at room temperature for 1 h. Primary antibodies against β -III tubulin (Abcam, Cambridge, UK; cat.# ab18207) and NeuN (Abcam; cat.# ab177487) were diluted to 1:1000 (v/v) in blocking buffer and incubated with the neuronal cultures at 4°C overnight. Next, Alexa Fluor 647 (Thermo Fisher Scientific; cat.# A21244) and Alexa Fluor 488 (Thermo Fisher Scientific; cat.# A11034) secondary antibodies were diluted 1:1000 (v/v) and incubated with the samples in blocking buffer at room temperature for 2 h. DAPI staining (1:1000 dilution; Thermo Fisher Scientific; cat.# D1306) was used to visualize nuclei. Alexa 488 (red) and Alexa 647 (green) staining of the immunostained OHS improved the visualization of the observed images (Figure 6, Movie S3, Supporting Information). Prior

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to confocal imaging, optical clearing solution (C Match, Crayon Technologies, Inc, South Korea; cat.# 50-3010) was placed dropwise onto the immunostained Mag-Neurobot and the OHS to match the refractive index of the samples and preserve maximum fluorescence signals for optical imaging.^[93] A confocal laser scanning microscope (LSM 780; Carl Zeiss) equipped with solid-state lasers (405, 488, 555, and 639 nm) was used for fluorescence imaging. Maximum intensity projection and 3D reconstruction were conducted using ZEN software (Carl Zeiss).

Magnetic manipulation: An electromagnetic coil system (OctoMag; Aeon Scientific AG, Schlieren, Switzerland) was used to control the position and orientation of the Mag-Neurobot. A rotating magnetic field, with a strength of 40 mT and a frequency of 3 Hz that resulted in a rolling motion, was used to accurately drive the Mag-Neurobot to the target location. The manipulations of the Mag-Neurobot in a 35-mm petri dish were recorded using a VZM 600i zoom imaging lens (Edmund Optics Inc., Barrington, NJ, USA) linked with a charge-coupled device camera (Grasshopper; Point Gray Research, Inc., Richmond, Canada) (Movie S1 and S2, Supporting Information).

Electrophysiological recordings. For patch-clamp recordings of PHNs, neurons that had been cultured for 15–16 days were transferred to a recording chamber with a continuous flow of artificial cerebrospinal fluid bubbled with carbogen (95% O₂, 5% CO₂) (Figure S11b, Supporting Information). The artificial cerebrospinal fluid contained (in mM): 119 NaCl, 2.5 KCl, 26 NaHCO₃, 1.25 NaH₂PO₄, 20 glucose, 2 CaCl₂, 1 MgSO₄, 0.4 ascorbic acid, and 2 pyruvic acid. In some recordings, biocytin (1 mg mL⁻¹) was included in the internal solution for subsequent immunocytochemistry. PHNs were visualized by infrared differential interference contrast microscopy using a BX51WI upright microscope (Olympus, Japan). Patch pipettes with 3–5-MΩ tip resistances were pulled using a horizontal puller (Sutter Instruments, USA) and filled with an internal solution that contained (in mM): 20 KCl, 125 K-gluconate, 10 HEPES, 4 NaCl, 0.5 EGTA, 4 adenosine triphosphate, 0.3 Tris-guanosine triphosphate, and 10 phosphocreatine (pH 7.2, ~290–300 mOsm).

PHN excitability was determined by measuring voltage changes in response to various 800-ms current pulses (–200 to 200 pA, in 50-pA increments). Passive membrane properties, including whole-cell membrane capacitance (C_m), RMPs, and input resistance (R_{in}), were determined 10 min after whole-cell analysis. The input resistance (R_{in}) was

calculated as the slope of the voltage change as a function of the injected current (– 200 pA to – 50 pA hyperpolarizing steps). The membrane potential was not adjusted with respect to the liquid junction potential. The AP threshold and width were measured based on the first AP evoked by the injection of a 100-pA depolarizing current (Figure 3e). The voltage clamp was used to record spontaneous synaptic events. sEPSCs and sIPSCs were recorded at holding potentials of – 70 mV and 0 mV, respectively.

All electrophysiological recordings were performed at 28–30°C and obtained using a Multiclamp 700B amplifier. The recordings were digitalized using a Digidata 1550A, controlled with the Multiclamp Commander and the pClamp10 (Molecular Devices, San Jose, CA, USA). Data acquisition and analysis were performed using Clampfit 10.4 (Axon Instruments, CA, USA), IGOR Pro software (Wavemetrics, OR, USA), and MATLAB (MathWorks Inc., Natick, MA, USA).

Electrophysiology analyses. Electrophysiological recordings of the neurospheroids (control and Mag-Neurobots) and organotypic slices with or without the Mag-Neurobot were obtained. Extracellular recordings using the HD-MEA system (MaxOne; MaxWell Biosystems AG, Basel, Switzerland) were acquired during activity scan (spike amplitude

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and firing rate) and network (raster plot and burst duration) assays and via stimulation-evoked signals inside the incubator. The HD-MEA chip was sterilized in 70% ethanol for 30 min, then coated with 0.07% polyethyleneimine (Sigma-Aldrich)^[94] and laminin (0.02 mg mL⁻¹, Sigma-Aldrich; cat.# L2020), before attachment of the spheroid or the slice on a membrane piece. All signals recorded on the HD-MEA were sampled at 20 kHz, and a threshold detection was applied (5× the standard noise deviation). All analyses described below were performed using MATLAB R2019b (MathWorks Inc.) with the "Signal Processing" and "Statistics and Machine Learning" toolboxes.

To extract extracellular APs, the signals of the control neurospheroid and the Mag-Neurobot recorded by HD-MEAs were measured at 8 DIV (about 2 weeks after cell isolation) and filtered with a digital band-pass (3–300 kHz) (Figure S11a, Supporting Information). As shown in Figure S11c (Supporting Information), for organotypic slices with or without the Mag-Neurobot, spontaneous spike recordings were measured at about 3 weeks after cell isolation and digitally band-pass filtered (1–3 kHz). The entire array with 26,400 electrodes was sequentially scanned with 27 electrode configurations for 20 s per configuration. For each measured result recorded in the activity scan assay, spikes were obtained when the negative voltage peak crossed a threshold, which was set

to fivefold greater than the standard deviation.^[95] Spontaneous spike amplitude maps were obtained to visualize the viability of the cultures and the positions of spontaneously active cells on the array (Movie S4, Supporting Information).

Neural activity propagation evoked by the electrical stimulation was analyzed to identify neuronal connections between the OHS and the control or the Mag-Neurobot. Electrodes near axon initial segments were selected as stimulation positions because, compared with other neuronal positions, this position required the lowest voltage for effective and selective activation of individual neurons in the network.^[96] Voltage pulses with phase durations of 200 μ s; amplitudes of $\pm$ 500, 800, and 1,000 mV; and 200 stimuli at a frequency of 10 Hz were applied. Nine electrodes were connected to a stimulation channel. Recorded stimulus-induced responses (200 trials) were temporally aligned according to the downswing of the biphasic stimulation. Aligned responses were used to generate the neural activity propagation results (Movie S5, Supporting Information).

Statistical analysis. Experimental data were represented as means $\pm$ standard deviations (S.D.s), standard errors of the mean, or minimum and maximum values through at least three separate experiments. Statistical significance was assessed by

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unpaired two-tailed Student's *t*-test with Welch's correction. A *p* value of 0.05 was selected as a significance level, and the data are indicated with **p* < 0.05, ***p* < 0.01, ****p* < 0.001; *ns* represents "no significant difference" between two groups, respectively. Statistical analyses were performed on GraphPad Prism 5 (GraphPad Software, San Diego, CA, USA).

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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Table of Contents (TOC)

Using an external rotating magnetic field, a magnetic neurospheroid consisting of primary hippocampal neurons and superparamagnetic nanoparticles can be precisely delivered to the target area in an ex vivo brain slice, where the neurospheroid subsequently establishes structural and functional neuronal connectivity. This method can be used to examine ex vivo functional neuronal connections and neuronal regeneration processes.

Eunhee Kim, Sungwoong Jeon, Yoon-Sil Yang, Chaewon Jin, Jin-young Kim, Yong-Seok Oh, Jong-Cheol Rah, and Hongsoo Choi

Title: A neurospheroid-based microrobot for targeted neural connections in a hippocampal slice

