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Constrained synaptic connectivity in functional mammalian neuronal networks grown on patterned surfaces

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Abstract

The use of ordered neuronal networks in vitro is a promising approach to study the development and the activity of small neuronal assemblies. However, in previous attempts, sufficient growth control and physiological maturation of neurons could not be achieved. Here we describe an original protocol in which polylysine patterns confine the adhesion of cellular bodies to prescribed spots and the neuritic growth to thin lines. Hippocampal neurons in these networks are maintained healthy in serum free medium up to 5 weeks in vitro. Electrophysiology and immunochemistry show that neurons exhibit mature excitatory and inhibitory synapses and calcium imaging reveals spontaneous activity of neurons in isolated networks. We demonstrate that neurons in these geometrical networks form functional synapses preferentially to their first neighbors. We have, therefore, established a simple and robust protocol to constrain both the location of neuronal cell bodies and their pattern of connectivity. Moreover, the long term maintenance of the geometry and the physiology of the networks raises the possibility of new applications for systematic screening of pharmacological agents and for electronic to neuron devices.

Keywords: Lithography; Neuronal growth control; Electrophysiology; Calcium imaging; Immunochemistry; Orientation of connectivity

1. Introduction

The ability to construct neuronal networks with a constrained synaptic connectivity and to maintain them over several weeks could open a way to new experiments which are not currently possible. Most approaches to understand information processing in biological neuronal networks use in vivo or brain slice preparations. A complementary approach consists in dissociated neuronal cell cultures (Goslin and Banker, 1991) in which the structural integrity of the tissue is lost but higher degree of biochemical and biophysical control is possible. For example, culture systems enable to simultaneously record the activity of groups of three to four connected neurons in an isolated network (Fitzsimonds et al., 1997; Bi and Poo, 1998) and to study the long term effect of molecules applied in the medium (Turrigiano et al., 1998). However, in standard cultures, the random spatial distribution, the overlap of neurites and the motion of neurons on the homogeneous substrate complicate the observation of a single neuron over long time (Lo and Poo, 1994).

Directed neuronal networks in vitro, obtained by confining soma location and neurite elongation to a prescribed pattern, should allow to circumvent these difficulties. By immobilizing cell bodies in defined positions, the technique would facilitate the study of individual neurons and of their interaction with their neighbors during the development of the network over weeks. It may be then feasible to monitor and stimulate neuronal activity by forming artificial neuronal circuits using neuron to electronic interfaces (Zeck and Fromherz, 2001). This development requires that neurons fulfil four essential conditions: (i) long term persistency of their growth pattern and low density survival in vitro, (ii) physiological integrity, (iii) presence of both inhibitory and excitatory neurons, (iv) maturity of synapses.

Patterning technologies use mainly lithography and self-assembled monolayers (Kleinfeld et al., 1988; Of-

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fenhausser et al., 1997; Ma et al., 1998; Wheeler et al., 1999). Photolithographic protocols consist usually in grafting two silane molecules with antagonistic action on cellular growth onto glass slides with a micron scale resolution. Some growth permissive molecules (e.g. an aminosilane or laminin-derived synthetic peptide, see Scholl et al., 2000; Matsuzawa et al., 1996) favor neuron adhesion and growth whereas antipermissive molecules such as fluorosilanes prevent it. In previous studies, several problems have emerged. In early studies, survival was only obtained in high density systems (Kleinfeld et al., 1988). In other attempts, synapses did not achieve a mature state (Offenhausser et al., 1997). Sometimes loss of order was observed when neurons gained excitability (Stenger et al., 1998). In a recent study, although synapses were observed, neuronal excitability was altered (as shown by the depolarized value of resting potentials and by the firing patterns showing a single action potential, Matsuzawa et al., 2000). Moreover survival was limited to less than 10 days: these neurons did not live beyond the critical survival step often described in low density cultures (Goslin and Banker, 1991).

The present work describes an original protocol for controlling neuronal growth on solid substrates allowing us to obtain mature and durable neural networks of controlled architecture. Its achievement is based on: (i) the use of polylysine instead of an aminosilane as the permissive molecule and (ii) the optimization of low density culture conditions in a defined medium in order to observe spontaneous activity and mature synapses up to 5 weeks in vitro. We designed with hippocampal neurons a wide variety of neuronal networks which maintain their spatial structure over a month. We restrained our study to neuronal networks grown between 2 and 3 weeks in vitro. At this stage, neuronal physiology is intact. Patch clamp recordings show that neuronal excitability is similar to the one observed in slices. Immunochemistry and electrophysiology reveal that both excitatory and inhibitory neurons form functional synapses. Whole cell recording and calcium imaging show that small networks express spontaneously collective activity. Characterization of neuronal connectivity with a double patch clamp approach demonstrates that connectivity is constrained by the geometry, since neurons preferentially form synapses to their first neighboring neurons.

2. Methods

2.1. Photolithography (Fig. 1A)

Cleaned coverslips are coated with hydrophobic fluorosilane $C_8H_4Cl_3F_{13}Si$ (Roth, France) in dichloromethane and *n*-decane for half an hour at 4 °C. After rinsing in chloroform, the silanized surfaces are spin-coated with a positive photoresist (Shipley, S1805). Each coverslip is pressed against a mask and exposed to UV light. Incubation in a development bath removes the exposed photoresist. The fluorosilane layer (no longer protected by the photoresist) is removed with an H_2O plasma and the glass surface is then coated with polylysine (Sigma P2636, 1 mg/ml overnight). The next day, unexposed photoresist is washed out with acetone. To optimize our protocol, several geometrical parameters have been tested. Survival is enhanced with large polylysine domains (typically 80–100 μm wide) in comparison with smaller ones (e.g. 40 μm wide or less). Connecting lines of width 2–5 μm provide the best guidance to neurites while preventing the attachment of cell bodies. Masks for lithography are easily prepared in the laboratory. Any desired pattern is drawn using a standard graphic software. A photograph of the printed pattern is used as a first reduction. It is then reduced to its final size by projecting it about 100 times onto a photoresist coated coverslip placed at the focal plane of a 20× Plan Neofluar Zeiss objective, by transient opening of a shutter and appropriate displacement of a motorized stage. After a standard metallization procedure, we obtain typically 100 chromium patterns on a coverslip with a final size for each pattern of 0.8 mm and with a 2–4 μm resolution.

2.2. Cell culture

Pyramidal neurons from rat hippocampus are grown on the substrates according to a protocol derived from Banker (Goslin and Banker, 1991). Patterned coverslips are incubated for 5 days in neuron plating medium (NPM) containing 10% Horse Serum (Invitrogen). Hippocampi from E18 rats embryos are dissociated chemically (0.25% trypsin, 20 min) and mechanically using fire polished Pasteur pipettes. Neurons plated on the patterned substrates (densities ranging from 1000 to 10 000 cells per cm^2) are maintained in a 5% CO_2 atmosphere at 37 °C. After 4 h, NPM is replaced by a serum free maintenance medium (NMM) and a feeding layer of glial cells is added to each dish. Glial cells proliferation in the culture is stopped by AraC after 2 days (1 μg/ml, Sigma). Standard glass coverslips (Marienfeld, Germany) used as controls are cleaned in nitric acid overnight, rinsed thoroughly in water and dried for 6 h at 220 °C. Coating with polylysine and incubation in NPM occur in the same conditions as for the patterned coverslips. We have also tested different conditions of maintenance and different glial cells contents. Hippocampal cells grown in serum containing medium together with glial cells (Wilcox et al., 1994), were not successfully constrained on the patterns after 3 days. Similarly, glial cells plated at high density (∼30 000 cells per cm^2) in serum containing medium

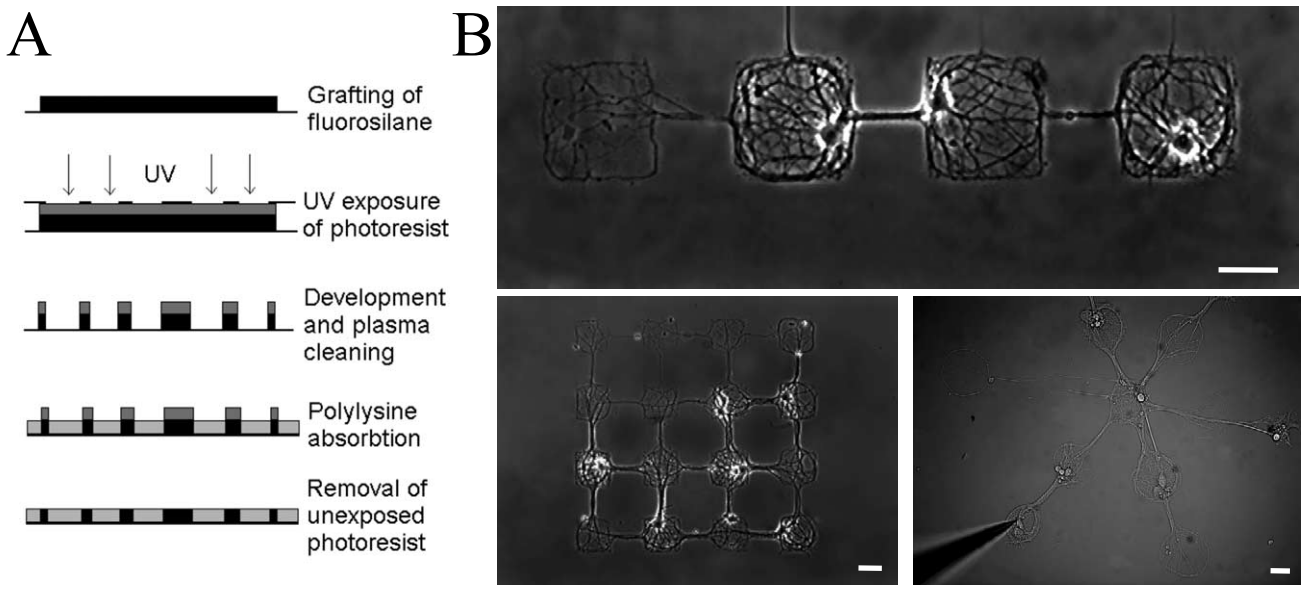

Fig. 1. Protocol and images of the pattern. (A) Protocol of photolithography. A glass coverslip (continuous line) is coated with domains of fluorosilane molecules (dark) and with regions of polylysine (light gray) according to the pattern designed on the mask (dashed line), using UV exposure of a spincoated photoresist (gray) (see Section 2). (B) Images of neural networks of controlled architecture. Top: linear network. Bottom left: matrix 4×4. Bottom right: star. Cell bodies of neurons are restricted to squares or disks of 80 μm and neurites to lines (80 μm length, 2–4 μm wide). Square and disk diameters are 80 μm for each figure. Scale bar is 50 μm.

will eventually spread out of the patterns and cover fluorosilane coated regions. But glial cells plated at low density (2500 cells per cm^2) in serum free medium remain confined on patterns. Our lithography protocol is hence well adapted to cultures of neurons and of dividing cells, as long as the growth medium is serum free. Serum is incompatible with long term patterning since (i) it favors cells division and growth and (ii) serum proteins may bind through hydrophobic domains to fluorosilanes.

2.3. Electrophysiology

Cultures were studied at room temperature after 2–3 weeks in vitro. Patch clamp records were performed using two Axopatch 200B. Bath solution contained in mM 145 NaCl/3 KCl/3 $CaCl_2$/1 $MgCl_2$/10 Glucose/10 HEPES/pH 7.25, and osmolarity adjusted to 315 mOsm. Pipette solution contained in mM 9 NaCl/136.5 KGlu/17.5 KCl/0.5 $CaCl_2$/1 $MgCl_2$/10 HEPES/0.2 EGTA/pH 7.25 and osmolarity equal to 310 mOsm. Patch pipettes were made of borosilicate tubes (Clarks, UK) and had a resistance of 3–4 MΩ when filled with the standard pipette solution. The series resistance was less than 10–12 MΩ, and was compensated up to 60% using a standard procedure. Resting membrane potentials were estimated directly after entering in whole cell configuration. In some experiments the following transmitter antagonists (from Sigma) were applied in the bath: 1 μM biccuculine methiodide to block $GABA_A$ receptor, 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) for non NMDA receptors and 50 μM amino-5-phosphopentanoic acid (AP5) for *N*-methyl-D-aspartate (NMDA) receptors. Firing patterns were assayed with all antagonists in the bath and no calcium buffer in the pipette.

2.4. Antibodies

Monoclonal antibody MAB363 specific for NMDAR1 subunit of the NMDA receptor was obtained from Chemicon (Temecula) and used at a 1:1000 dilution. MAb GAD-6 specific for GAD 65 (Boehringer Mannheim) was used at a 1:400 dilution. Mab specific for synaptotagmin was a gift from E. Jover (Strasbourg) and used at a 1:4000 dilution. Rabbit polyclonal antibodies against synaptophysin and GABA kindly given by L. Stoeckel (Strasbourg) were used at the dilution 1:1000 and 1:10 000, respectively. Secondary antibodies obtained from Molecular Probes were IgG anti mouse coupled to Alexa Green or anti rabbit coupled to Cy3 both used at 1:400 dilution.

2.5. Immunocytochemistry

Neurons were fixed with 4% formaldehyde, 4% sucrose in PBS for 15 min at 37 °C (except for NR1 staining where 10 min methanol fixation at −20 °C is used) and permeabilized with 0.25% Triton X-100 for 5 min. After preincubation in 10% serum of secondary antibodies species for 30 min, cultures were incubated with primary antibody(ies) overnight at 4 °C. For

double labeling experiments, both primary antibodies were incubated together. Controls include incubations with no primary antibody and comparisons of double- and single-labeled samples to ensure that the labelling was independent in the double-label experiments.

2.6. Calcium imaging

Cultures were loaded with 5 μM of the membrane-permeant acetoxymethyl ester of Fura-2 AM (Molecular Probes, Oregon) for 30 min at room temperature and then rinsed for 30 min. A 100 W Xenon lamp filtered at 380 nm ensured excitation of the probe, emission was filtered at 510 nm. Images obtained with a CCD (Roper Scientific) were acquired at 50 Hz, stored and analyzed using Metamorph in order to measure the fluorescence intensity variation in all cellular bodies selected in the network.

All values are given ±standard deviation (S.D.).

3. Results

3.1. Adhesion, growth and morphology of neurons

Polylysine, a polymer of basic amino acids, is commonly used to promote cell adhesion. It was thought to be difficult to pattern by lithography because of its size and the resulting thickness of the layer on glass (Stenger et al., 1998). Our strategy to pattern polylysine (see Section 2) relies on the use of an H_2O plasma to clean the surface prior to polylysine deposit and on the removal of the unexposed photoresist with acetone which does not degrade the coated polylysine.

We obtain micron scale patterns of polylysine which target cellular bodies and guide neurites to form spatially directed neuronal networks (Fig. 1A). A wide diversity of patterns has been designed to form autapses, linear networks, matrices and stars (some shown Fig. 1B). In the patterns (Fig. 1B), neuronal somata adhere to the 100 μm wide spots of polylysine (square or disk) but not to the polylysine lines connecting two spots because of their thinness (2–4 μm wide). Neurite elongation occurs in spots and thin lines (100 μm long). We can direct cell body adhesion and neuritic growth within certain geometrical conditions (see Section 2). Somatic adhesion sites have to be at least ∼80 μm in diameter. The width of polylysine lines should be inferior to 10 μm in order to allow neurites elongation and to prevent cell body adhesion. Their length was set to ∼100 μm to allow formation of connections and to maintain good neuronal survival (Ikegaya et al., 2000).

The number of cells per domain is determined through the plating density, thereby fluctuates from one pattern to the next. We adjusted plating density in order to have an average of one cell per spot (Fig. 1B). The pattern is respected by neurons in serum free medium in vitro for 5 weeks. During that period cells do not escape from polylysine spots, so that each neuron can be followed in a given pattern over time. The neuronal morphology (Fig. 1B) is similar to the one observed in classical low density cultures (Goslin and Banker, 1991). We studied neurons between 2 and 3 weeks in vitro, when they exhibit a large number of neurites (Fig. 1B).

3.2. Electrophysiological characteristics of neurons are intact

Electrophysiological properties of neurons grown on patterned substrates are similar to those observed in classical 'random' low density cultures. Using patch clamp (in the whole cell configuration), we evaluated and compared membrane properties and firing patterns of neurons in both systems. As shown in Fig. 2A, there is no difference in resistance, capacitance and time constant of the membrane between culture types. In all cases, membrane resistance ranges approximately between 100 MΩ and 1 GΩ ($239 = \pm 17$ MΩ) as observed in standard cultures ($217 = \pm 28$ MΩ; see also Evans et al., 1998). To evaluate cells excitability, resting potential and firing patterns were compared in standard and patterned cultures. Resting membrane potentials vary between −50 and −60 mV in both culture types (-55.6 ± 2.3 mV for standard low density cultures and -58.7 ± 1.8 mV for patterned cultures) and the firing patterns are similar (Fig. 2B). In response to a 800 ms depolarizing pulse, neurons usually discharge multiple action potentials which habituate. A smaller fraction (18% for patterned cultures ($n = 52$) and 17% for classical cultures ($n = 31$), see Fig. 2B) exhibits a single spike response. All current-evoked action potentials disappear after extracellular application of 0.5 μM tetrodotoxin (TTX, $n = 7$). The similarity of these results with those obtained in acute slices (Staley et al., 1992) demonstrates the integrity of the membrane and of the excitability of our neurons.

3.3. Inhibitory and excitatory cells are both present

We observe both GABAergic, identified by immunoreactivity for GABA (Craig et al., 1996), and non GABAergic, presumably glutamatergic, neurons (Fig. 2C). The patterned neuronal networks are slightly enriched in GABAergic cells (27% of the overall population in patterns ($n = 432$) while 22% in standard cultures ($n = 513$)). We conclude that both inhibitory and excitatory neurons grow on patterned networks. But do they form an interconnected neuronal network? The presence of synapses was assayed by immunostaining with antibodies against marker proteins of presynaptic specializations, synaptophysin and synapto-

A

CULTURES	Resting potential	Membrane resistance	Membrane capacity	Membrane time constant	Days in vitro
low density culture (n=14)	-55,6 +/- 2,3mV	217 +/- 28MΩ	19 +/- 4,2pF	4,2 +/- 1,9ms	17 +/- 3,6
pattern (n=36)	-58,7 +/- 1,8mV	239 +/- 17MΩ	17 +/- 3,9pF	4,0 +/- 1,3ms	17+/- 2,4

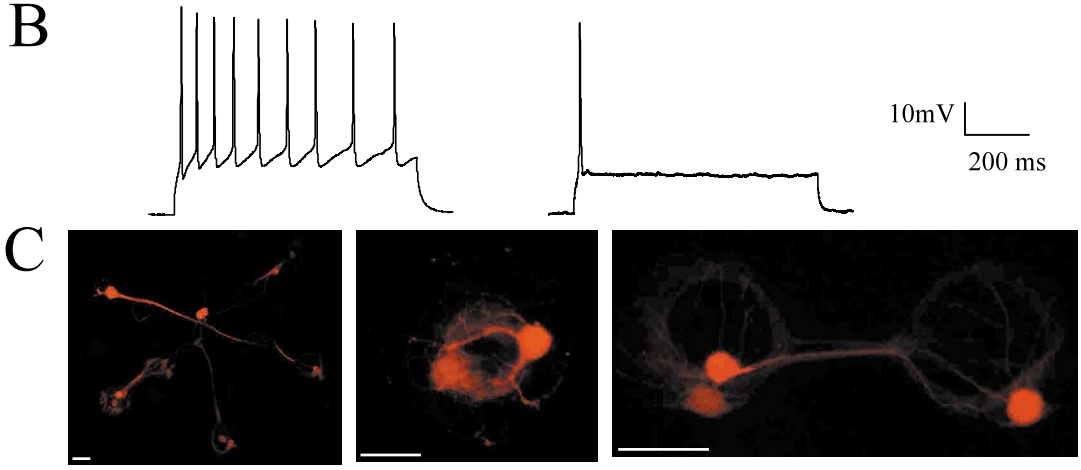

Fig.2

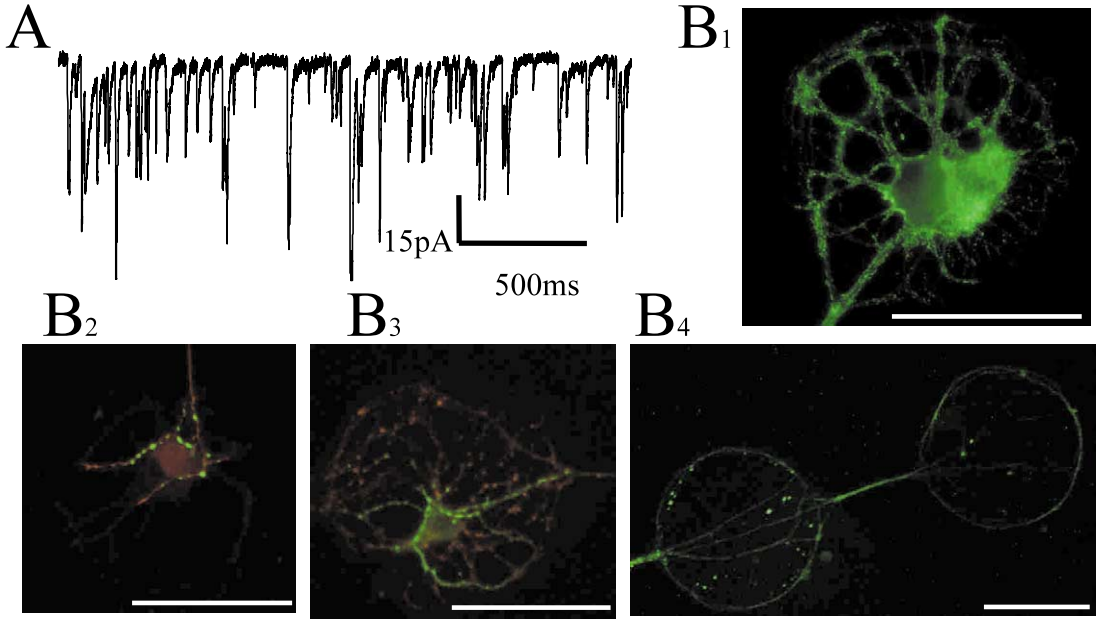

Fig.3

Fig. 2. Physiology of neurons grown on polylysine patterns is similar to the one observed for neurons grown in standard low density cultures. (A) Membrane biophysical properties. (B) Firing patterns observed in both cultures after a depolarizing step of 800 ms with CNQX and bicuculline added to the bath. (C) Detection of inhibitory neurons by immunocytochemistry using anti-GABA antibody. Scale bar is 50 μm.

Fig. 3. Evidence for mature synapses between neurons. (A) Spontaneous synaptic currents recorded in voltage clamp mode (voltage holding −60 mV). (B) Localization of inhibitory and excitatory synapses in the networks with B1, Anti-synaptotagmin; B2, Anti-GAD 65 (green) and anti-GABA (red); B3, Anti-GAD 65 (green) and anti-synaptophysin (red); B4, Anti-NR1. Scale bar is 50 μm.

tagmin. Immunoreactivity for each protein is distributed in clusters, corresponding presumably to synapses spread all over the networks (Fig. 3B). We now show evidence for functional synapses by recording spontaneous synaptic currents.

3.4. Neurons form mature inhibitory and excitatory synapses

Whole cell recordings reveal spontaneous (excitatory and inhibitory) synaptic currents (Fig. 3A) distinguished by their reversal potential and by specific pharmacological agents sensitivity. Inhibitory Post Synaptic Currents (IPSCs) are sensitive to the $GABA_A$ receptor antagonist bicuculline methiodide (1 μM) and reverse polarity near −45 mV. Their decay time constant is 45 ± 7 ms. Excitatory Post Synaptic Currents (EPSCs) are sensitive to 10 μM CNQX, which blocks α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor. These currents reverse near 0 mV (1.1 ± 2.3 mV) and their decay time constant is 2.0 ± 0.7 ms. The reversion values are coherent with the ionic concentrations (see Section 2) and the currents kinetics with those observed in slices (Pettit and Augustine, 2000) or dissociated cultures (Wilcox et al., 1994; Evans et al., 1998).

We attempted to compare qualitatively the distribution of inhibitory and excitatory synapses. Immunochemistry for Glutamate Acid Decarboxylase 65 (GAD 65, which stains cell bodies and synaptic terminals of inhibitory cells) shows that inhibitory synapses are concentrated around the cellular bodies of excitatory neurons (Fig. 3B) more than on neurites (inhibitory synapses on cell bodies of inhibitory neurons can not be identified since GAD 65 staining is very dense in these cell bodies). On the other hand, NMDA receptor positive synapses revealed by staining for NR1 (NMDA receptor subunit 1 common to all NMDA receptors, expressed by all neurons) are widely distributed on neurites (Fig. 3B). Both GAD and NR1 staining match the labeling observed on 'mature' neurons in culture (Pickard et al., 2000).

3.5. Orientation of connectivity between neurons in designed networks

Neurons do form mature synapses on the patterned surface: does cellular location influence synaptic connectivity? In particular, do neurons form synapses preferentially with their nearest neighbors located on adjacent spots in the patterned networks? To answer this question, double patch recordings were made from pairs of neurons, either first (Fig. 4A1) or second (Fig. 4A2) order neighbors, to evaluate the probability of connection in both cases. The pre-synaptic neuron was recorded in current clamp mode so that an action potential could be elicited with a depolarizing pulse. The post-synaptic cell was initially voltage clamped at −60 mV.

We first performed double recordings of first order neighboring neurons (Fig. 4A1). In 37 out of 42 neurons tested, we observe evoked synaptic currents (Fig. 4B1, B2, C). In 11 neurons (Fig. 4B2) IPSCs are systematically recorded and characterized by an inversion potential near −43 mV and a reversible blockage by the bicuculline methiodide (1 μM). The time delay of the response (4.1 ± 1.3 ms) and the absence of failure demonstrate their mono-synaptic nature. Action potential induced in 26 neurons elicits with almost no failure EPSCs which reverse near 2.0 ± 3.1 mV (Fig. 4B1). EPSCs occur with a constant latency (3.9 ± 0.8 ms) and are abolished by application of 10 μM CNQX revealing an AMPA-type receptors component. In 23 records, we observe after a monosynaptic EPSC synaptic currents with a longer time delay (> 10 ms), a low occurrence probability (39%) and usually a complex shape (Fig. 4C). These presumably poly-synaptic currents are always abolished by 10 μM CNQX application revealing the excitatory relais.

Regarding second order neurons (Fig. 4A2), no evoked currents were observed in 62% of the cases ($n = 8$). In the other cases (38%), we measured either inhibitory ($n = 2$) or excitatory ($n = 3$) mono-synaptic connections. The probability for mono-synaptic connections between first order network (87%) is thus very high and about twice the probability for second order connections (38%). Therefore, the connectivity between neurons in the patterned network does not occur randomly but is orientated by the geometry of the pattern. This last feature is the most fascinating property of our networks since it makes connectivity between neurons predictable to some extent considering the geometry of the networks.

We asked whether connectivity could be strictly restricted to first neighbor at earlier stage of development of the networks. We tested connectivity between three pairs of second neighboring neurons in 1–2 weeks old cultures and observed that monosynaptic connectivity between second order neighboring neurons could be already observed at this stage (in three cases out of six pairs tested). Moreover time lapse experiments in a 5% CO_2 37 °C chamber over the first week of culture has revealed that after 5–7 days of culture—when we start to record spontaneous currents neurons in the networks—neurons can already grow neurites to first, second and third neighboring neurons. These observations suggest that the time dependencies of large scale neurite elongation and synapse formation explain why we have not been able to observe, even after 1 week of culture, restricted monosynaptic connectivity to first neighbors. Therefore, it is indeed very satisfactory that

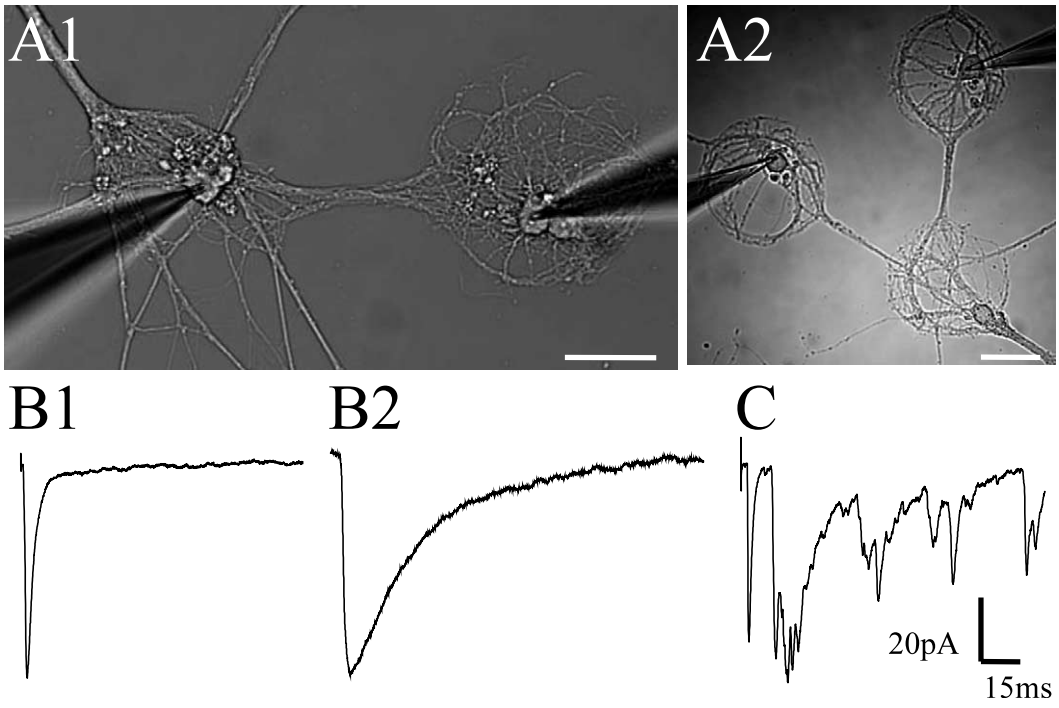

Fig. 4. Evoked synaptic currents between first order neighboring neurons in the patterns. (A) Double patch of first (A1) and second (A2) order neighboring neurons. (C) Example of typical excitatory (C1) and inhibitory (C2) mono-synaptic evoked current in the post synaptic cell. (D) Example of a response with mono-synaptic and poly-synaptic components. Scale bar is 50 μm.

connectivity is still orientated by the architecture in mature networks.

3.6. Spontaneous activity during development

Current clamp experiments show that TTX sensitive spikes could be induced after 3 days in vitro. Spontaneous postsynaptic potentials appear between 4 and 7 days. Cell attached experiments show that both the number of spontaneously active cells and their rate of discharge increased with time. After 3 weeks in vitro, spontaneous action potentials often appear in bursts (Fig. 5B). This bursting activity is blocked by application of 10 μM CNQX (not shown).

To probe whether spontaneous activity occurs simultaneously throughout the network, we used calcium imaging with Fura-2 AM probes (Fig. 5A, Smetters et al., 1999; Peterlin et al., 2000). Single action potential triggers an increase in intracellular calcium concentration in the soma which causes a decrease of fluorescence intensity (with a time to peak of 10–20 ms and a time decay of about 1 s). We measured fluorescence intensity over all neurons of the network every 20 ms (Fig. 5A). We observed that calcium increases in all neurons of the network during a burst of action potentials (Fig. 5C). These data suggest that all neurons are usually active during a burst.

We have seen that in patterned networks spontaneous activity evolves with time from random isolated spikes to bursting spike formation. Bursts of spontaneous action potentials cause simultaneous calcium transients in all neurons of the network. This might be compared with what has been seen for cortical dissociated cultures (Muramoto et al., 1993) and in young slices (see Feller, 1999).

4. Discussion

Our protocol is a robust and very accessible way to produce *mature ordered* neuronal networks with intact neuronal physiology. Indeed, for at least 5 weeks, the geometry of the network and the membrane properties of both excitatory and inhibitory cells are maintained and functionally mature synapses do form. Different observations indicate that inhibitory and excitatory synapses are mature: (i) clusters of presynaptic markers; (ii) specific localization of inhibitory and excitatory synapses; (iii) voltage clamp recordings of spontaneous EPSCs and IPSCs with standard current kinetics and pharmacology. Synaptic connectivity between pairs of neurons and spontaneous activity are similar to what has been observed between pyramidal neurons in slices (Markram et al., 1997; Feller 1999) and may reflect a general property of neuronal networks organization.

Moreover synaptic connectivity can be easily assayed in designed networks, as shown in this paper, and appeared to be constrained by geometry. Because of the simplicity of identification and study over time of given connected neurons, we believe that the design of

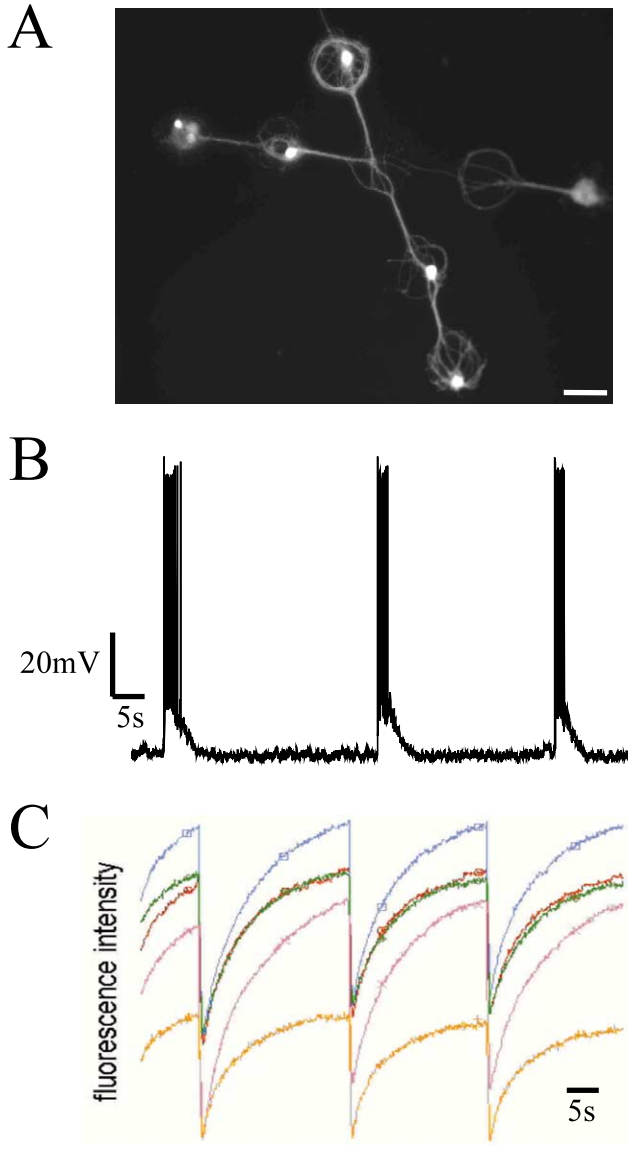

Fig. 5. Spontaneous activity in neurons of a mature (23 days old) network. (A) Image of neurons in a network after Fura-2 AM loading. (B) Whole cell recording of one neuron in the pattern showing repetitive bursts of action potential. (C) Bursting activity occurs in all neurons of the network revealed by the simultaneous decrease of fluorescence. Whole cell recording and calcium imaging in these examples were not simultaneously aquired.

these mature ordered networks may constitute a novel tool to study over long period interactions between multiple neurons in a network.

Our technique maintains about a hundred networks per glass coverslip in a defined medium. The main advantages of these neuronal chips are the fixed position of the cellular bodies, the maturation of the neurons, the simplicity of the connectivity patterns and finally the long term maintenance of the networks in a serum free media. These properties could be of great interest to systematically screen new pharmacological compounds at different stages of neuronal development.

Two main perspectives could be reached by successive lithographies: (i) the grafting of different molecules onto the substrates could permit further refinement of constructed networks (to orientate inhibitory or excitatory cells adhesion or axonal or dendritic growth); (ii) the coating of electrodes on the surface in order to stimulate and record from all neurons of the network over time. The realization of neuron to electronic devices (Zeck and Fromherz, 2001) with mammalian neurons may be then greatly facilitated by our technique.

Finally our protocol has succeeded in designing patterns with non dividing cells (neurons) as well as dividing cells (astrocytes) thus making them suitable to form hybrid assemblies and study specific interactions between different types of mammalian cells (such as motoneurons and muscular fibers or tumoral and non-tumoral cells considered to be more easy to maintain in vitro).

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