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Generation of Patterned Neuronal Networks on Cell-Repellant Poly(oligo(ethylene glycol) Methacrylate) Films

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Abstract: The utilization of non-biofouling poly(oligo(ethylene glycol) methacrylate) (*p*OEGMA) films as a background material for the generation of neuronal patterns is reported here. Our previously reported method, which was surface-initiated, atom transfer radical polymerization of OEGMA, and subsequent activation of terminal hydroxyl groups of *p*OEGMA with disuccinimidyl carbonate, was employed for the generation of activated *p*OEGMA films on glass. Poly-L-lysine was then microcontact-printed onto the activated polymer films, followed by backfilling with poly(ethylene glycol) moieties. E18 hippocampal neurons were cultured on the chemically patterned substrate, and the resulting neuronal networks were analyzed by phase-contrast microscopy and whole-cell patch clamp method. The results indicated that the *p*OEGMA films played an important role in the generation of good-quality neuronal patterns for up to two weeks without any negative effects to neurons.

Keywords: biological activity · cell adhesion · neurochemistry · polymers · surface chemistry

Introduction

Patterning neurons in vitro to form an ordered neuronal network,[1–7] has a tremendous impact on neural engineering as well as fundamental studies of neural sciences. In the field of neural engineering, neuronal patterns have been applied to neuroelectronic interfaces,[8–10] and neuron-based sensors[11] for improving the quality of signals, and obtaining isolated network systems. Neuronal patterns have also been used as a model system for studying network connectivity,[12,13] axonal[14–16] and neuronal regeneration,[17] and network plasticity.[18]

The neuron-patterning techniques generally require chemical micropatterning of neuron-adhesive regions or neuron-repellent regions, or both. Extracellular matrix (ECM) proteins,[6,12,13] positively charged organosilane molecules,[1,3] and positively charged polymers, such as polylysine[2,4,5,7,8] and polyethyleneimine,[19] utilizing biospecific or electrostatic interactions between surfaces and cell membranes of neurons, have been reported to be effective in the adherence of neurons onto surfaces. The hydrophobic organosilanes,[3] single-chain poly(ethylene glycol) (PEG),[4] and even commercialized bare Petri dish surfaces made of polystyrene,[6,12,13] on the other hand, have been used for repelling neuronal adhesion at unwanted regions.

The high-quality neuron-repellent background is imperative if long-term, high-precision neuronal patterning is required in experiments. While neuron-adhesive molecules determine the viability and functionality of neurons, the quality of the cell-repellent background plays a crucial role in terms of the fidelity and longevity of neuronal patterns. Wheeler et al. showed that the long-term, high-fidelity patterning could be achieved with a relatively simple cell-adhe-

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sive material, polylysine, by engineering the cell-repellent background region.[4]

Conventionally, ethylene glycol (EG) groups have mostly been used as a cell-repellent moiety because of their good biocompatibility, low toxicity, non-immunogenicity, and high water solubility.[20–23] Recently, surface-initiated, atom transfer radical polymerization (SI-ATRP) of oligo(ethylene glycol) methacrylate (OEGMA), leading to the formation of *p*OEGMA films, has been introduced as a new method for generating high-density and high-coverage EG brushes on gold and silicon oxide surfaces by Chilkoti et al.[24,25] According to their results, the *p*OEGMA films could repel protein absorption and cell adhesion effectively due to the high surface coverage of EG groups. In addition, the *p*OEGMA films were further derivatized by our group for biological applications including highly specific biomolecular interaction study,[26] protein and cell adhesion study using surface plasmon resonance (SPR) and quartz crystal microbalance (QCM),[27] and microarray-type bioassay platform construction,[28] taking advantage of post-modifications of the terminal hydroxyl group of *p*OEGMA. In comparison with the single-chain PEG, the *p*OEGMA film exhibited superior cell-repelling ability and possessed more robust and reproducible structure with controllable thickness.

Herein, we report the generation of neuronal patterns based on the selective functionalization of the *p*OEGMA film. The objectives of this work were (1) to suggest an application of *p*OEGMA polymer films as a neuron-repellant background material, and (2) to demonstrate a neuron-patterning technique using conjugation chemistry targeting terminal hydroxyl groups of the polymer film. Micropatterns of poly-L-lysine (PLL) were generated on the functionalized *p*OEGMA film by microcontact printing (μCP), and E18 rat hippocampal neurons were cultured on the patterned polymer films up to two weeks. The results showed that in vitro neuronal networks were successfully formed on the *p*OEGMA films with high precision and fidelity for two weeks, and the patterned neurons were electrophysiologically functional for two weeks. To the best of our knowledge, this is the first report using EG-based polymers grown directly from a substrate as a cell-repellent background for neuronal patterning.

Results and Discussion

Formation of PLL Patterns on *p*OEGMA Films

The chemical reaction to form the *p*OEGMA film followed by the DSC activation (Figure 1a) was confirmed by X-ray photoelectron spectroscopy (XPS). After 1 h of polymerization, we obtained the *p*OEGMA film with the thickness of 10 nm. The thickness was highly reproducible in every reaction, and uniform throughout the entire surface of the substrate. The dramatic increase of the ratio of C 1s peak to Si 2s, Si 2p, Br 3p, and Br 3d peaks after the polymerization confirmed the formation of the *p*OEGMA film on the surface (Figure 1b). To immobilize PLL onto the *p*OEGMA films, the terminal hydroxyl groups should be converted into the groups which could readily be coupled with amine groups in PLL. For that, the terminal hydroxyl groups were replaced with *N*-hydroxysuccinimide (NHS) groups by immersing the samples in a DMF solution of DSC and DMAP.

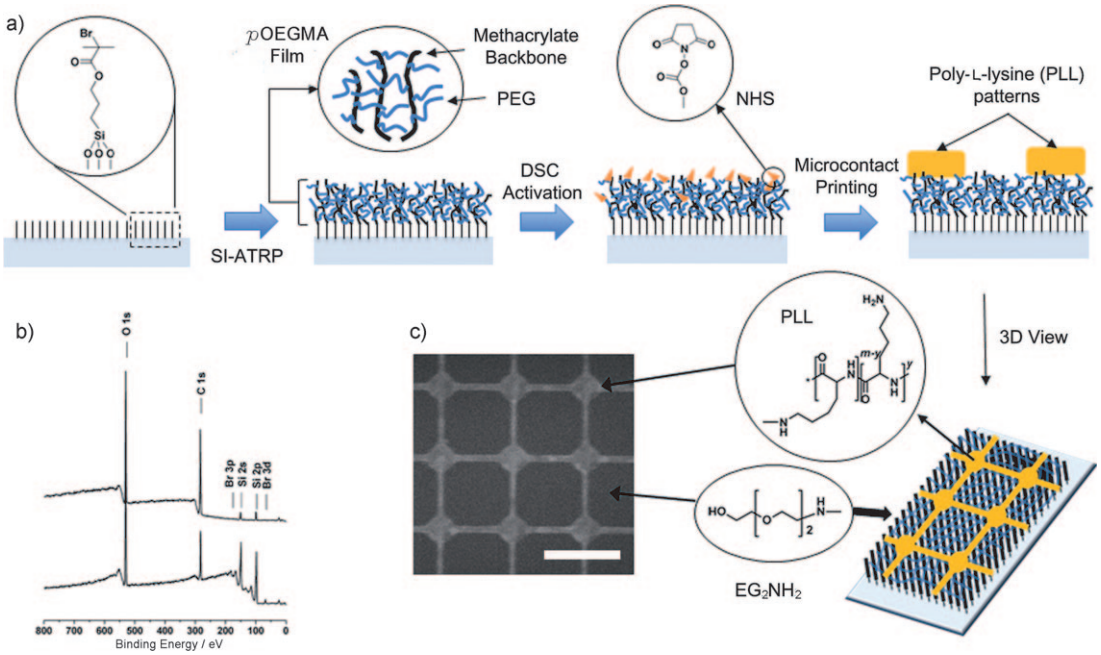

Figure 1. a) Schematic illustration of the procedures for generating PLL patterns on *p*OEGMA films. b) Wide-scan XPS spectra of the gold substrates: (lower trace) initiator-presenting SAMs, (upper trace) intact *p*OEGMA films. c) Conformational illustration of stamped grid patterns of PLL. d) Fluorescence micrograph of the gird pattern generated by stamping PLL-FITC on a *p*OEGMA film. Node diameter is 40 μm, line width is 10 μm, and spacing between nodes is 100 μm. Scale bar is 100 μm.

A previous report on this reaction[26] proved that almost all of the hydroxyl groups of *p*OEGMA were converted to NHS groups under the same reaction condition, according to polarized infrared external reflectance spectroscopy (PIERS) spectra. Therefore, by combining the polymer formation and subsequent coupling chemistry, the properties from both polymer and specific terminal functional groups could be introduced into one substrate.[27] We also confirmed by IR and XPS spectra that this reaction effectively generated the NHS moiety on the surface of *p*OEGMA films (see the Supporting Information).

The PLL patterns were successfully generated on the *p*OEGMA films by using microcontact printing. Figure 1c represents a fluorescence image of the stamped FITC-conjugated PLL pattern on the activated film. After stamping, the remaining regions of the films were backfilled with short PEG (EG_2NH_2) moieties. These short PEG moieties were used to replace remaining NHS groups from the film surfaces. Because these short PEG moieties were added to the original PEG brushes from the *p*OEGMA films, the chemical conformations between unmodified films and backfilled films were basically identical (Figure 1a). The advanced non-biofouling ability of *p*OEGMA films was not affected by terminal modifications, because the effect was due to the densely packed PEG brushes, not terminal hydroxyl groups. As expected, designed patterns (line and grid patterns) were successfully delivered to the surfaces of the NHS-modified films and maintained during stamping and backfilling procedures. The fluorescence patterns were further maintained for more than a week in culture medium (data not shown). From this result, we could speculate that amide bonds between amine groups in PLL and NHS groups from the surfaces were firmly formed.

Most of the previously reported methods led to the generation of micropatterns directly on SAM-terminated or oxide-based surfaces. In these cases, the uniformity of the surfaces is relatively low and varies depending on the materials of which substrates are composed. In addition, there are possibilities for inherent defects from the substrates, which may deteriorate the chemical properties of the surfaces. In contrast, in our case, the surface-grafted polymer films were grown from the entire substrate as a background platform, which results in the formation of uniformly functional (cell-repellent) surfaces. These polymer-based surfaces exhibit more uniform and controllable chemical and physical properties. Because the polymer films are relatively thicker, they cover and weaken inherent defects, and make the chemical properties of the surfaces independent of the substrate materials. Previously, more robust structure and controllable thickness of the *p*OEGMA films were reported.[24] This robustness enabled the *p*OEGMA films to yield the advanced non-biofouling effect, although the exact mechanism of this effect is still not claimed yet. Combining superior physical properties and various conjugation chemistries, *p*OEGMA films are expected to play a versatile role in the applications of precisely constructed neuronal patterns as a background platform.

In Vitro Neuronal Network Formation

A simple linear neuronal network was tested by stamping 50 μm wide PLL patterns on the *p*OEGMA films. The cultured neuronal network was observed periodically using a phase-contrast microscope. Neurons were well confined to the PLL lines that were spaced by 100 μm for two weeks (Figure 2). As shown in Figure 2a, somata adhered on the PLL lines, and neurites tended to follow the edge of the lines for the first week. There was rarely any direct cell adhesion or neurite growth or extension on the background EG-based repulsive region at seven days in vitro (DIV). By two weeks after the plating, there were some occurrences of neurite bridging between neighboring lines, but the majority of neurites and somata remained in the lines (Figure 2b).

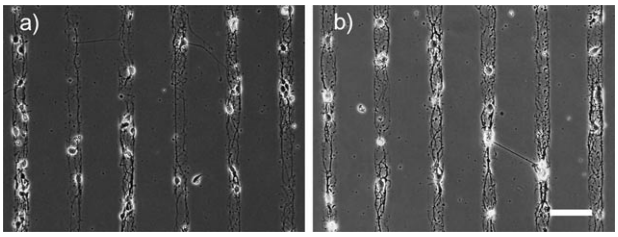

Figure 2. Phase-contrast optical micrographs of line patterns of E18 hippocampal neuron cells after a) 7 DIV and b) 13 DIV. Scale bar is 100 μm.

A grid shape of neuronal networks was constructed to investigate the longevity of the resulting neuronal patterns and the compliance of the overall network morphology to the underlying PLL patterns. The pattern was composed of thin lines (width: 10 μm) and relatively large nodal area (diameter: 50 μm). The grid pattern was more complex than the line patterns, and the quality of the resulting neuronal patterns could be affected by mechanical forces between neurons, culture density, the degree of neuronal aggregation, and the quality of the cell repellent background materials.[4,29,30] Figure 3a shows a representative neuronal network cultured on the PLL-patterned *p*OEGMA film at 3 DIV. In three days, most of the somata (cell bodies) were located at the nodal areas in contrast to thin lines connecting the nodes, and there were rich neurite outgrowths to thin lines. Single neurites and growth cones following the underlying

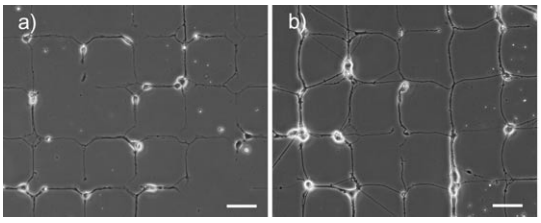

Figure 3. Phase-contrast optical micrographs of grid patterns of E18 hippocampal neuron cells after a) 3 DIV and b) 11 DIV. Node diameter is 40 μm, line width is 10 μm, and spacing between nodes is 100 μm. Scale bar is 100 μm.

PLL patterns were clearly observed as well. After 11 DIV, neuronal clusters that were formed by a few neurons nearby and the fasciculation of neurites forming neurite bundles were frequently observed in many parts of the patterned neuronal networks (Figure 3b). Neurites were bridging over the background squares, and the compliance of the neuronal network to the pattern seemed to be compromised.

We analyzed the change of the pattern compliance by following the quantitative measure used by Branch et al.[4] First, the percentage of the somata located at the nodal area was calculated to measure the compliance of the neurons to the underlying grid pattern. As shown in Table 1, nearly

Table 1. Fidelity of the neuronal patterns.[a]

	3 DIV ($N=6$)	7 DIV ($N=12$)	11 DIV ($N=9$)
Neurons on nodes [%]	86.60±6.41	87.30±5.55	88.67±4.55
Crossed regions [%]	2.03±0.43	10.35±1.13	20.87±2.96

[a] Mean±SD; N: the number of analyses.

86% of neuron cell bodies (somata) in the pattern were located on the nodes up to 11 DIV, which was the longest period that the culture was maintained in this study. Although there were neuronal clusters after 11 DIV, they were still highly localized at the nodal areas. Second, the percentage of the off-pattern squares (the *p*OEGMA film) free of neural processes (neurites and somata) was measured. At the early stage of the culture (3 DIV), 98% of the squares were free of any neurites or somata. This matched well with our qualitative observation that nearly all neurites and somata were growing on the PLL patterned areas. The percentage decreased to 80% at 11 DIV (Table 1), which was mostly due to the cross-bridging of neurite bundles. The measured high compliance of neuronal networks at 3 DIV was due to the neuronal repulsiveness of the *p*OEGMA film in the off-pattern area, while the degradation of the compliance at 11 DIV were mostly due to the complicated dynamic nature of the cell-cell interaction in neuronal cultures.[31]

Electrophysiology

The whole-cell patch clamp recording was used to characterize the electrophysiological properties of neurons in the patterned networks at 10 DIV. The resting potential of the neurons was −46.6±2.6 mV ($n=6$, mean ±S.E.), which was similar to other reports.[30,32] Action currents and potentials triggered by membrane depolarization are shown in Figure 4. These measurements implied that the neuronal networks on the *p*OEGMA films were active after 10 days, and voltage-sensitive sodium and rectifying potassium channels were normally expressed in the patterned neuronal networks, since transient inward sodium currents and delayed outward potassium currents were clearly exhibited (Figure 4a). Action potentials were also observed in current clamping modes with a threshold at about 350 pA (Figure 4b).

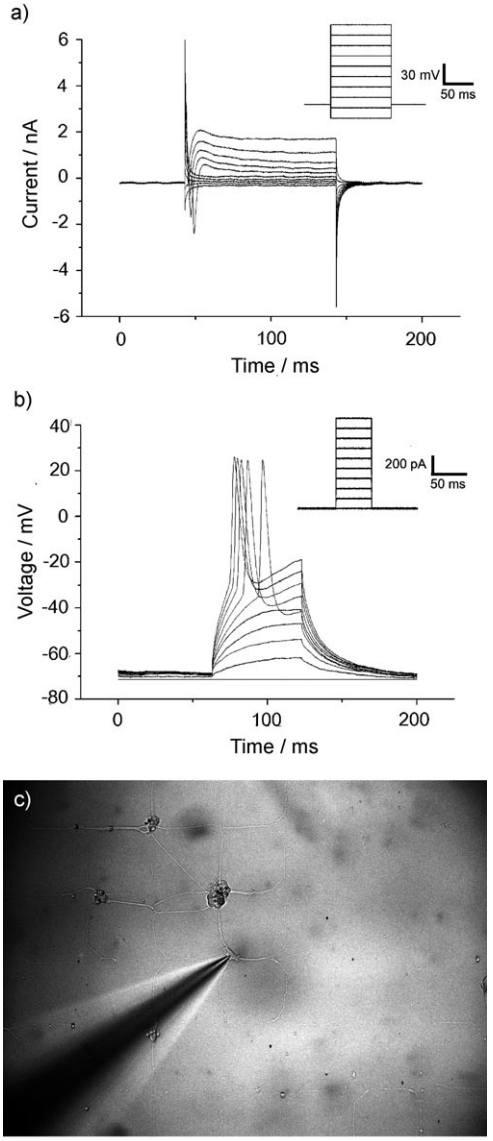

Figure 4. Electrophysiological recordings of patterned hippocampal neurons at 10 DIV. a) Voltage clamping recordings. The neurons were set to 10 different voltages starting from −100 mV sequentially (inset), and their currents were recorded. Clamping time was 100 ms. b) Current clamping recordings. Sequential 9 different currents starting from −150 pA were injected into the neurons (inset), and their membrane potentials were recorded. Injecting time was 50 ms. c) A representative image of the patched neurons.

This result also implied that we could construct a minimal but behaving neuronal circuits on *p*OEGMA films for biomedical engineering applications, such as neuron-based sensors and neuronal chips.[7,11,32–35]

Conclusions

In summary, we demonstrated a possibility of the *p*OEGMA applications for in vitro neural tissue engineering. We obtained the well-ordered in vitro neuronal net-

works using the *p*OEGMA films as a background material, and the viability and functional normality of patterned neurons were confirmed by optical microscopy and whole-cell patch clamp recording, respectively. The *p*OEGMA film constantly maintained good cell-repelling ability without any collapse of conjugated polylysines, as informed by the viability and functionality of neurons on top, and therefore a solid network of neurons could be obtained. The combination of chemistry based on *p*OEGMA polymers and in vitro nerve cell patterning is expected to be useful in neuron-based sensor applications and studying fundamental neural science, which require more stable and precisely patterned neuronal networks.

Experimental Section

PLL-patterned *p*OEGMA films on glass substrates were prepared according to the literature (Figure 1a).[26] For confirming the polymerization, Si/SiO_2 substrates were used.

Materials

Absolute ethanol (EtOH, 99.9+%, Merck), absolute methanol (MeOH, 99.9+%, Merck), anhydrous *N,N′*-dimethylformamide (DMF, 99.8+%, Aldrich), toluene (J. T. Baker), 3-(trimethoxysilylpropyl)-2-bromo-2-methylpropionate (ATRP initiator, Gelest Inc.), copper(I) bromide (Cu^IBr, 99.999%, Aldrich), 2,2′-dipyridyl (bpy, 99+%, Aldrich), *N,N′*-disuccinimidyl carbonate (DSC, Aldrich), 4-(dimethylamino)pyridine (DMAP, Fluka), 2-(2-aminoethoxy)ethanol (EG_2NH_2, 98%, Aldrich), and poly-L-lysine (PLL, Sigma) were used as received. Poly(ethylene glycol) methacrylate (OEGMA, M_n: ca. 360, Aldrich) was passed through a column of activated, basic aluminum oxide to remove inhibitors.

Preparation of ATRP Initiator-Coated Glass Substrates

Glass substrates were immersed for 2 min in piranha solution, which was a 3:7 mixture of 30% hydrogen peroxide and sulfuric acid by volume. This process cleaned organic residues off from the substrates and hydroxylated most regions of substrates, which made the substrates extremely hydrophilic. For further hydroxylation of the substrates, the substrates were treated with O_2 plasma for 1 min. After hydroxylation, the substrates were immersed in an anhydrous toluene solution of ATRP initiator ($0.33\,L\,mL^{-1}$) for 18 h. The substrates were subsequently rinsed with toluene and ethanol, and dried under a stream of Ar gas.

SI-ATRP of OEGMA

Before the polymerization reaction, deionized water and methanol was bubbled with Ar gas for at least 2 h in order to minimize the oxygen concentration in the solution, which could seriously inhibit the polymerization by oxidizing Cu^I ions. According to the literature,[26] the concentration of the OEGMA solution was chosen to be 1 M so that 1 h reaction could grow a *p*OEGMA film of about 10 nm thickness, which was reported to show the best non-biofouling effect against non-specific bindings of proteins. To 10 mL of this solution, were added deionized water (bubbled, 1.34 mL), methanol (bubbled, 5.39 mL), CuBr (143 mg), bpy (312 mg), and OEGMA (3.26 mL). The resulting dark brown solution was mixed for 15 min, and poured into a degassed Schlenk tube containing ATRP initiator-modified glass substrates. The reaction was kept under Ar gas at room temperature, and lasted for 1 h. After 1 h, the reaction was terminated, and the substrates were washed with deionized water and methanol sequentially, and dried in a stream of Ar gas.

Activation of pOEGMA films with N,N′-Disuccinimidyl Carbonate (DSC) and Subsequent Patterning with PLL by Microcontact Printing (μCP)

The *p*OEGMA films were immersed in anhydrous DMF solution containing 0.1 M DSC and 0.1 M DMAP for 14 h. With the DSC treatment, the polymer films were activated so that they could be easily coupled with amine-containing molecules. The substrates were then rinsed with DMF and dried in a stream of Ar gas. For patterning the modified *p*OEGMA films with PLL moieties, poly(dimethylsiloxane) (PDMS) was used as a stamping material. PDMS stamps were prepared as reported previously.[27] In short, a negative photoresist (SU8-50, Microchem) was spin-coated onto a cleaned silicon wafer. Patterns were then generated by photolithography and subsequently developed on the wafer. The patterned wafer, "master", was then silanized with (tridecafluoro-1,1,2,2,-tetrahydrooctyl)trichlorosilane under vacuum for 2 h. This step enabled the master to be easily separated from cured PDMS stamps. For the PDMS stamps, a 1:10 (v/v) mixture of Sylgard 184 silicon elastomer curing agent (Dow Corning) and Sylgard 184 silicon elastomer base (Dow Corning) was cast, cured for 6 h at 60 °C, and carefully peeled off from the master. The PDMS stamp was wetted by spin-coating with an aqueous solution of PLL ($1\,mg\,mL^{-1}$) and stamped to the activated *p*OEGMA films. Remaining succinimidyl moieties on the *p*OEGMA films were subsequently passivated with an aqueous solution of EG_2NH_2 ($0.1\,mg\,mL^{-1}$) by immersing the substrates to the solutions for 1 h. To confirm that PLL was transferred successfully, fluorescein isothiocyanate (FITC)-modified PLL was stamped.

Cell Culture

Hippocampal tissues were dissected from E-18 Sprague/Dawley rat embryos. Tissues were treated with 2.5% trypsin (Gibco) at 37 °C for 15 min, washed several times with Hank's Balanced Salt Solution (HBSS) to remove trypsin, and mechanically dissociated using narrow ends of sterilized Pasteur pipette tips. Cell suspensions were plated onto the patterned substrate at the density of 5000 to 12000 cells cm^{-2} with the plating medium consist of serum-free Neurobasal/B27 medium (Gibco, Gibco) supplemented with 2 mM L-glutamine (Gibco) and 12.5 μM L-glutamate (Sigma). After 1 h from the cell plating, the substrate was washed by dropping 1 mL of plating medium slowly using a pipette to remove non-adhered cells. After two days in vitro (DIV), the entire medium was changed to maintaining medium which was serum-free Neurobasal/B27 medium supplemented with 2 mM L-glutamine. Cultures were maintained in an incubator with 5% CO_2 at 37 °C, and half of the maintaining media was periodically renewed once every four days.

Electrophysiology of Cultured Neurons

Whole-cell rupture patch recordings from 10 DIV hippocampal neurons were performed in voltage and current clamping modes using micropipettes made from borosilicate glass capillaries (Warner Insturments, Hamden, CT, USA) with resistances in the range of 3–5 MΩ. The internal solution contained the following (in mM): K-gluconate (130), KCl (10), $MgCl_2$ (5), HEPES (5), EGTA (0.6), $CaCl_2$ (0.06), Mg-ATP (2), GTP (0.2), leupeptine (0.2), phosphocreatine (20), creatine-phosphokinase ($50\,ug\,mL^{-1}$), pH 7.2.[36] The external bath solution was a HEPES-buffered saline containing the following (in mM): NaCl (119), KCl (5), $CaCl_2$ (2), $MgCl_2$ (2), HEPES (20), glucose (30), glycine (0.001), pH 7.3, osmolarity adjusted to 330 mosM with sucrose. While recording, neurons were visualized by differential contrast interference (DIC) mode using an upright microscope (Olympus BX61WI), and stimulation was performed using a patch-clamp amplifier (MultiClamp 700B, Axon Instruments) interfaced with a PC via a DIGIDATA 1322 A data acquisition system (Axon Instruments). Signals, filtered at 10 kHz, were sampled at 20 kHz, and analyzed using pClamp software (Axon Instruments).

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