ABSTRACT
Optimal formation of neuronal networks and synapses are essential for accomplishing higher brain functions. Extracellular molecules and nano-structures are considered to play important roles for in vitro studies of neuronal patterns. By using microcontact printing (μCP) and microchannel techniques, we found that neurons extended their neurites on μCP-defined biochemical patterns, whereas axons could preferentially elongated along no-coated microchannels. As expected, synapses could be formed at the crossing points of microchannel-guided axons and biochemically guided neurites. These results demonstrated that both topographic and biochemical patterning are necessary for the control of well-designed neuronal networks.

KEYWORDS: Primary neuronal culture, Synapse, Microcontact printing, Microchannel

INTRODUCTION
For the realization of higher brain functions, the finely-formed neuronal networks, their optimum activation and the signal transmission through synapses are of great importance. It is considered that the extracellular molecules and nano-structures play important roles in the regulatory mechanisms of neuronal network formations. Primary cultures, which make the extracellular environments highly-controllable, are widely performed to study this machinery; however, it is difficult to control the pattern of the network formation under normal conditions, because neurites outgrow randomly. Recent studies show that neurites can outgrow in a desired fashion on a patterned substrate defined by microcontact printing (μCP) [1] or photolithographic [2] techniques. In order to make more exquisite networks and to study them more precisely, not only the neurite outgrowth, but also the extension of each axon and dendrite as well as the regulation of the synapse formation have to be controlled. Thus, we studied these with an aim to accomplish more elaborate neuronal networks in a desired way, by using both μCP and microchannel techniques.

EXPERIMENTAL
Primary hippocampal culture. Hippocampal neurons from 18-day-old C57BL/6J mice embryos were cultured onto coverslips at a density of 2.5 × 10⁴ cells/cm². Cultures were maintained in serum-free Neurobasal medium supplemented with B27 and L-glutamine (2 mM), at 37 °C in a 5 % CO₂ humidified incubator. All experiments were performed in accordance with Animal Care and Use Committee institutional and national guidelines and regulations for approved protocols.

Immunocytochemistry. After 2-3 weeks, neurons were fixed for 15 min in 4 % paraformaldehyde and permeabilized for 2 min with 0.3 % Triton X-100. After 30 min incubation at room temperature in 3 % bovine serum albumin (BSA) in PBS, they were incubated for 30 min at room temperature with primary antibodies in 3 % BSA. After washing, they were incubated for 30 min at room temperature with secondary antibodies, washed, and mounted on slides with Vectashield. The primary antibodies used were monoclonal anti-MAP2 (1:500), polyclonal anti-Tau (1:1000), polyclonal anti-VGluT1 (1:5000), monoclonal anti-PSD-95 (1:200), polyclonal anti-GAD65/67 (1:200) and monoclonal anti-gephyrin (0.5 μg/ml). Secondary antibodies were FITC-conjugated goat ant-mouse (1:500), Cy3-conjugated goat anti-rabbit (1:500), FITC-conjugated goat anti-guinea pig (1:500), Cy5-conjugated rabbit anti-mouse (1:500) and Alexa Fluor 488-conjugated goat anti-rabbit (1:500). Synaptic structures were visualized by confocal microscopy.

Microcontact printing (μCP). Poly-dimethylsiloxane (PDMS) stamps were inked with 1 mg/ml of Cy3-labeled poly-Ornithine (PORN) or 25 μg/ml of laminin and dried for 15 min. The PDMS stamps were placed onto the coverslips for 1 min and then removed, resulting in well-defined biochemical patterns.

Microchannel fabrication. Patterns were defined on fused silica substrates by standard photolithography, lift-off and reactive ion etch techniques.
RESULTS AND DISCUSSION

Biochemical patterns are fundamental for the control of neurite outgrowth and synapse formation. These molecules show different effects on neurons in a dose-dependent manner. First, the effects of the PORN concentrations (in μg/ml: 20, 80 and 100) were studied and we found that high concentration (100 μg/ml) facilitated dendritic outgrowth, while low dose (20 μg/ml) concentrated axonal formation. Next, μCP-defined network regulation was examined with the patterns as shown in Figure 1A. It was revealed that neurons extended their axons and dendrites along the defined pattern strips. Excitatory and inhibitory synaptic formations were also confirmed in these circuits (Figure 2).

Figure 1: μCP-defined patterns regulate the neurite outgrowth. (A, B) PDMS stamps used for μCP (A) and a printed pattern on a substrate (B). (C, D) Neurons extended their dendrites (C) and axons (D) along the printed patterns (laminin, red).

After that, microchannel techniques were used. To facilitate the axonal outgrowth, PORN printing was applied only on the top surface of the device so that there is no molecular coating on the bottom of the channels (Figure 3B). Immunocytochemical analysis revealed that axons could elongate along the no coated microchannels (Figure 3C).

Finally, μCP and microchannel-integrated technique was conducted to form higher functional neuronal circuits designed with fully controlled axonal and dendritic outgrowths as well as synaptic formations (Figure 4). It was confirmed that synapses were formed at the crossing points of the microchannel-guided axons and neurites.

Figure 2: Synapse formation on the μCP defined neuronal networks. (A) The excitatory presynaptic terminals (VGluT1, green) and the excitatory postsynaptic scaffolding proteins (PSD-95, red) were visualized by immunostaining. (B) Immunostaining of the inhibitory presynaptic terminals (GAD65/67, green) and the inhibitory postsynaptic scaffolding proteins (Gephyrin, red). Higher magnification of the indicated regions was shown on the bottom.
CONCLUSION
These results demonstrated that μCP and microchannel-integrated techniques could control the axonal and dendritic outgrowths, and also synapse formations. Furthermore, it was suggested that this technique can be used to effectively achieve higher network formations.

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CONTACT
* Correspondence should be addressed to Dr. Antoine TRILLER,
Tel.: +33 1 44 32 35 47; Fax: +33 1 44 32 36 54; E-mail: triller@biologie.ens.fr