

# Statistical properties of information processing in neuronal networks

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## Abstract

Information processing and coding were analysed in dissociated hippocampal cultures, grown on multielectrode arrays. Multisite stimulation was used to activate different neurons and pathways of the network. The neural activity was binned into firing rates and the variability of the firing of individual neurons and of the whole population was analysed. In individual neurons, the timing of the first action potential (AP) was rather precise from trial to trial, whereas the timing of later APs was much more variable. Pooling APs in an ensemble of neurons reduced the variability of the response and allowed stimuli varying in intensity to be distinguished reliably in a single trial. A similar decrease of variability was observed pooling the first evoked APs in an ensemble of neurons. The size of the neuronal pool ( $\sim 50$ – $100$  neurons) and the time bin ( $\sim 20$  ms) necessary to provide reproducible responses are remarkably similar to those obtained in *in vivo* preparations and in small nervous systems. Blockage of excitatory synaptic pathways mediated by NMDA receptors improved the mutual information between the evoked response and stimulus properties. When inhibitory GABAergic pathways were blocked by bicuculline the opposite effect was obtained. These results show how ensemble averages and an appropriate balance between inhibition and excitation allow neuronal networks to process information in a fast and reliable way.

## Introduction

Understanding the neural code requires the identification of electrical events occurring consistently and reliably from trial to trial. These electrical events could correspond to the exact timing of action potentials (APs) in individual neurons or to the firing rate averaged over a population of neurons. These two neural codes are usually referred to as the temporal and the rate code, respectively (Georgopoulos *et al.*, 1986; Hopfield, 1995; DeCharms & Merzenich, 1996; De Ruyter van Steveninck *et al.*, 1997; Nicolelis *et al.*, 1998; Parker & Newsome, 1998; Panzeri *et al.*, 2001; Zoccolan *et al.*, 2002; Johansson & Birznieks, 2004). Whether the computational unit in the nervous system is a single neuron or a neuronal assembly is still an open debate (Cohen & Nicolelis, 2004; Johansson & Birznieks, 2004; Osborne *et al.*, 2004).

The analysis of neuronal networks has been carried out by intracellular recordings from a small number of neurons (Silberberg *et al.*, 2004) or by using multielectrode arrays (MEA) either implanted in the cortex (Nicolelis *et al.*, 1997), or in the hippocampus (Harris *et al.*, 2003). Optical methods have also been used to analyse the dynamics of cortical neurons and to characterize the global states of cortical networks (Jancke *et al.*, 2004) and to visualize plasticity in the hippocampal CA1 area (Aihara *et al.*, 2004). Recording the electrical activity from neuronal networks *in vivo* provides valuable information on how the brain works, but has a number of limitations. Firstly, given the high convergence of inputs to the cortex it is difficult to control exactly, in every trial performed with the same stimulation, the

effective input reaching the neuronal network. Secondly, it is not easy to change in a controlled way the properties of the stimulus applied to the cortical network and its chemical environment. These limitations are overcome by studying neuronal cultures grown over MEAs (Gross, 1979; Pine, 1980; Jimbo *et al.*, 1999; Potter, 2001; Eytan *et al.*, 2003). These cultures with random connections, provide a more general view of neuronal networks and assemblies, not depending on the circuitry of a neuronal network *in vivo*, and allow a more detailed and careful experimental investigation.

In order to investigate statistical properties of information processing in neuronal networks, rat hippocampal neurons were cultured on a MEA. After a few weeks in culture the network established synaptic contacts, showed spontaneous activity (Van Pelt *et al.*, 2005; Maeda *et al.*, 1995) and was largely composed of excitatory glutamatergic neurons with a few per cent of inhibitory GABAergic neurons. Extracellular electrical stimuli were delivered to the network, through the MEA. The neural activity was binned into firing rates over time windows of different length and its statistical properties were analysed. The variability of firing of individual neurons and of the whole population was first studied. In individual neurons, the timing of the first AP was rather precise whereas the timing of the following APs was much more variable. Pooling evoked APs in an ensemble of neurons reduced the variability of the response across different trials. As a consequence, it was possible to distinguish at the level of a single trial, stimuli varying in intensity. A similar decrease of variability was observed counting the first evoked APs in an ensemble of neurons. Blockage of excitatory synaptic pathways mediated by NMDA receptors improved the mutual information between the evoked response and the stimulus. The opposite effect was observed when inhibitory GABAergic pathways were blocked by bicuculline. The estimations of the size of the neuronal pool and time bin necessary to

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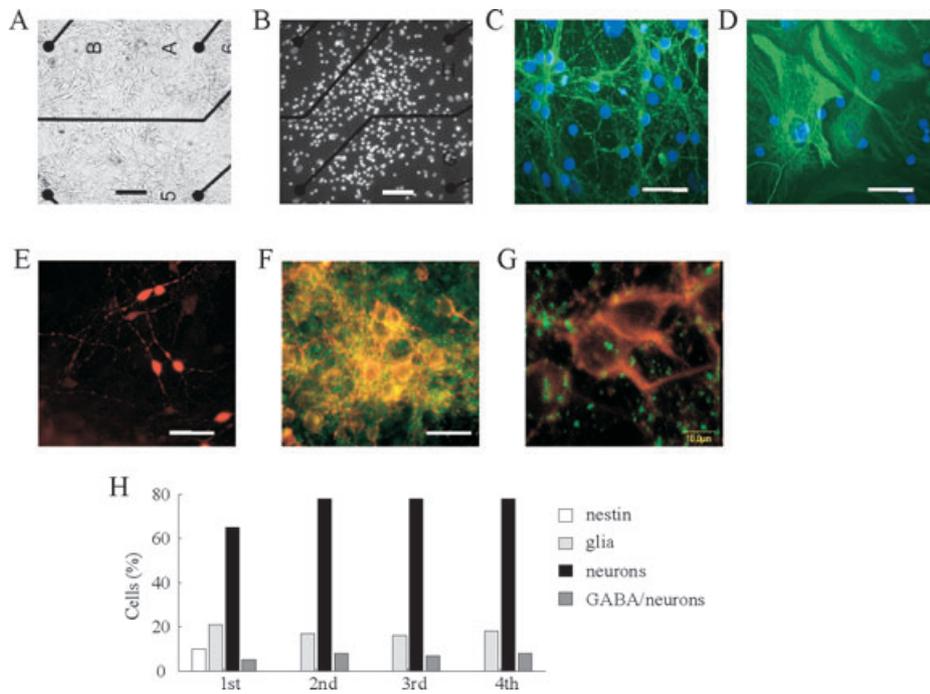


FIG. 1.

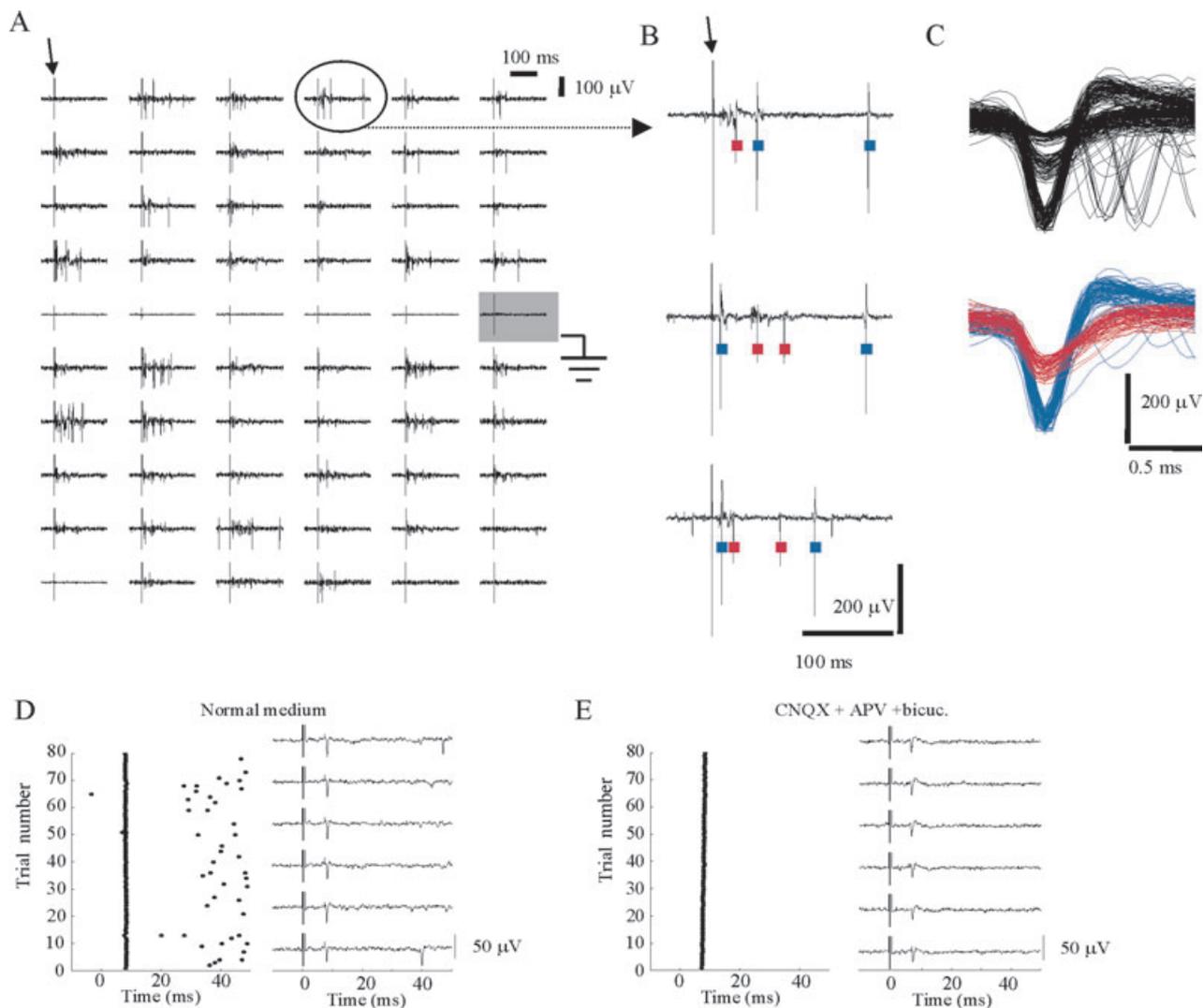


FIG. 2.

process information in a reliable way are remarkably similar to those obtained in *in vivo* preparations (Nicoletis *et al.*, 1998; Shadlen & Newsome, 1998) and in small nervous systems (Lewis & Kristan, 1998; Zoccolan *et al.*, 2002). These results show how an appropriate ensemble average and a balanced presence of inhibition and excitation allow neuronal networks to process information in a fast and reliable way.

## Materials and methods

### Neuronal culture preparation

For hippocampal culture preparation, rats were anesthetized with CO<sub>2</sub> prior to decapitation. Hippocampal neurons from Wistar rats (P0–P2) were prepared as described previously (Ruaro *et al.*, 2005). Cells were plated on polyornithine/matrigel precoated MEA (Ruaro *et al.*, 2005) at a concentration of  $8 \times 10^5$  cells/cm<sup>2</sup> and maintained in Minimal Essential Medium with Earle's salts (Gibco-Brl) supplemented with 5% fetal calf serum, 0.5% D-glucose, 14 mM Hepes, 0.1 mg/mL apo-transferrin, 30 µg/mL insulin, 0.1 µg/mL D-biotin, 1 mM vitamin B12, and 2 µg/mL gentamycin. After 48 h, 5 µM cytosine-β-D-arabino-furanoside (Ara-C) was added to the culture medium, in order to block glial cell proliferation. Half of the medium was changed twice a week. Neuronal cultures were kept in an incubator providing a controlled level of CO<sub>2</sub> (5%), temperature (37 °C) and moisture (95%).

### Immunohistochemistry

Cells were fixed in 4% paraformaldehyde and 0.15% picric acid, and were stained with the following primary antibodies: mouse monoclonal antibodies against nestin (Chemicon) glial fibrillary associated protein (GFAP; Sigma-Aldrich), class III beta-tubulin (TUJ1; Covance), glutamate (Sigma-Aldrich). Rabbit polyclonal antibodies against CaMKII and GABA (Sigma-Aldrich) serotonin (Sigma-Aldrich) and tyrosine hydroxylase (TH; DiaSorin). Appropriate FITC-labelled and TRITC-labelled secondary antibodies and Hoechst 33345 nuclei counterstain were used for visualization. Images were acquired under a Zeiss AxioShop2 fluorescent microscope using an Optronics camera.

### Density of neuronal cultures

Cell density on the MEA after 4 weeks in culture was evaluated by incubation of the culture with 2 µg/mL of Hoechst 33342 in PBS, which stains all nuclei (Latt & Stetten, 1976), for 10 min at room temperature. After washing with PBS the cells were covered with a round coverslip and observed under a fluorescent microscope.

Images of microscope fields covering 500 µm<sup>2</sup> (the region comprised among a square of four electrodes on the MEA, as shown in Fig. 1B) were taken and the number of cell nuclei counted. The mean was obtained counting four fields of five different preparations. To evaluate the relative composition of neurons and glial cells in the preparation, parallel cultures were obtained at the same density on a coverslip and double immunofluorescence assays were performed with the primary antibody TUJ1, which binds to a neuron-specific class III beta-tubulin (Geisert & Frankfurter, 1989; Fig. 1C) and with anti-GFAP (Fig. 1D; Debus *et al.*, 1983). Total nuclei were counterstained with Hoechst 33342 dye. The percentage of neurons and glia in the culture was therefore evaluated by counting the number of TUJ1 or GFAP labelled cells over the total number of nuclei. The graph in Fig. 1F shows the results of these counts after 1, 2, 3 and 4 weeks culture. At each time point ~20% of the cells were positive for GFAP. The percentage of neurons increased from the first to the second week in culture and remained stable in the following weeks. The remaining cells in the first week samples were positive to nestin, a marker for neural precursors (Renfranz *et al.*, 1991). In similar cultures, the neuronal composition was evaluated by double immunofluorescence assays. Neurons were recognized by the antibody TUJ1. GABAergic, glutamatergic, or serotonergic neurons were revealed by specific antibodies binding to their neurotransmitters; dopaminergic neurons were revealed with specific antibodies against the enzyme tyrosine hydroxylase (TH), which is required for the production of catecholamine neurotransmitter. (Molinoff & Axelrod, 1971). The percentage of neurotransmitter specific neurons relative to the total number of neurons was therefore counted under the fluorescence microscope. To estimate the number of synaptic contacts between cells an immunofluorescence assay against the synaptic vesicle marker protein SV2 and the neuron-specific class III beta-tubulin (TUJ1) was performed. Five fields were photographed under the normal fluorescent microscope and the confocal microscope, then the number of dots per field were counted and divided by the number of cells in the field to give an approximate number of synapses per neuron.

### Electrical recordings and electrode stimulation

The MEA system used for electrophysiology was commercially supplied by Multi Channel Systems (MCS). MEA dishes had 10 × 6 TiN electrodes with an interelectrode spacing of 500 µm and each metal electrode had a diameter of 30 µm. The MEA is connected to a 60 channel, 10 Hz–3 kHz-bandwidth preamplifier/filter-amplifier (MEA 1060-AMP) which redirects the signals towards a further electronic processor (i.e. amplification and AD conversion), operated

FIG. 1. Characterization of the neuronal culture. (A) Image of dissociated hippocampal neurons on the MEA. (B) Cell nuclei of the dissociated culture stained with Hoechst 33342. (C) Neurons expressing type III tubulin recognized by TUJ1 antibody (green); total nuclei (blue). (D) Glial cells expressing GFAP (green); total nuclei (blue). (E) GABAergic neurons recognized by anti-GABA specific antibody. (F) Synaptic vesicles stained by the protein specific for synaptic vesicle SV2 (green) and neurons expressing type III tubulin recognized by TUJ1 antibody (red). (G) Confocal image of the same preparation shown in F. Scale bars 100 µm (A and B); 50 µm (C, D and E) and 25 µm (F). Images in C–G are from cells after 3 weeks in culture. (H) Cell composition of neuronal cultures after 1–4 weeks of culture.

FIG. 2. Electrical recordings, AP sorting and APs antidromically evoked. (A) Electrical recordings obtained with a MEA composed by an array of 10 × 6 electrodes. The electrical artifact is indicated by the black arrow. A bipolar voltage pulse was applied to the fifth row of electrodes. The electrode framed in the grey box was used as ground. The stimulation evoked APs of different amplitude in most of the MEA electrodes. (B) Magnified electrical recordings obtained from the circled electrode in panel A. Red and blue squares mark APs from two identified neurons. (C) AP sorting. Upper panel shows all events exceeding a threshold of –50 µV. Lower panel shows APs sorted from two different neurons in red and blue. The same colour in B and C indicates the same neuron. (D) Raster plots (left panel) and electrical recordings (right panel) from one electrode in normal conditions showing a highly reliable AP with a low temporal jitter (less than 0.25 ms) which is suppressed in presence of synaptic blockers (10 µM bicuculline, 100 µM CNQX and 50 µM APV). (E) Raster plots (left panel) and electrical recordings (right panel) from one electrode in the presence of synaptic blockers (10 µM bicuculline, 100 µM CNQX and 50 µM APV), showing a highly reliable AP with a low temporal jitter (less than 0.25 ms) which is not evoked in normal conditions.

by a board lodged inside a high performance PC. Signal acquisitions are managed under software control and each channel was sampled at a frequency of 20 kHz. One electrode was used as ground (see Fig. 2A). Sample data were transferred in real time to the hard disk for later processing. In order to keep the desired environmental conditions during the electrical recordings, the dish was moved to a different incubator providing only a controlled level of CO<sub>2</sub> (5%) and temperature (37 °C) and it was sealed by a cap distributed by MultiChannelSystem in order to eliminate evaporation and contamination. The neuronal culture was then allowed to settle for ~1 h in order to reach a stationary state. After conclusion of the experiment, usually after 3–10 h, the medium was changed and the dish was moved back to the original incubator. Recordings were performed, in culture medium, from 3 weeks after seeding for up to 6 months. The same culture could be used repeatedly for other experiments for up to one month. Each metal electrode could be used either for recording or for stimulation. Voltage stimulation was used and consisted of bipolar pulses lasting 100 µs at each polarity injected through the STG1004 stimulus generator. The voltage pulse generated by the STG1004 was applied in parallel to the set of electrodes manually selected for stimulation (simultaneous multisite stimulation). A thermostat (HC-X) maintained the temperature at 37 °C underneath the MEA.

The amplitude of the voltage pulse varied between 200 and 900 mV. The minimum amplitude required to evoke an electrical response varied between 200 and 300 mV depending on the responsiveness of the culture and on the geometry of the stimulus. Analogously the lower amplitude giving the maximum response varied between 750 and 900 mV. In order to avoid invasive effects due to the stimulus itself, intensities higher than 900 mV were not applied. Once the lowest and highest intensity for a specific culture were selected, two intermediate values were chosen to complete the experiment. In most of the experiments the intensities applied were 300, 450, 600 and 900 mV. For each different spatial stimulus, once the amplitude of the voltage pulse was selected, the neuronal culture was usually stimulated for 100 trials with a fixed interpulse interval, selected between 2 s and 4 s. In general, every 30 min, test stimulation was repeated in order to test the stability of the response.

### Pharmacology

The following chemicals were used as synaptic blockers: 2-amino-5-phosphonovalerate (APV; Sigma-Aldrich), bicuculline (Sigma-Aldrich) and 6-cyano-7-nitroquinoxaline-2,3-dione disodium salt (CNQX; Sigma-Aldrich). Synaptic blocker/s was/were added to the extracellular medium in the required amount. After completion of the planned measurements, blockers were washed out by four medium replacements, and the original extracellular medium was restored.

### Data analysis

Acquired data were analysed using MATLAB (The Mathworks, Inc.). An artifact lasting 5–20 ms, caused by the electrical stimulation, was induced on the recording and was removed during data analysis (Wagenaar & Potter, 2002; Ruaro *et al.*, 2005). As the artifact removal was not reliable during the first 2 ms following the stimulation, we excluded this time bin from the analysis. Antidromically evoked APs were identified in two different ways. First, all APs observed in the presence of a cocktail of synaptic blockers (50 µM APV, 10 µM bicuculline and 100 µM CNQX) were classified as antidromic APs, and second, in agreement with Wagenaar *et al.* (2004), all APs with a reliability close to 100% and with a temporal jitter of less than 0.25 ms were classified as antidromic (Wagenaar

*et al.*, 2004). In our analysis of the variability of neuronal firing we excluded those APs classified as antidromic. For each individual electrode, we computed the standard deviation ( $\sigma$ ) of the noise, which ranged from 3 to 6 µV, and only APs crossing the threshold of  $-5\sigma$  were counted as APs used for data analysis. AP sorting was obtained using principal component analysis and open source toolboxes for the analysis of multielectrode data (Egert *et al.*, 2002) with MATLAB. For the analysis on the first evoked APs, for each electrode, only the timing of the first evoked AP was considered. Taking a pool of neurons, we define first the AP response as the number of neurons within the pool firing at least an AP in the time bin  $t$  after the stimulation (FAPR<sub>*t*</sub>, Fig. 7B). In order to calculate the average firing rate (AFR) of the neurons, peristimulus time histograms (PSTHs) were calculated for the sorted neurons (Fig. 3A) using a 10-ms time bin, where time 0 ms corresponds to the delivery of the stimulation. When APs recorded from sorted neurons were pooled, the PSTH over the population of neurons (PPSTH) was similarly calculated, counting in each time bin the APs of all neurons (Fig. 3B). In the same way, when APs recorded from the whole array of electrodes were pooled, the population PSTH (APSTH) was calculated (Figs 3C, 8A and 9A). We define single neuron response (SR<sub>*t*</sub>) as the number of APs fired in a single trial by a single neuron in the time bin  $t$  after the stimulation. Summing the SR<sub>*t*</sub> of a pool of neurons, a population response (PR<sub>*t*</sub>) is defined. Analogously, when all the APs recorded by an electrode or by a population of electrodes are counted in the time bin  $t$  in a single trial, respectively, an electrode response (ER<sub>*t*</sub>) and an electrode population response (EPR<sub>*t*</sub>) are obtained. The coefficient of variation (CV) of any analysed variable, is the standard deviation over the mean of the variable. If  $CV_i$  is the CV of the single neuron #*i* response (SR<sub>*t*</sub>),  $\langle CV \rangle$  is the average  $CV_i$  over a population of  $M$  selected neurons. See Table 1 for definitions of main variables.

### Calculation of correlation and statistical independence of neuron firing

The degree of correlation and of statistical independence of neuronal firing was measured on short (i.e. few ms) and longer time scales (i.e. few tens of ms). On short time scales, i.e. with binwidth of 2 ms as in Fig. 4C, for each pair of neurons #*i* and #*j* the product of the probability of firing  $p_i p_j$  was compared to the joint probability of firing  $p_{ij}$ , as described by Pinato *et al.* (2000). The same procedure was used to measure the statistical independence of the neuronal firing recorded for each pair of electrodes #*i* and #*j* (Fig. 4F). On longer time scales, i.e. with a time bin of 50 ms, the cross correlation between pairs of single neuron responses (SR<sub>*t*</sub>) were calculated (CSN<sub>*t*</sub>) as shown in Fig. 4B. The same procedure was applied to calculate the cross correlation between single electrode responses (ER<sub>*t*</sub>) for a pair of electrodes (CSE<sub>*t*</sub>), as shown in Fig. 4E.

### Calculation of the mutual information

In order to decode the stimulus intensity, we analysed and compared neural codes based on the firing rate of single neurons (SR<sub>*t*</sub>), on the firing rate of an ensemble of neurons (ER<sub>*t*</sub>, EPR<sub>*t*</sub>) and on the first evoked APs in an ensemble of neurons (FAPR<sub>*t*</sub>). We used the information theory (Shannon & Weaver, 1949) and in particular mutual information to estimate the amount of information that can be decoded by the different neural codes in different time bins (i.e. varying  $t$ ) and for different extent of pooling (i.e. different number of

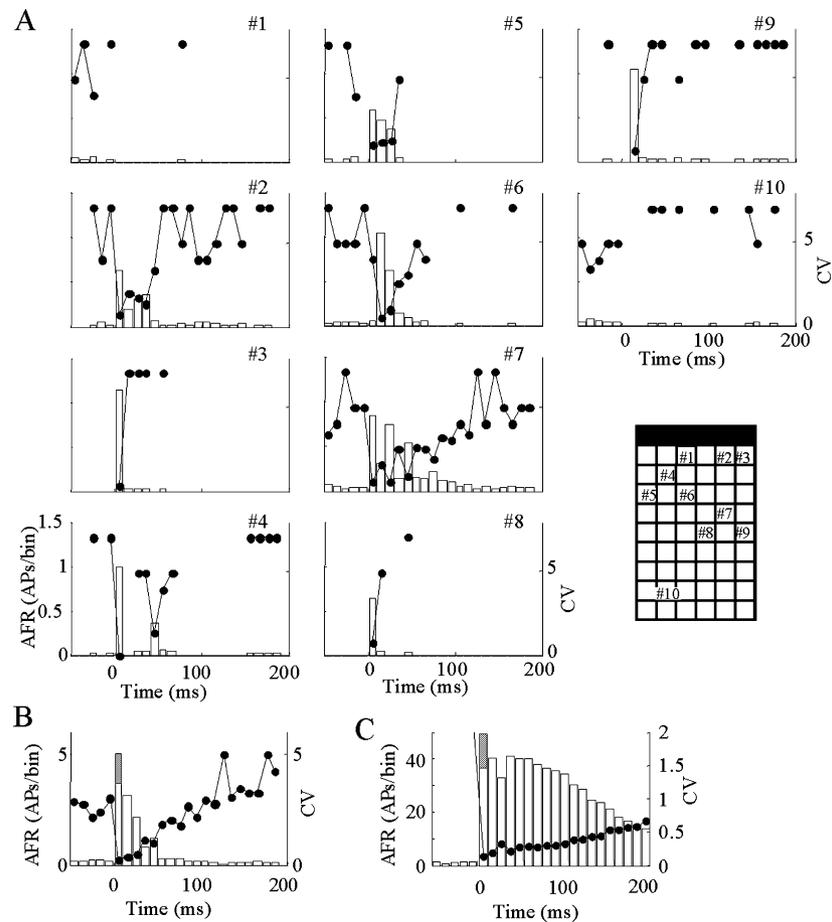


FIG. 3. Reproducibility of neuronal firing. (A) Each panel reproduces the AFR (open bars) and the corresponding CV (black symbols) for ten neurons identified in the electrodes indicated in the grid (#1 to #10), where the black bar corresponds to the electrodes used for stimulation. (B) AFR (open bars) and its CV (black symbols) when all APs of the ten neurons identified in A were pooled. (C) AFR (open bars) and its CV (black symbols) when all the APs recorded by the MEA were pooled. In B and C the shaded bar corresponds to antidromic APs, i.e. APs that were observed also in the presence of APV, CNQX and bicuculline.

electrodes considered). In particular, the mutual information was calculated as follows:

$$I_t \equiv I_t(R, S) = \sum_{s \in S} p(s) \sum_{r \in R} p_t(r|s) \times \log_2(p_t(r|s)/p_t(r)) \quad (1)$$

where

$$p_t(r) = \sum_{s \in S} p(s) \times p_t(r|s) \quad (2)$$

$I_t$  quantifies in bits the amount of information that a single response,  $r$  (i.e.  $SR_t$ ,  $ER_t$ ,  $EPR_t$ , or  $FAPR_t$  depending on the different neural code) provides about the intensity of the stimulus  $s$ ;  $p_t(r)$  is the total probability of observing the response  $r$  considering the time bin 1 to  $t$  ms after the stimulus, averaged over all stimuli. In our case, all stimuli occurred with equal probability,  $p(s)$ . In order to minimize the effects of finite sample size on our estimates of information, the real response  $r$  have been binned into different intervals, following the methods of Panzeri & Treves (1996).

## Results

### Characterization of the neuronal culture

Hippocampal neurons from neonatal rats were grown over a  $10 \times 6$  MEA (Fig. 1A). After 4 weeks in culture, the density of

neurons was evaluated by counting the cell nuclei on the surface delimited by four adjacent electrodes on the MEA (Fig. 1B). The obtained density was  $1756 \pm 255$  cells/mm<sup>2</sup> ( $n = 4$ ). By immunofluorescence assays, it was possible to clearly identify neurons (Fig. 1C) and astrocytes (Fig. 1D). The neuronal culture composition was analysed over a period of 4 weeks. As shown in Fig. 1, during the first four weeks, the fraction of glial cells was always  $\sim 20\%$ . The percentage of neurons increased between the first and the second week in culture and remained stable afterwards. The remaining cells during the first week were positive to nestin, a marker for neural precursors (Renfranz *et al.*, 1991) (Fig. 1H). Neurons had long dendrites extending in all directions and often it was possible to recognize an axonal structure. Due to the difference in cell body size, although much less abundant in number, glial cells occupied most of the space of the culture and neurons appeared to be growing over and beneath glial cells. Approximately 8% of neurons were GABAergic (Fig. 1E) and 90% were glutamatergic. No serotonin or dopaminergic neurons were detected and we were unable to determine the nature of the few remaining neurons. With double immunofluorescence assay against the synaptic vesicle marker protein SV2 and the neuronal marker TUJ1 (Fig. 1F and G) we estimated the number of synapses between 450 and 500 per cell, in agreement with the data published for cortical neurons in culture (Tateno *et al.*, 2002).

TABLE 1. Definition of variables

Abbreviation	Full name of variable	Definition
FAPR <sub>t</sub>	First AP response	Number of neurons within the pool firing at least one AP within time <i>t</i> after the stimulus
SR <sub>t</sub>	Single neuron response	Number of APs fired in a single trial by a single neuron within time <i>t</i> after the stimulus
PR <sub>t</sub>	Population response	Sum of SR <sub>t</sub> over a pool of neurons
ER <sub>t</sub>	Electrode response	Number of APs recorded by an electrode after the stimulus within time <i>t</i>
EPR <sub>t</sub>	Electrode population response	Sum of ER <sub>t</sub> over a pool of electrodes

AP, action potential.

### Electrophysiology, single neuron detection and antidromically evoked APs

MEAs allow the recording of the extracellular voltage signals, produced by APs of all neurons establishing a good electrical contact with MEA electrodes (Fig. 2A). Often extracellular APs larger than 100  $\mu$ V were measured. The neuronal culture was stimulated by a brief bipolar

voltage pulse applied to a set of electrodes. The electrical stimulation produced an artifact lasting 5–20 ms (removed off-line, see Materials and methods) in all electrodes (indicated by an arrow in Fig. 2A and B), followed by clear evoked APs. Extracellular signals larger than five times the standard deviation of the voltage noise ( $5\sigma$ ) were considered as reliable APs. The number of neurons recorded by different MEAs ranged from a few dozen to hundreds of units. APs clearly produced by the same neuron (indicated in red and blue in Fig. 2C lower panel) were identified using AP sorting algorithms (see Materials and methods) and the reproducibility of their firing during repetitive stimulations was studied. The population of neurons that could be identified on different MEAs ranged between 10 and 50 units. As neurons of our cultures had long axons (see Fig. 1), some detected APs were produced by a direct antidromic stimulation of the neuron and were not evoked through synaptic pathways (Wagenaar *et al.*, 2004). In order to identify these antidromic evoked APs, we compared recordings in the absence and in the presence of a cocktail of synaptic blockers (50  $\mu$ M APV, 10  $\mu$ M bicuculline and 100  $\mu$ M CNQX). In addition to that reported by Wagenaar *et al.* (2004), we observed highly reliable APs with a low temporal jitter (less than 0.25 ms) that were abolished by the cocktail, indicating that they were travelling through synaptic pathways (Fig. 2D). We also observed APs with a low temporal jitter (less than 0.25 ms) detected only in the presence of synaptic blockers (Fig. 2E). These APs are likely to be antidromically evoked but in control conditions are blocked by inhibitory synapses.

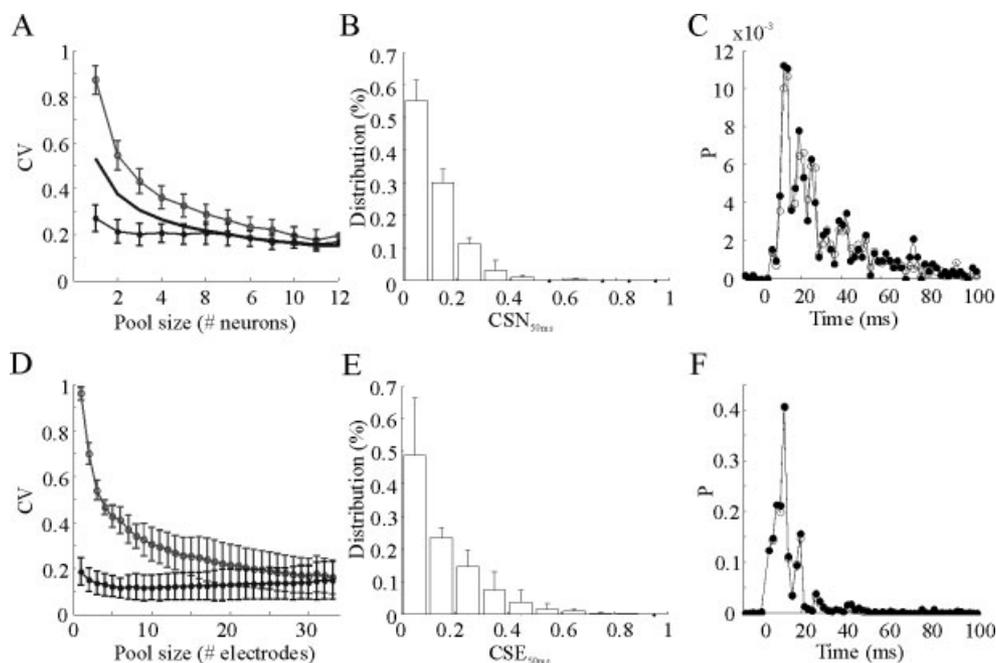


FIG. 4. Coefficient of variation of the population response and correlation of neuron firing. (A) CV of the population response in the first 50 ms poststimulus (PR<sub>50ms</sub>) as a function of the number of neurons pooled (*N*). Neurons with a similar CV (i.e. <1) were pooled. In the grey (black) plot, neurons with an increasing (decreasing) CV were added one by one. The data are averaged over five different preparations. The thick black line represents  $\langle CV \rangle / \sqrt{N}$ , where  $\langle CV \rangle$  is the average CV of the neurons. (B) Distribution of the coefficient of correlation of single neuron response in the first 50 ms poststimulus (CSN<sub>50ms</sub>). For each different preparation, all the possible pairs of neurons were considered. The data were averaged over five different preparations. (C) Average joint probability of firing of all possible pair-wise combinations of neurons. Considering all the possible pairs of neurons #*i* and #*j*, the average joint probability of firing  $p_{ij}$  (filled circles) is compared with the average product of individual probabilities  $p_i p_j$  (open circles). The data shown are from a single preparation and identical results were obtained in the other four preparations considered. (D) CV of the electrode population response in the first 50 ms poststimulus (EPR<sub>50ms</sub>) as a function of the number of pooled electrodes. In the grey (black) plot, electrodes with an increasing (decreasing) CV were added one by one. The data were averaged over five different preparations. (E) Distribution of the coefficient of correlation of single electrode response in the first 50 ms poststimulus (CSE<sub>50ms</sub>). For each different preparation, all the possible pairs of electrodes were considered. The data were averaged over five different preparations. (F) Average joint probability of firing of all possible pair-wise combinations of electrodes. Considering all the possible pairs of electrodes #*i* and #*j*, the average joint probability of firing  $p_{ij}$  (filled circles) is compared with the average product of individual probabilities  $p_i$  and  $p_j$  (open circles). The data shown are from a single preparation and identical results were obtained in the other four preparations considered.

### Reproducibility, pooling and statistical independence of neuronal firing

When the same voltage pulse was repetitively applied to the same electrodes, the number of evoked APs in a single neuron varied, but often the first evoked AP was reliable with a jitter varying from just a few hundreds  $\mu$ s to some ms. The evoked AFR and the associated coefficient of variation (CV, see Materials and methods) computed on a time window of 10 ms, for ten individual neurons, at progressively longer distances from the stimulated electrodes are shown in Fig. 3A.

The ten neurons were recorded at distances varying between 500  $\mu$ m to 4000  $\mu$ m from the row of electrodes used for stimulation (black bar in the grid). Neurons #1 and #10 were not activated and their firing was almost abolished by the electrical stimulation. The CV of their firing increased following the electrical stimulation. On the contrary, the firing of neurons #2–9 increased significantly, whereas their CV decreased. Neuron #4 responded to the stimulation by firing an AP in the time window between 1 and 11 ms, in each of the 50 trials, with a jitter of less than 0.25 ms and the corresponding CV was 0. Other identified neurons, were less reliable, their firing was more distributed over time and, at the peak of the evoked response, their CV was between 0.2 and 0.8. When antidromically evoked APs

were excluded (see Materials and methods) and the firing of the ten identified neurons was pooled, the CV was  $\sim 0.25$  and remained less than 0.4 for at least 20 ms at the peak of the evoked response (Fig. 3B). Similar results were obtained from five additional neuronal cultures. When we pooled together all APs recorded from all electrodes on the MEA, the CV was transiently lower than 0.1 and remained lower than 0.3 for at least 40 ms (Fig. 3C). When antidromically evoked APs were included in the pool, the CV did not change significantly, as antidromic APs are extremely reliable (Fig. 3B and C).

When pooling single neuron responses ( $SR_{50\text{ms}}$ ) with a similar CV (Fig. 4A), the CV of the pooled activity decreased as  $\langle CV \rangle / \sqrt{N}$  (thick black line), where  $\langle CV \rangle$  is the average CV and  $N$  is the number of pooled neurons. Figure 4A shows how the CV varies when neurons with the lowest (black symbols) and the highest CV (grey symbols) were progressively pooled one by one. Single neurons had an average CV of 0.5 and, when 12 neurons were pooled, the CV approached 0.15. Therefore at least two dozens of neurons are needed to obtain a CV of  $\sim 0.1$ , i.e. a very reliable response. Pooling the electrical activity from different neurons reduces the corresponding CV only if their evoked firing is not correlated (Gawne & Richmond, 1993;

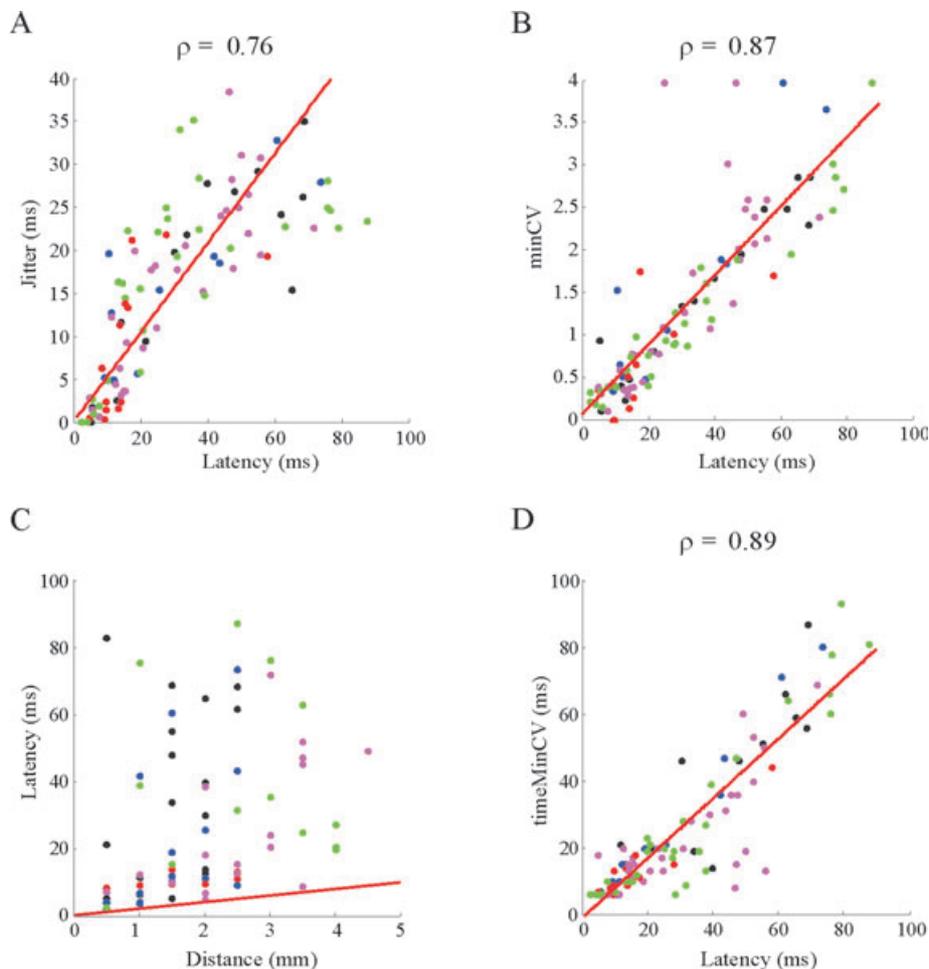


FIG. 5. Statistical properties of single neuron firing, when a single bar of electrodes was stimulated. APs antidromically evoked are considered only in the statistic shown on panel C. (A) Relation between jitter and latency of first evoked AP. (B) Relation between the minimum CV ( $\text{min}_{\text{CV}}$ ) of the AFR and latency of first evoked AP. (C) Relation between latency of first evoked AP and distance between stimulating and recording electrodes. The slope of the line estimates the maximum speed of propagation of action potentials in the cultures, which is  $\sim 350$  mm/s. (D) Relation between the time of the minimum CV ( $\text{time}_{\text{minCV}}$ ) and latency of first evoked AP. The bin width considered is 10 ms. Only neurons that had a CV of the evoked AFR lower than four were analysed. A population of 99 neurons identified in five neuronal cultures was considered (different colours correspond to different cultures). In panel A, B and D the coefficient of correlation  $\rho$  is reported on the top. The red line is the linear regression.

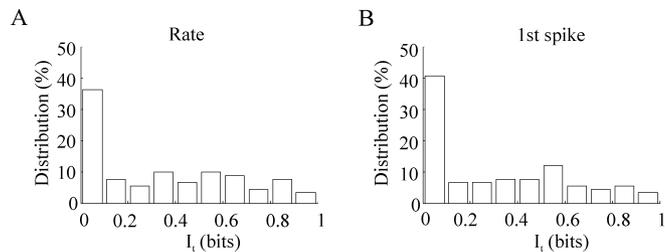


FIG. 6. The maximal mutual information  $I_t$  carried by single neurons computed considering the highest and the lowest intensities of stimulation. Five different preparations were considered. For each of the 91 neurons analysed, the time bin  $t$  was varied in order to maximize the mutual information. (A) Distribution of the mutual information provided by the first evoked AP (FAPR<sub>*t*</sub>). (B) Distribution of the mutual information provided by the single neuron response (SNR<sub>*t*</sub>).

Shadlen & Newsome, 1994, 1998). The correlation between single neuron responses (SR<sub>50ms</sub>) of pooled neurons, was on average  $0.11 \pm 0.02$  (mean and standard deviation of the mean for five preparations; Fig. 4B) considering all possible pairs of neurons. Also on shorter time scales, i.e. at a binwidth of 2 ms, neuronal firing was very poorly correlated as shown in Fig. 4C; for each pair of neurons #*i* and #*j* the product of the probability of firing  $p_i p_j$  was very similar to the joint probability of firing  $p_{ij}$ , of neurons #*i* and #*j* firing simultaneously. As a consequence, on short time scales the binned firing of pairs of neurons is close to statistical independence and therefore is almost uncorrelated.

Similar results were obtained when all APs recorded in a single electrode were counted in the first 50 ms following the stimulation (ER<sub>50ms</sub>) and pooled (Fig. 4D). In this case, when APs from

approximately ten electrodes were pooled, the CV approached 0.1. As each electrode of the MEA detects APs from different neurons (usually between two and five), a pool of 20–50 neurons provides a reliable response.

The correlation between pairs of ER<sub>50ms</sub> was  $0.17 \pm 0.05$  (Fig. 4E). Similarly, at a binwidth of 2 ms, APs recorded by pairs of electrodes were almost statistically independent and therefore not correlated (Fig. 4F). These results, although obtained on a small fraction of neurons forming the network under investigation, suggest that coactivated neurons on a time scale of some milliseconds are poorly correlated and therefore averaging their electrical activity reduces the variability of the evoked response.

Statistical properties of neuron firing, when a single bar of electrodes was stimulated, for a population of 99 neurons identified in the five neuronal cultures analysed (different colours correspond to different cultures) are reproduced in Fig. 5. The jitter of the first evoked AP on its average latency were significantly correlated with coefficient of correlation  $\rho = 0.76$  (Fig. 5A). As the latency of an evoked AP is primarily determined by the number of synapses between the stimulating site and the neuron, the significant correlation between jitter and latency shows that the reliability of the firing of a single neuron decreases with the number of synapses that the signal has to cross. The minimum CV (min<sub>CV</sub>) of the AFR and the time of its occurrence (time<sub>minCV</sub>) were correlated to the latency of the first evoked AP, respectively, with  $\rho = 0.87$  and  $\rho = 0.89$  (Fig. 5B and D), indicating that the firing of the first evoked AP is the most reliable part of the neural response. The average latency increases with the physical distance ( $d$ ) between the recording electrode and the bar of stimulating electrodes (Fig. 5C). The slope of the line provides the maximum speed of APs propagation in the cultures, which is  $\sim 350$  mm/s, in agreement with our previous report (Ruaro *et al.*, 2005).

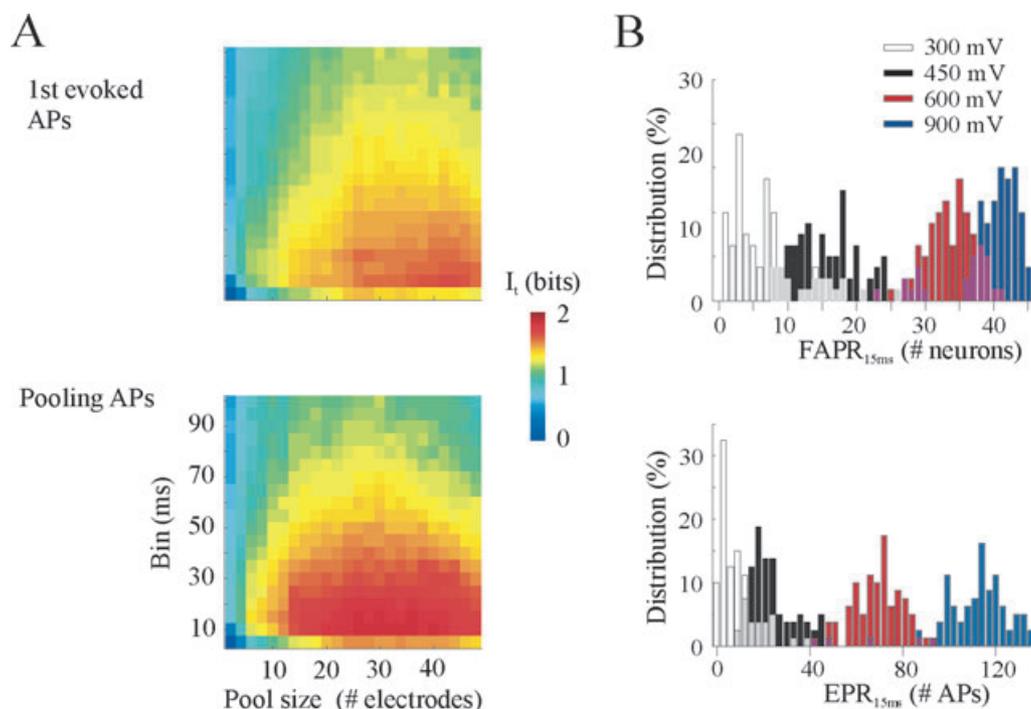


FIG. 7. Coding stimulus intensity by pooling neuron firing. The statistics shown in the upper and lower panels were carried out, respectively, by pooling the first evoked APs (FAPR<sub>*t*</sub>) and by pooling all the APs evoked (EPR<sub>*t*</sub>) in the time bin  $t$  after the stimulus. (A) Mutual information  $I_t$  calculated for four different stimulus intensities as function of bin width ( $t$ ) and of the number of pooled electrodes. (B) Distribution of the population response to the four different intensities of stimulation. The APs evoked in the first 15 ms poststimulus in all the electrode arrays were pooled. The data shown refer to one preparation.

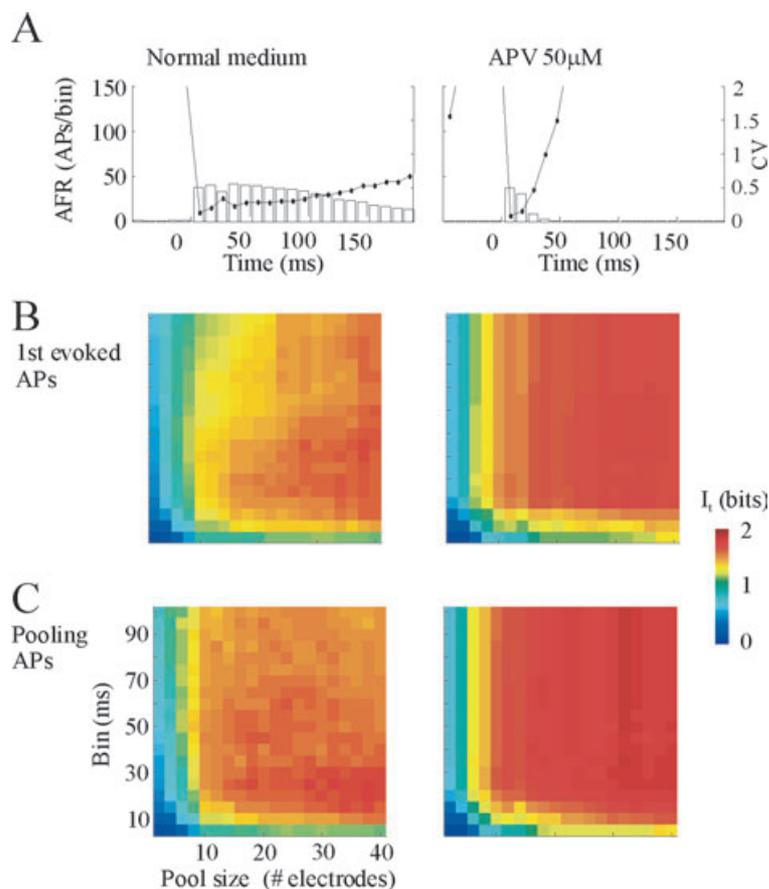


FIG. 8. The effect of APV on information processing. The statistics shown on the left panels and on the right panels were calculated in normal conditions and in the presence of 50  $\mu$ M APV. The statistics shown in the upper and lower panels were carried out, respectively, by pooling the first evoked APs (FAPR<sub>*t*</sub>) and by pooling all the APs evoked (EPR<sub>*t*</sub>) in the time bin *t* after the stimulus. (A) AFR (solid bars) and CV (black dots) of all APs recorded by the MEA. (B and C) Mutual information  $I_t$  calculated for four different stimulus intensities as functions of bin width *t* and of the number of pooled electrodes.

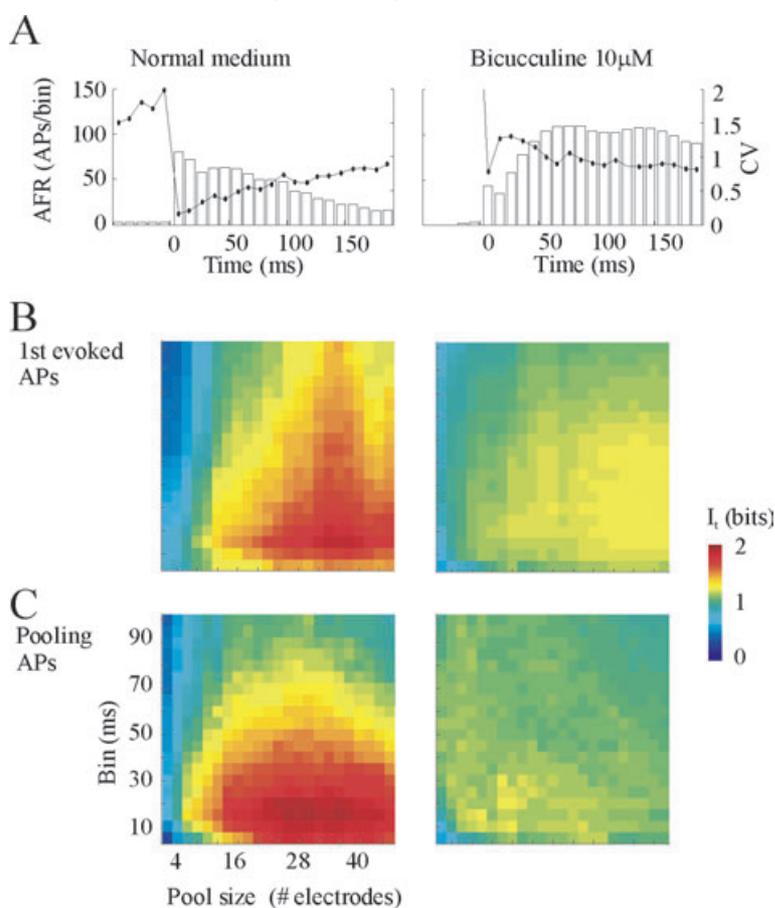


FIG. 9. The effect of bicuculline on information processing. The statistics shown on the left panels and on the right panels were calculated in normal conditions and in the presence of 10  $\mu$ M bicuculline. The statistics shown in the upper and lower panels were carried out, respectively, by pooling the first evoked APs (FAPR<sub>*t*</sub>) and by pooling all the APs evoked (EPR<sub>*t*</sub>) in the time bin *t* after the stimulus. (A) AFR (solid bars) and CV (black dots) of all APs recorded by the MEA. (B and C) Statistics of the mutual information  $I_t$  calculated for four different stimulus intensities as functions of bin width (*t*) and of the number of pooled electrodes.

### Processing information – coding stimulus intensity by pooling neuron firing

The neural code is expected to distinguish, and therefore to encode, important features of the stimulus, such as its intensity. Therefore, the coding of stimulus intensity was investigated and compared at the level of a single neuron and when APs were pooled from a population of neurons. More precisely it is assumed that  $N$  stimulus intensities can be distinguished in a reliable way, and therefore properly coded, if, by analysing features of the response, it is possible to determine in almost all trials the exact stimulus intensity. In this case, the response distributions for the  $N$  stimuli do not overlap appreciably and it is possible to code  $\log_2 N$  bits of information.

The first evoked AP is usually highly reliable and these APs may carry most of the relevant information for recognition (Abeles, 1991; Delorme & Thorpe, 2001; Thorpe *et al.*, 2001; Van Rullen & Thorpe, 2001; Delorme, 2003) and later processing. The first evoked APs are possibly the fastest signals achievable within the nervous system and are ideal for a fast neural code.

Voltage pulses of different intensities were applied to an electrode row. By increasing the voltage pulse, APs recorded on each electrode became more frequent and often APs with a new shape, produced by a different neuron, appeared (Ruaro *et al.*, 2005). Usually, no APs were evoked by voltage pulses below 200 mV and a saturating maximal response was evoked with a voltage of  $\sim 1$  V. Four voltage pulses, varying between 200 mV and 900 mV, were considered (see Materials and methods) and coding was measured by computing the mutual information  $I_t$  (see Materials and methods) between the evoked response in each trial and the stimulus intensity.

The maximal mutual information  $I_t$  carried by binned firing rates of single neurons (see Materials and methods), was on average 0.34 bits. The analysis was performed on 91 neurons from five different preparations (Fig. 6A). In the great majority of these neurons,  $I_t$  was much less than 1 bit and only in ten neurons  $I_t$  approached to 1 bit. Therefore, by counting the number of evoked APs in single neurons, it is not possible to reliably distinguish two stimulus intensities. Very similar results were obtained, computing  $I_t$  carried by the first evoked AP (FAPR<sub>t</sub>, see Materials and methods) (Fig. 6B). In this case  $I_t$  was, on average, 0.32 bits and in seven neurons out of 91 the value of  $I_t$  approached 1 bit.

As binned firing rates of single neurons cannot reliably distinguish the stimulus intensity, we investigated population coding in which APs from different neurons were pooled. Each electrode of the MEA usually detects APs from different neurons (between 2 and 5, data not shown) and therefore we pooled all APs recorded by a single electrode (ER<sub>t</sub>) and by an ensemble of electrodes of the array (EPR<sub>t</sub>).  $I_t$  was maximal ( $1.48 \pm 0.22$  bits, mean and standard deviation over five cultures) when APs recorded by a population of at least 20 electrodes were pooled in a time bin of  $\sim 30$  ms. Similar results were obtained when the first evoked APs in all electrodes were counted, in this case  $I_t$  was  $1.35 \pm 0.18$  bits in a similar time bin.

Figure 7 shows, in one preparation, the dependence of  $I_t$  on the number of electrodes pooled and on the time bin  $t$ , when the first evoked APs were counted (Fig. 7A upper panel) and when all APs were pooled (Fig. 7A lower panel). In this case, the network distinguishes clearly four stimulus intensities, both when the first evoked APs were counted (Fig. 7B upper panel) and when all APs were pooled (Fig. 7B lower panel).

### The effect of APV and bicuculline

Hippocampal neuronal cultures are composed of GABAergic and of glutamatergic neurons (Fig. 1). Therefore, we analysed the effect of

blockers of these synaptic pathways such as APV and bicuculline on the mutual information. APV is a well known blocker of excitatory synaptic transmission mediated by NMDA receptors and bicuculline blocks inhibitory pathways mediated by GABAergic synapses.

Increasing the extracellular concentration of APV from 1  $\mu\text{M}$  to 50  $\mu\text{M}$ , the second component of the evoked response progressively decreased and in the presence of 50  $\mu\text{M}$  APV was completely blocked (Fig. 8A). In the presence of 50  $\mu\text{M}$  APV, the initial component of the AFR was almost unaltered, but the second component was significantly depressed leading to an increase of the corresponding CV. For single neurons, the late evoked response was also depressed but the timing and the occurrence of the first evoked AP was unaltered and had approximately the same latency and jitter (data not shown). The correlation between the electrode responses in the first 50 ms post stimulation (ER<sub>50ms</sub>) did not significantly change.

The value of  $I_t$  when the first evoked APs were counted (Fig. 8B) and when all APs were pooled (Fig. 8C), significantly increased in the presence of APV by 15% ( $n = 5$ ,  $t$ -test,  $P < 0.01$ ).

The opposite effect was observed when bicuculline was added to the extracellular medium. Increasing the concentration from 200 nM to 10  $\mu\text{M}$ , the spontaneous electrical activity increased and became progressively synchronized.

While the late component of the evoked response increased, the correspondent CV decreased (Fig. 9A). In the presence of 10  $\mu\text{M}$  bicuculline, the correlation between the electrode responses (ER<sub>50ms</sub>) of two electrodes increased drastically ( $0.61 \pm 0.19$ ). The mutual information based on first evoked APs (Fig. 9B) and on rate coding (Fig. 9C) significantly decreased in the presence of 10  $\mu\text{M}$  bicuculline, respectively, by 25% and 23% ( $n = 5$ ,  $t$ -test,  $P < 0.01$ ).

These results show that blockage of excitatory synaptic pathways mediated by NMDA receptors increases the mutual information and improves neuronal coding. We observed instead the opposite effect when inhibitory pathways mediated by GABA receptors are blocked.

## Discussion

In order to investigate the statistical properties of information processing in neuronal networks, the reproducibility of the electrical activity evoked by multisite stimulation in a neuronal culture from rat hippocampal neurons was investigated in single neurons and in neuronal assemblies. The neural activity was binned into firing rates over time windows of different length and its statistical properties were analysed. Our analysis reaches three major conclusions. Firstly, although individual neurons are noisy and unreliable elements carrying a low amount of information, by averaging APs from a neuronal assembly of  $\sim 25$ –100 neurons, it is possible to decode at a single trial level a stimulus in  $\sim 20$  ms. Secondly, a reliable information processing is obtained by averaging evoked APs or by counting the first evoked APs, provided that enough neurons are pooled. Thirdly, information processing depends critically on the balance between excitation and inhibition.

### Variability of firing of individual neurons and of neuronal populations

When a neuron was postsynaptically excited by the stimulation, the evoked firing was composed by a first reliable AP followed by less reliable APs (Fig. 3). The degree of correlation on short binwidths (i.e. less than 50 ms) between coactivated individual neurons and between all APs recorded by individual electrodes was usually small, often less than 0.1 (see Fig. 4). These results, although obtained on a small fraction

of the neurons forming the neuronal network, suggest that coactivated neurons fire almost independently on a short time scale. This is not surprising as vesicle release at different synapses is an uncorrelated process at short time scales. As a consequence of this statistical independence, when the electrical activity was pooled over  $N$  coactivated neurons, the CV of the pooled electrical activity decreased as  $1/\sqrt{N}$  (see Fig. 4) (Papoulis, 1984; Pinato *et al.*, 2000). As neurons had an average CV of 0.5, a good reproducibility, with a CV of less than 0.1, was obtained by pooling the electrical activity of 25 neurons or more.

These conclusions, drawn from an investigation carried out in a dissociated culture of rat hippocampal neurons, are remarkably similar to those obtained in intact neuronal tissues or small nervous systems. In the leech nervous system (Arisi *et al.*, 2001; Zoccolan *et al.*, 2002) motoneurons coactivated during the same behavioural reaction and fired APs in an almost statistically independent way. As a consequence of statistical independence, pooling the electrical activity over all coactivated motoneurons makes AP trains underlying reproducible motor reactions. Shadlen & Newsome (1998) suggested that in the cortex quantities are represented as rate codes in ensembles of 50–100 neurons, i.e. a column-like ensembles, providing a reliable estimation of rate in just one interspike interval (10–50 ms). Using multisite recordings, Nicolelis *et al.* (1998) showed that in the primate somatosensory cortex, the electrical activity from a small neural ensemble, of ~30–40 neurons, coded correctly the location of a single tactile stimulus on a single trial.

#### Processing information by ensemble averaging

The large variability of firing of individual neurons is reflected in the low amount of information carried by binned firing rates of the single neurons (Fig. 6). As the first evoked AP is the most reliable, in the large majority of neurons the information carried by the first evoked AP is almost identical to the information carried by the firing rate (Fig. 6). In order to decode a larger amount of information, it is necessary to average the response of a neuron population (Fig. 7). By pooling the electrical activity of 50–100 neurons evoked in the first 20–50 ms, it was possible to extract more than 1.5 bits (Fig. 7). Pooling over an ensemble of neurons the first evoked APs or the number of evoked APs (Fig. 7) allowed the extraction of approximately the same amount of information (Fig. 7). Therefore, pooling first evoked APs or firing rates is almost equivalent. First evoked APs also represent the fastest signal in the nervous system. *In vivo* experiments on the somatosensory cortex (Panzeri *et al.*, 2001) have shown the prominent role of the first AP, which contained ~83% of the total information. Recently, Johansson & Birznieks (2004) have shown that first APs in ensembles of ~30 neurons of human tactile afferents can code, within 40 ms after the stimulus onset, complex spatial fingertip events.

Recognition of complex scenes and images occurs within 180 ms (Thorpe *et al.*, 2001) likely obtained in several steps, each completed within a short time interval of 10–20 ms. In this case, the best procedures are either averaging APs in a short time window or considering the first evoked APs in an ensemble of neurons. A faster and reliable processing such as that necessary in the auditory system requires an appropriate neuronal network composed of specific neurons and synapses (Koppl, 1997). For different tasks it may be necessary to have a different amount of temporal and spatial averaging. For instance in the leech nervous system, in order to guarantee reliability of important behavioural reactions, such as the escape from a noxious stimulus, a longer integration time is used – some hundreds of ms – but on 10–20 motoneurons (Zoccolan *et al.*, 2002).

#### The role of inhibition and excitation – variability, reliability and information processing

Inhibition and excitation seem to play a fundamental role in the mechanisms underlying variability and reliability of the evoked response and they influence the network's potential to process information. Blocking the excitatory pathways mediated by NMDA-receptors had several major effects, in agreement with previous investigations (Kamioka *et al.*, 1996; Jimbo *et al.*, 2000). In the present investigation, it is shown (Fig. 8) how the blockage of these synaptic pathways reduced and almost eliminated the second component of the evoked response and, most relevant for the present investigation, increased the mutual information between the evoked response and the stimulus, allowing a greater recovery of information. Blocking inhibitory pathways mediated by GABA-receptors had opposite effects as previously described (Jimbo *et al.*, 2000; Arnold *et al.*, 2005). The present investigation shows how, blocking these synaptic pathways, the mutual information between the evoked response and stimulus decreases and very little information can be recovered. This remarkable deterioration of information processing was caused by an increased variability of the early phase of the evoked response and by the occurrence of large spontaneous bursts of synchronized electrical activity. Under these conditions, the noise in the neuronal network became correlated and could not be eliminated or reduced by averaging or pooling. These results support the idea (Shadlen & Newsome, 1998; Wehr & Zador, 2003; Zhang *et al.*, 2003; Turrigiano & Nelson, 2004; Mariño *et al.*, 2005) that, in order to process information in a reliable way, neuronal networks require an appropriate balance of excitation and inhibition, so that a stimulus can generate a reliable neuronal response, distributed through the network.

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#### Abbreviations

AFR, average firing rate; AP, action potential; GFAP, glial fibrillary associated protein; MEA, multielectrode arrays; PSTHs, peri-stimulus time histograms; TH, tyrosine hydroxylase; TUJ1, class III beta-tubulin.

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