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LOW-FREQUENCY STIMULATION ENHANCES BURST ACTIVITY IN CORTICAL CULTURES DURING DEVELOPMENT

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Abstract–The intact brain is continuously targeted by a wealth of stimuli with distinct spatio-temporal patterns which modify, since the very beginning of development, the activity and the connectivity of neuronal networks. In this paper, we used dissociated neuronal cultures coupled to microelectrode arrays (MEAs) to study the response of cortical neuron assemblies to low-frequency stimuli constantly delivered over weeks *in vitro*. We monitored the spontaneous activity of the cultures before and after the stimulation sessions, as well as their evoked response to the stimulus. During *in vitro* development, the vast majority of the cultures responded to the stimulation by significantly increasing the bursting activity and a widespread stabilization of electrical activity was observed after the third week of age. A similar trend was present between the spontaneous activity of the networks observed over 30 min after the stimulus and the responses evoked by the stimulus itself, although no significant differences in spontaneous activity were detected between stimulated and non-stimulated cultures belonging to the same preparations. The data indicate that the stimulation had a delayed effect modulating responsiveness capability of the network without directly affecting its intrinsic *in vitro* development.

Key words: micro-electrode array, rat cortical neurons, multisite stimulation, network burst.

The use of dissociated cortical neurons cultured onto microelectrode arrays (MEAs) allows for the investigation of neuronal network dynamics at a mesoscopic scale. Cultures represent a useful experimental model to characterize both the spontaneous behaviour of neuronal populations and their activity in response to pharmacological and/or electrical conditioning. Several studies performed long-term monitoring of the electrical activity of cortical primary neurons by exploiting the distinctive features of extracellular non-invasive multielectrode recordings. By using this technique it was shown that the primary networks self-organize and rewire following plating (Pasquale et al., 2008), adapt to external stimuli (Eytan et al., 2003), respond to pharmacological manipulations (Eytan et al., 2004), learn through a set of external stimuli (Shahaf and Marom, 2001) or act as an effective computational device (Ruaro et al., 2005). However, most studies were focused on the analysis of neuronal dynamics in mature cultures once achieved a stable morphological and functional connectivity, while only few investigations were aimed at the characterization of the network spontaneous activity over time (Van Pelt et al., 2004a; Chiappalone et al., 2006; Wagenaar et al., 2006).

During development, while synapses and neural connectivity build up, neuronal network activity starts to organize in synchronized patterns. The latter activity is observed since the early stages of development and accounts for the majority of the spontaneous electrical activity for a period extending from days to weeks (Ben-Ari, 2001; Corner et al., 2002; Corner, 2008). As brain circuits develop through processes of synapse formation and elimination (Goodman, 1996; Katz and Shatz, 1996; Feller, 1999), the early activity shown during network development is believed to play a paramount role in the structuring of its functionality (Opitz et al., 2002). Developing *ex-vivo* cultured neurons show the same properties of brain tissue, although at a simplified level of organization (Fields and Nelson, 1992; Chub and O'Donovan, 1998; Chiappalone et al., 2006).

Virtually all previous studies, focusing on the spontaneous activity during development of cultured networks, left a number of unanswered questions such as: (i) How does a culture react if stimulated at different stages of its growth? (ii) When does it acquire the capability of responding in a stable way? (iii) Is the development of a neuronal culture affected by an early delivered electrical conditioning? (iv) Does a culture stimulated over time differ from a never stimulated mate? To address these questions, in the present work we monitored a population of rat neocortical primary cultures for at least 4 weeks from early stages to *in vitro* maturation and chronically administered constant low-frequency stimuli twice a week starting from the second week *in vitro*. To understand the effects of electrical stim-

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Abbreviations: COM, centre of mass; DIV, days *in vitro*; DT, differential threshold; IBI, interburst interval; II, increment index; MBR, mean bursting rate; MEA, micro-electrode array; MFR, mean firing rate; *mrd*, mean relative deviation; NB, network burst; NBD, network burst duration; NBFP, network burst falling phase; NBPV, network burst peak value; NBRP, network burst rising phase; PLP, peak lifetime period; *pmrd*, population mean relative deviation; PSTH, post stimulus time histogram; *rd*, relative deviation; RP, refractory period.

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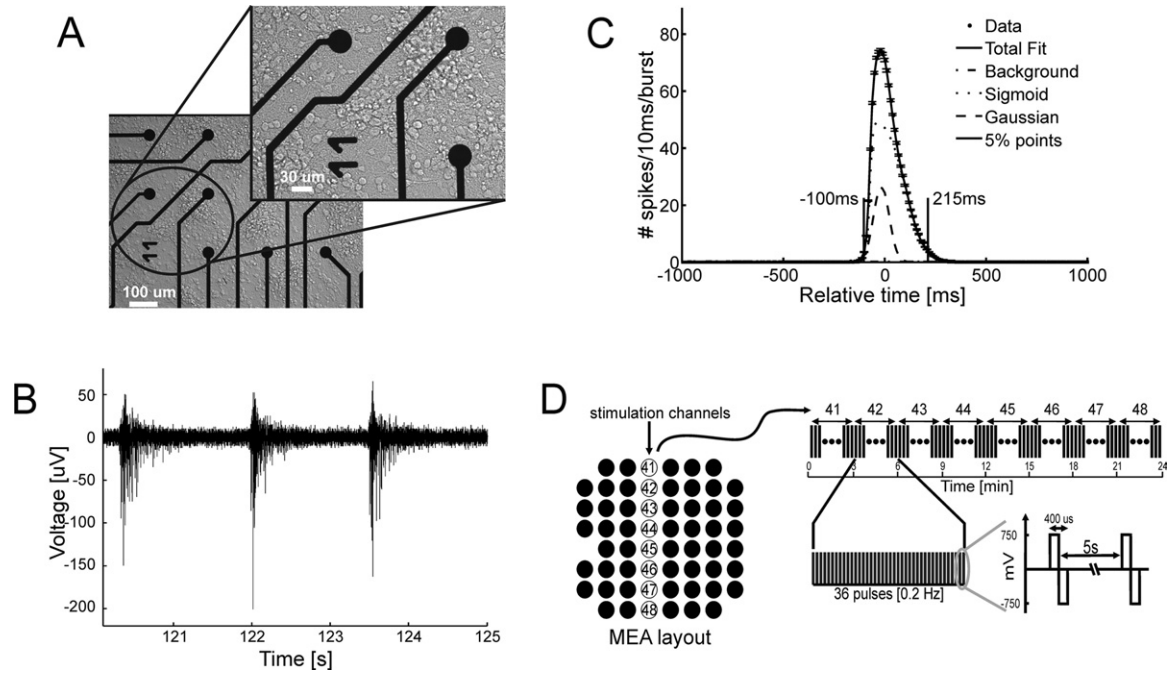

Fig. 1. Overview: (A) Example of neuronal cells coupled to MEAs. (B) An example of a burst recorded through a single channel. The early and central phase of the burst is clearly denser with spikes than the tail in which isolated spikes can be distinguished. (C) An example of the network burst fitting procedure: the network burst profile is fitted by using three curves, that is, a background, a double sigmoid and a gaussian. For further details, cf. *Experimental procedures*. (D) Sketch of the stimulation protocol: the central column of the MEA layout is used to deliver the stimuli from top to bottom (left). Every channel delivers a set of 36 stimuli at 0.2 Hz, for 3 min (top right). A single pulse was 1.5 V peak to peak and biphasic (positive phase first) and lasted 400 μs (bottom right).

ulation on the spontaneous activity of developing neuronal populations, we monitored spontaneous activity before and after the stimulation and compared stimulated and non-stimulated cultures under the same conditions.

Although the networks started to reliably respond to the electrical conditioning at various stages of the development, the stimulation paradigm drove the cultures to significantly burst more frequently than before stimulation. The changes in activity lasted for at least 30 min and all active electrodes were involved, showing that the effect impacted on the entire culture. In addition, a coherent behaviour was found between the changes in the bursting activity and the post-stimulus time histogram area, indicating a long-term effect of the stimulation.

EXPERIMENTAL PROCEDURES

Cell culture preparation and maintenance

Primary neuronal cultures were obtained from cortices of Sprague–Dawley rats at day 18 of gestation. Embryos were extracted by caesarian section from anesthetized pregnant dams in accordance with the European Community Council directive approved by the Italian Ministry of Health. Culture preparation was performed as previously described (Chiappalone et al., 2008). Briefly, rat embryonic cerebral cortices were dissected out from the brain and dissociated first by enzymatic digestion in trypsin solution (20 min at 37 °C) and subsequently by mechanical dissociation with a fine tipped Pasteur pipette. The resulting tissue was resuspended in Neurobasal medium supplemented with 2% B-27 and 1% Glutamax-I (Invitrogen, Carlsbad, CA, USA) at the final concentration of 1600∼2000 cells/μl. Cells were afterwards plated onto MEAs previously coated with poly-D-lysine and laminin to promote cell adhesion (see Fig. 1A). Cultures were kept in an incubator at 5% CO_2 at 37 °C and pulled out only during the experimental sessions or for medium change. To reduce thermal stress of the cells, MEAs were kept at 37.2 °C by means of a controlled thermostat (MCS, Reutlingen, Germany). Culture medium was changed weekly, immediately after the recording sessions. All the animal experiments were made in accordance with the Italian and UE legislation and special care was made to reduce animal suffering. Additionally the use of dissociated cultures intrinsically helped to reduce the number of animals used.

Culture dishes and experimental setup

Microelectrode arrays (Multichannel systems, MCS) consisted of 59 TiN/SiN planar round electrodes (30 μm diameter; 200 μm center-to-center interelectrode intervals) arranged in a square grid excluding corners (see Fig. 1D). One recording electrode was replaced by a bigger ground electrode. All dish chambers were sealed with a gas permeable Teflon membrane to prevent contamination and evaporation (Potter and DeMarse, 2001). The activity of all cultures was recorded by means of either a MEA60 or MEA120 System (MCS). After a 1200× amplification, signals were sampled at 10 kHz and acquired through the data acquisition card and MCRack software (MCS). Electrical stimuli were delivered through a four or eight channel stimulator (MCS STG2004 or 1008). Data analysis was performed off-line by a custom software tool developed in MATLAB© (The Mathworks, Natick, MA, USA; Vato et al., 2004).

Experimental protocols

(a) Spontaneous activity recording protocol. The spontaneous activity of 11 cultures belonging to three independent preparations was measured twice a week. All recordings started 20–30

min after positioning the dishes upon the heated plate of the amplifier to let the cultures recover from the mechanical and thermal stress due to transfer from the incubator.

(b) Stimulation protocol. The activity of 13 cultures belonging to three independent preparations (the same as in (*a*)) was recorded and stimulated twice a week. The complete protocol consisted of the following steps:

(1) Spontaneous activity was recorded for 30 min after a 20–30 min recovery period;

(2) cultures were then stimulated through the eight channels belonging to the central column in the grid layout (i.e., channels 41 to 48 according to the MCS MEA nomenclature. See Fig. 1D). A train of stimuli was delivered at 0.2 Hz for 3 min through each channel (36 stimuli per channel, on the whole). The stimulation was administered sequentially to the eight electrodes in the column and always in the same order. The delivered stimulus was a biphasic pulse, lasting 200 μs per phase (positive phase first) and with a peak-to-peak amplitude of 1.5 V.

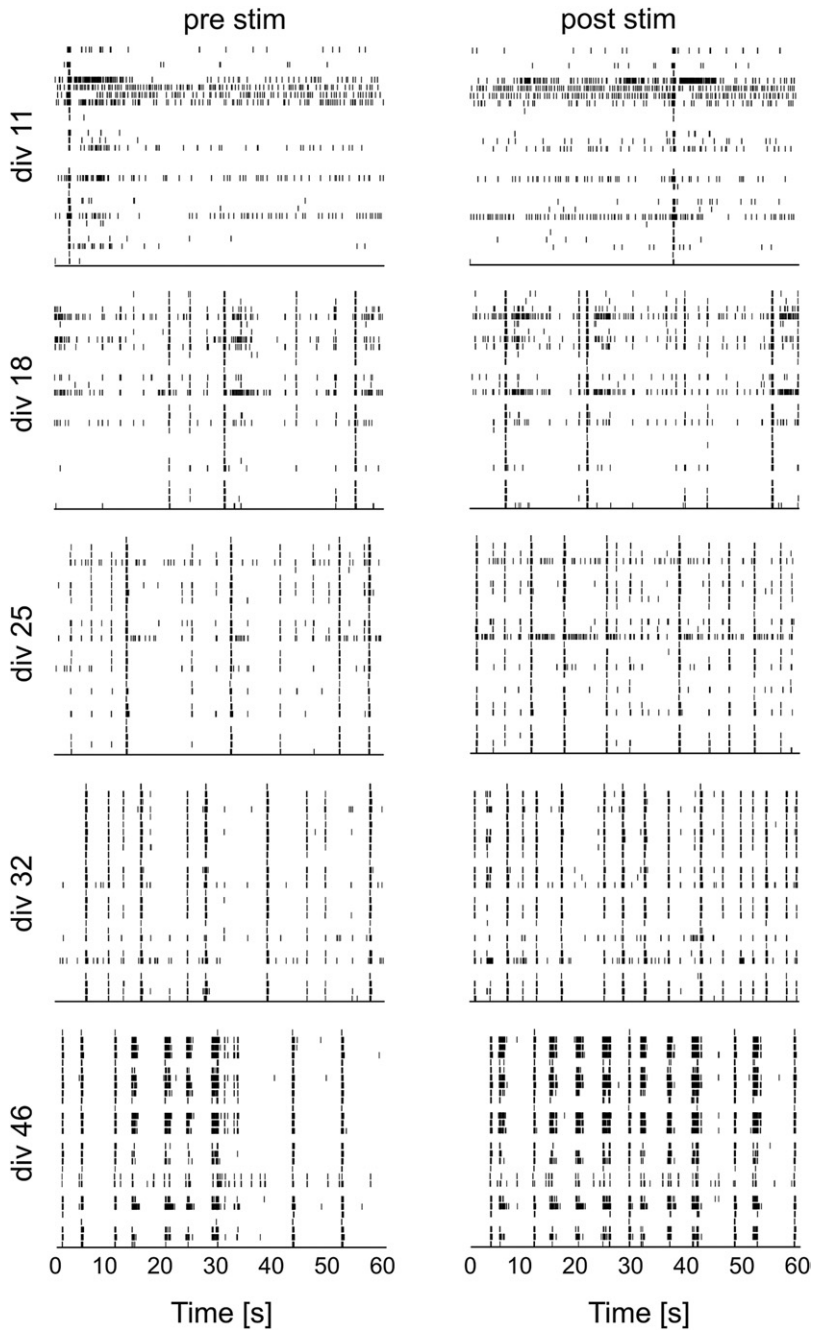

Fig. 2. Example of raster plots extracted from a single experiment over development, before and after the stimulation phase (29 channels shown). After the third week *in vitro*, the bursting activity is more frequent and robust and the increase in the number of network bursts is much more evident than during the first weeks.

(3) Spontaneous activity was recorded for further 30 min after the stimulation.

An example of raster plots collected before and after the stimulation delivery is shown in Fig. 2. The stimulation protocol described above was chosen in order to stimulate the cultures since their first DIV, in contrast with previous works in which the low-frequency stimulation was administered only at later stages of *in vitro* development (Vajda et al., 2008). Moreover, by considering that the cell density generally decreases towards the periphery of the culture, the choice of the central column was taken in order to increase the probability of stimulating a populated area of the array.

Spike detection

A recently developed spike detection algorithm was used to discriminate population spike events (Maccione et al., 2009). Briefly, the algorithm is based on the use of three parameters: (1) a differential threshold (DT) set independently for each channel and computed as eightfold the standard deviation of the biological and thermal noise of the signal; (2) a peak lifetime period (PLP) set to 2 ms; (3) a refractory period (RP) set to 1 ms. The algorithm scans the raw data to discriminate the relative minimum or maximum points. Once a relative minimum point is found, the nearest maximum point is searched within the following PLP window (or vice versa). If the difference between the two points is larger than DT, a spike is identified and its timestamp saved.

Experiment selection

A 2 h-long recording session goes across hardly predictable environmental and physiological changes which can compromise the robustness of its outcome. Thus, we used a method to assess the stability of the networks before selecting the final set of experiments to analyse. The method consists in the comparison of the individual electrodes Mean Firing Rate (MFR, expressed in spikes/s) of two consecutive recording epochs. As previously described (Chiappalone et al., 2008) we assumed a Poisson process underlying the recorded spike trains (Rieke et al., 1997) and realized a two-logarithmic plot, in which the MFR of each channel in the two epochs was reported (for a collection of such plots for a single experiment over development see Fig. 4). If the points lie on the diagonal, it means that the activity recorded on the respective channels had the same MFR in both periods. The dotted lines in the plot are computed as follows:

$$\text{upper boundary: } \text{diag} + k_L \cdot \sigma$$

$$\text{lower boundary: } \text{diag} - k_L \cdot \sigma$$

where diag is the MFR on the diagonal, k_L and k_U are the boundary coefficients ($k_L = k_U = 7$) and σ is the standard deviation of a Poissonian spike train computed as $\sigma = (MFR \cdot T_{REC})^{1/2}/T_{REC}$, where T_{REC} is the recording time. We refer to the area within the boundaries as the confidence region. In order to quantify the variation between two recording periods, the *relative deviation* (*rd*) of each point in the plot from the diagonal (d) was similarly calculated as previously described (Vajda et al., 2008) by dividing the signed distance to the diagonal by the $7 \cdot \sigma$ spread of the Poisson distribution (s). The *rd* of a point lying on the lower (upper) boundary equals -1 ($+1$). Values $> +1$ indicate an increase in firing, while values < -1 indicate a decrease. All points lying within the confidence region show no significant changes in the MFR. Finally, the *mean relative deviation* (*mrd*) between the two considered epochs was calculated by averaging over all signed *rd*s. The only difference in the method we used with respect to the one used by Vajda et al. (Vajda et al., 2008) is that we considered the sign of the deviation (instead of its absolute value), given our interest in the collective behaviour of the neuronal population. Thus, a $mrd < -1$ indicates a global decreasing trend in activity, while a $mrd > 1$ is observed in cultures whose global MFR tends to increase.

The choice of seven as the multiplicative factor is in line with a recently presented work (Chiappalone et al., 2008) and was mainly adopted because it allowed to recruit a higher number of experiments according to the applied stability criteria (cf. *Supplementary material*, section SM.1).

The selection of a single experiment for the final analysis was based on the following criteria:

(1) *Spontaneous activity protocol.* We split the 30 min recordings into two epochs of 15 min. We then computed the *mrd* as previously described, by comparing the two periods obtained and kept the recordings whose *mrd* absolute value was smaller than 1. We finally selected 77 recording sessions out of 98.

(2) *Stimulation protocol.* We split each of the two 30 min recordings preceding and following the stimulation into two epochs of 15 min. We refer to the four "sub-phases" as to *pre1, pre2* (obtained before the stimulation) and *post1, post2* (obtained after the stimulation). We then computed the *mrd* of the pairs *pre1-pre2, pre2-post1* and *post1-post2* and obtained the mrd_{pre}, mrd_{prepost} and mrd_{post} values, respectively. For the sake of completeness, we performed the Network Burst analysis on two subsests of data. On the one hand, we applied the statistics on the recording sessions in which $|mrd_{\text{pre}}| < 1$, $|mrd_{\text{post}}| < 1$ and $|mrd_{\text{prepost}}| > 1$. This criterion was chosen in order to exclude the recordings in which the stimulation did not induce significant changes (mostly the ones performed during the first DIV). On the other hand, we counted the number of network bursts (and evaluated its percentage variation) for all stable recordings (i.e., with $|mrd_{\text{pre}}| < 1$, $|mrd_{\text{post}}| < 1$) and we subsequently focused only on the subset performed after DIV 21. In such a situation, only unstable recordings were discarded and excluding the ones done during the very first DIV strengthened our previous claim according to which the data collected during the early periods of culture lifetime are the less significant. Thus, we finally selected 51 recording sessions out of 98 stable for the first subset, all stable recording sessions for the second and we finally focused on all 54 stable recordings performed after DIV 21.

Stability evaluation

To further validate that the observed changes in activity after the stimulation were actually due to the stimulation and not to physiological changes or activity drifts induced by environmental factors, we also performed a set of long spontaneous activity recordings in a sample of seven cultures over development ($n = 63$ recordings), up to DIV 42. We recorded the activity for 2 h immediately after positioning the dish on the amplifier in order to reproduce the very same experimental conditions of the stimulation protocol experiments, except for the application of the stimulus. We then subdivided the recordings in eight epochs of 15 min (*long experiment period, lep*) and named these periods *lep1, lep2, . . . , lep8*. We selected experiments showing stability in the pairs *lep3-lep4* and *lep7-lep8* and finally extracted, as previously described for the stimulation protocol, the *mrd* of the pairs *lep3-lep4, lep4-lep7, lep7-lep8* that we indicated as mrd_{lpre}, mrd_{lprepost} and mrd_{lpost}. The latter parameters corresponded to the mrd_{pre}, mrd_{prepost} and mrd_{post} values of a single stimulation experiment, respectively, since they were computed for the corresponding periods after the positioning of the MEA on the amplifier (the *lep1* and *lep2* periods corresponded to the 30 min of recovery adopted in the stimulation protocol).

Finally, to quantify the *mean relative deviation* of all the experiments, we extracted the *population mean relative deviation* (*pmrd*, computed as the mean between all *mrd*) and evaluated it for the same periods considered for a single experiment. Thus, we

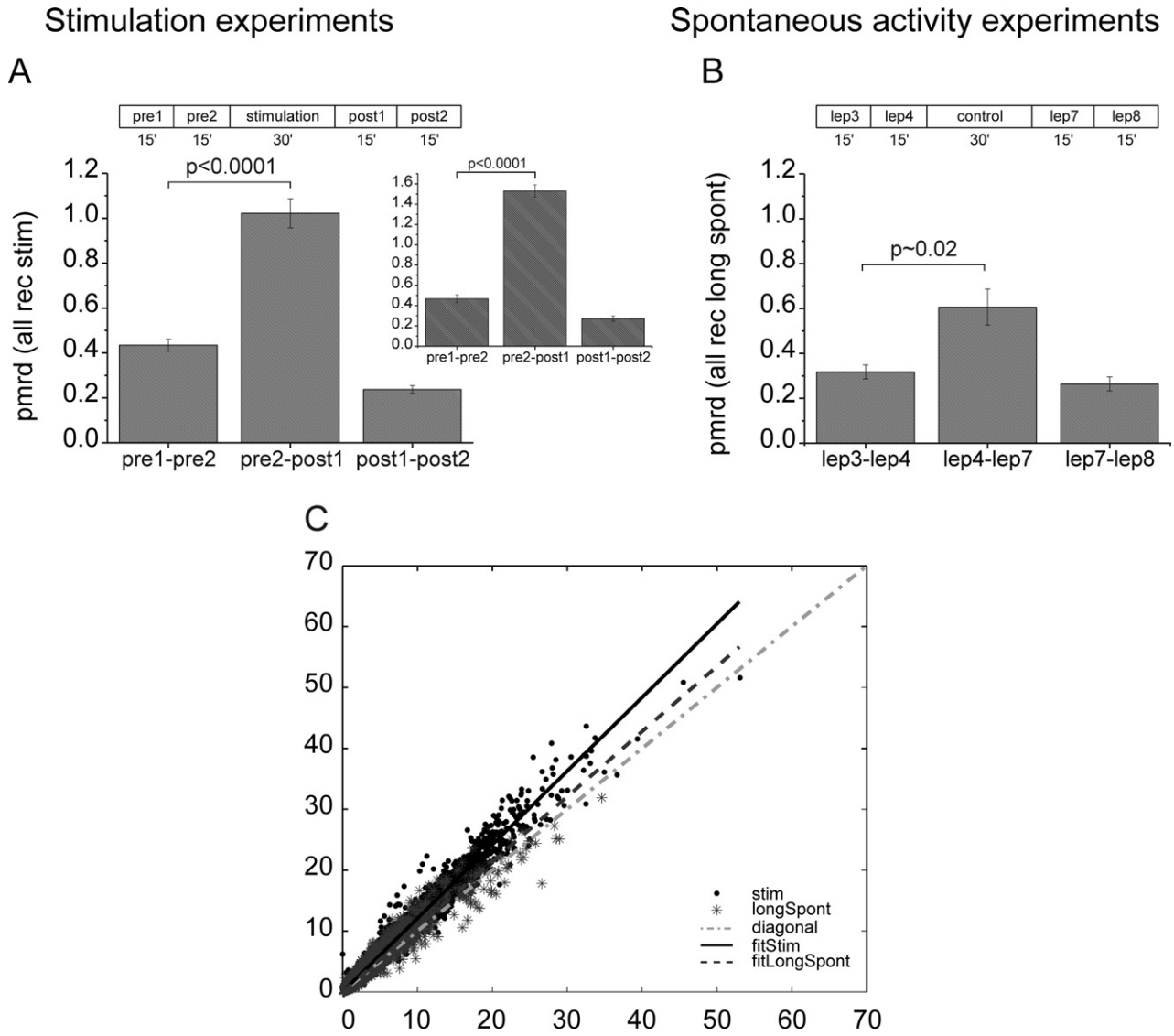

Fig. 3. Results of the stability analysis for the stimulation protocol experiments and for the long spontaneous protocol experiments are shown. (A) We computed the *pmrd* (population mean relative deviation) of all the stable experiments and of the experiments whose *mrd* was >1. Both groups showed a significant change in the firing rate between the *pre1-pre2* and *pre2-post1* periods ($P<0.0001$, Wilcoxon's rank sum test). (B) The changes observed in the *pmrd* for the *lep4-lep7* periods were less significant ($P\sim0.02$, Wilcoxon's rank sum test). Furthermore, a *pmrd* mean value (0.60 ± 0.08, mean±sem) much smaller than the one observed in the stimulation protocol experiments (1.02 ± 0.06, mean±sem) suggests that the change in network activity was the result of the stimulation. (C) We plotted the MFR of all channels over all cultures and DIV. For stimulated cultures we plotted *pre2*- versus *post1*-MFR while for the non-stimulated ones we plotted *lep4*- versus *lep7*-MFR. Linear fitting is shown for both groups together with "no change" reference diagonal. A significantly higher deviation from the diagonal of the stimulated cultures (slope 1.209, R^2 0.97) is observed with respect to the non-stimulated ones (slope 1.069, R^2 0.94).

obtained the $pmrd_{pre}$, $pmrd_{prepost}$ and $pmrd_{post}$ values for the stimulation experiments and the $pmrd_{lpre}$, $pmrd_{lprepost}$ and $pmrd_{lpost}$ values for the corresponding non-stimulated experiments and compared them (see Fig. 3A, B). As stated above, the long spontaneous activity experiments were performed as an additional control to verify the effectiveness of the stimulation delivered over development. We choose to split the recordings into 15 min epochs because we considered that such a duration was able to collect a sufficient deal of activity, given that the cultures we monitored were usually very active (showing a network MFR of at least 3–5 spikes/s since the first DIV). Thus, computing mrd_{pre}, mrd_{post} and mrd_{lpre}, mrd_{lpost} for the stimulated and long spontaneous experiments respectively was considered an appropriate choice for assessing the stability of the cultures before and after the stimulation. At the same time, $mrd_{prepost}$ and $mrd_{lprepost}$ were chosen as valuable indices of a significant change in the activity, since they evaluated the firing rate variation in the periods surrounding the stimulation and in the corresponding periods of the non-stimulated experiments, respectively. The comparison of $pmrd_{pre}$ and $pmrd_{lpre}$ is the natural choice for the validation of the stimulation effects on a culture with respect to a control, non-stimulated one.

As a further approach to the study of the significance of the MFR changes, we collected the MFR recorded from all electrodes of all stable cultures (both stimulated and non-stimulated), regardless of the DIV of observation. We then plotted the MFR recorded in period pre2 versus post1 for the stimulated cultures and *lep4* versus *lep7* for the non-stimulated ones. In other terms, we compared the activity in the periods surrounding the stimulation (and

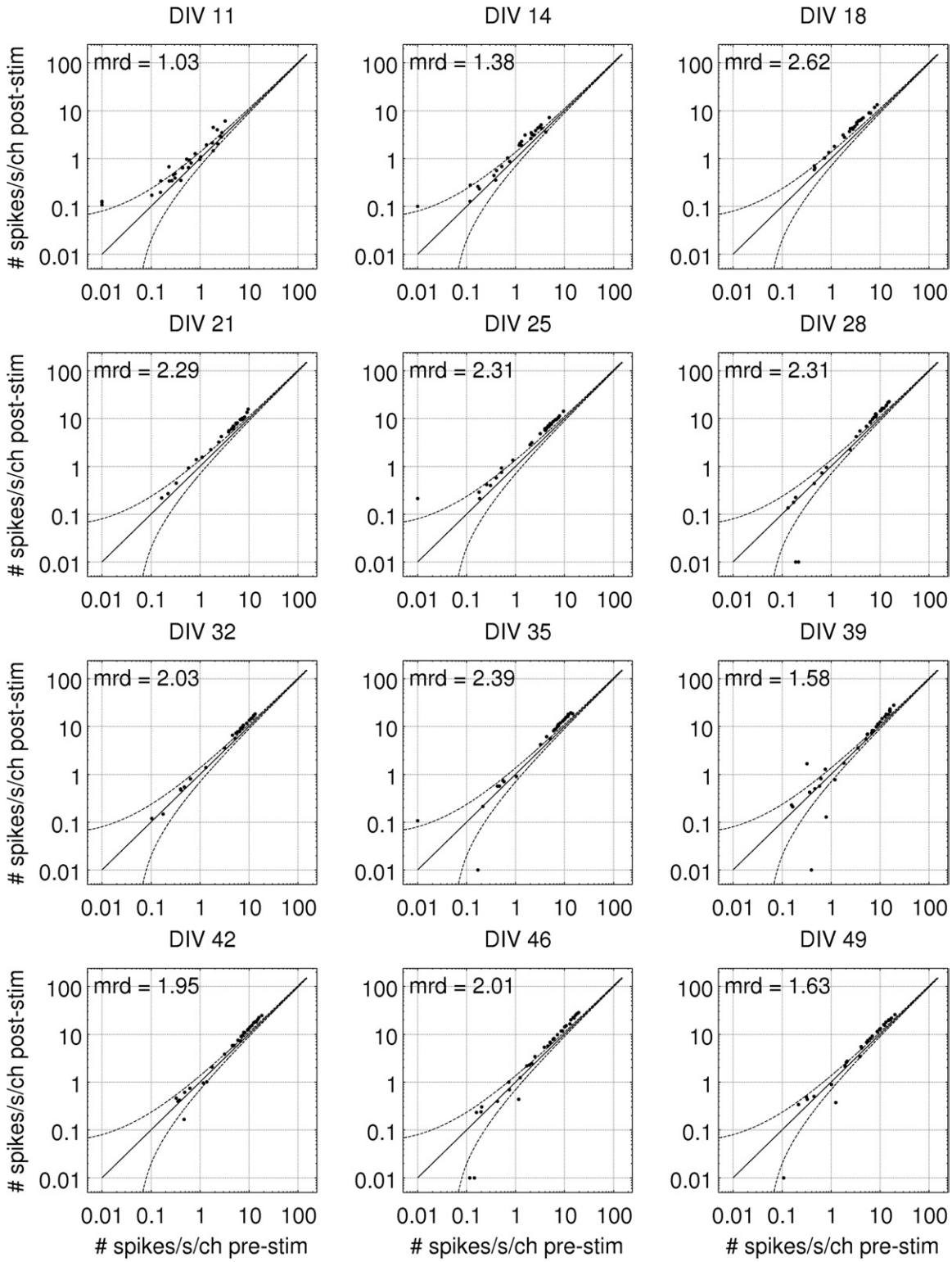

Fig. 4. Spontaneous firing rates extracted from individual channels over development of a representative culture. The spontaneous phases preceding and following the stimulation lasted 30 min. The log-log plots show the changes in the firing rates after the stimulation with respect to the confidence interval drawn from a Poissonian distribution. The mean relative deviation (*mrd*) is reported for each plot and indicates that the culture started to show a significant increase in the firing rate after stimulation, starting from DIV 18 and reached its peak during the central part of its development (3rd–5th wk).

corresponding periods for the spontaneous activity) and look at the deviation from the diagonal. The higher the deviation, the stronger the increase/decrease of firing rate in the second period of observation with respect to the first one.

Network burst detection

To characterize the dynamics of the cultures over time, we mainly focused on their bursting activity. A burst (see Fig. 1B) consists of a barrage of high frequency spikes and usually involves all the active channels of the array. Nonetheless, a *consensus* definition of burst has not been adopted in the literature yet (Maeda et al., 1995; Beggs and Plenz, 2003; Eytan and Marom, 2006; Wagenaar et al., 2006). Because of our interest in the collective activity of the network and in the shape of the bursts involving the entire culture, we decided to analyze the spike trains of all channels to detect network burst events (NB), as previously described (Van Pelt et al., 2005). Briefly, the onset of a network burst is detected whenever the collective activity of the network strongly increases, with respect to its basal activity, in terms of number of spikes fired in a reasonably short time window. To detect the timestamp at which a network burst occurred, the temporal activity of all channels was subdivided into 25 ms time bins and the product of the number of active sites and the number of spikes was computed per each bin. Whenever the product exceeded an appropriately chosen threshold a network burst was detected. Due to the very high level of activity displayed by our cultures, after a series of tests, the threshold was set to 400 (i.e., 20 channels firing at least one spike in the 25 ms bin), significantly higher than that previously used in the literature (Ben-Ari, 2001; Van Pelt et al., 2004b). The choice of such a threshold is explained in the Supplementary material, section SM.2. Once the onset of a burst was identified, the bin in which the maximum product occurred was chosen as the centre bin of the burst. Then, the preceding and the following five time bins were analyzed to detect the Centre of Mass (COM) of the product distribution which served as the centre time of the burst. Subsequently, network bursts were aligned according to their COMs and summed using a 10 ms bin. The entire profile was traced over a window of 2 s centred on the timestamp used as the alignment point.

NB profile was then fitted by a curve consisting of three components: a background, a double sigmoid and a Gaussian. The previously reported curve fitting procedure (Vajda et al., 2008) allowed us to better describe the network burst in terms of rising and falling phases, duration and peak value (NBRP, NBFP, NBD and NBPV, respectively). The NBRP was defined as the time needed by the burst to reach its peak (expressed in number of spikes) from the point in which its profile exceeds the 5% of the peak itself. Similarly, the NBFP was defined as the time the burst profile takes to go down from the peak to below the 5% of the peak value. The NBD is given by the sum of the rising and falling phases, while the NBPV is given by the maximum value of its profile (see Fig. 1C).

Post stimulus time histogram (PSTH)

To evaluate the capability of the network to respond on a short-term basis to the applied stimuli, the number of evoked spikes after each stimulus was computed and the histogram of their distribution plotted (Rieke et al., 1997). The first 4 ms after the stimulus were not considered in the computation due to interference of the stimulus artefact. The spike counts were performed within the first 600 ms after the stimulus and normalized with respect to the number of delivered stimuli (Chiappalone et al., 2008). To better identify the spiking probability over development of the cultures, the PSTH area computed for a single culture in individual recording sessions was normalized with respect to the sum of all the PSTH areas computed for that culture.

Network burst changes over development

To analyze the influence of the stimulus delivery on the bursting activity pattern, the Inter Burst Interval (IBI) distribution of the spontaneous activity before and after the stimulation was calculated. We evaluated the changes in network burst activity by computing also the percentage variations between the pre-stimulus and the post-stimulus phase of the following parameters over time: (1) number of network bursts, (2) rising phase duration, (3) falling phase duration and (4) overall duration of the network burst. We took into account only the recording sessions in which a significant variation of the firing rate after stimulation was present, as previously described (cf. *Experiment selection* section).

Comparison between spontaneous and stimulus-driven activity: increment index

To compare the development of cultures belonging to the same batch, but treated differently over development (i.e., either stimulated or not), we introduced the Increment Index (II), defined as follows:

$$\mathrm{II} = (p1 - p2)/(p1 + p2)$$

where $p1$ and $p2$ are the values the parameter p assumes at DIV $n+1$ and at DIV n, respectively. p could be any of the parameters previously computed, such as the MFR, the number of network burst, the rising/falling phase duration or the overall duration of the network burst. Given that the value of p is always positive, the II approaches 1 (-1) as $p1$ ($p2$) greatly exceeds $p2$ ($p1$). We evaluated the II for both the MFR and all the network burst parameters. When computing the II with respect to stimulated cultures, we took into account only the spontaneous activity (showing stability as previously described) preceding the stimulation, with the purpose of evaluating whether the low-frequency stimulation delivered over weeks affected the spontaneous activity of the cultures up to a few days later.

RESULTS

In this section we show results and statistical analysis of 112 recordings obtained from 11 cultures (related to three different preparations) for the stimulation experiments and, for comparison, results and analysis of 63 recordings from seven cultures for non-stimulated experiments. All the cultures were monitored at least for 6 weeks and only stable recordings were considered for quantitative analysis. As reported in the introduction section, the main aim is to find out whether chronically stimulated cultures (cf. *Experimental procedures*) undergo changes in the dynamic developmental profile or if the introduced stimulation protocol induces differences in the short-term evoked response over development.

Stimulation significantly changes the mean firing rate of the networks

About 87.5% of the recordings (i.e., 98 out of 112) in the stimulation experiments were stable before and after stimulus delivery. About 50% of the experiments (i.e., 51 out of 98) showed a significant change in the MFR after stimulus (cf. *Experimental procedures* for the stability criteria). This fact should not be confused with the chance (50%) to find a significant change in the MFR after stimulus delivery; instead the large amount of recordings showing “unchanged” MFR is due to the fact that during the first period of development the delivered electrical stimulation is largely ineffective. In fact, when the same analysis was

performed on the recordings carried out after DIV 21, significant changes were observed in 72.2% of the experiments, confirming the previous considerations on the low response capability of the cultures during the first DIV.

To further demonstrate the induced changes, when the $pmrd_{pre}$ and $pmrd_{prepost}$ of all stable experiments (i.e., 98 recording sessions) were compared, we found that a statistically significant difference was present ($P<0.0001$, Wilcoxon's rank sum test; 0.4348 ± 0.0266 versus 1.02 ± 0.06, means$\pm$SEM). At the same time a much larger variation was present when comparing the above mentioned *pmrds* of the 51 experiments showing $|mrd_{\text{prepost}}|>1$ ($P<0.0001$, Wilcoxon rank sum test; 0.4688 ± 0.0373 versus 1.53 ± 0.06, means$\pm$SEM) (see Fig. 3A).

An additional validation of the previous results was carried out by performing 2 h recordings from seven non-stimulated cultures (cf. *Experimental procedures* for details). About 81% (i.e., 51 out of 63) of long spontaneous activity experiments was stable. This result is comparable with the one observed for the stimulation experiments. Nonetheless, only 21.57% (i.e., 11 out of 51) of recording sessions showed a $|mrd_{\text{lprepost}}|>1$, indicating a higher stability when no stimulus was delivered. Moreover, the $pmrd_{lprepost}$ displayed a smaller increase over the $pmrd_{lpre}$ value with respect to that computed for the stimulated cultures ($P<0.05$ Wilcoxon rank sum test, 0.60 ± 0.08 versus 0.3177 ± 0.0310, mean$\pm$SEM; see Fig. 3B). No statistical test was done for the long recordings with $|mrd_{\text{lprepost}}|>1$ because of the very low number of experiments (i.e., 11).

A representative example of the variation in the firing rates and *mrds* values after stimulation is reported in Fig. 4 as a function of the development in culture. The deviation from the Poissonian confidence interval reached its highest values during the central period of the culture's development (3rd–5th week) and was constantly >1 since the first days after plating. Interestingly, all channels were clearly involved in the network MFR changes, indicating that the stimulation affected the network as a whole and that the bursting activity change was a network dynamics process.

In order to further validate the difference observed in the MFR change of stimulated and non-stimulated cultures in the two periods of observation (i.e., pre and post), we followed the approach explained in section *Stability evaluation* of the *Experimental Procedures*. The results are shown in Fig. 3C. As one can clearly observe, the fitting of stimulated data deviates from the diagonal much more than the fitting of non-stimulated ones. More quantitatively, for the first group (i.e., stimulated) we obtained a slope of 1.209 with 95% confidence interval slopes of 1.205 and 1.213, and a coefficient of determination $R^2=0.97$. For the second group (i.e., non-stimulated) we obtained a slope of 1.069 with 95% confidence interval slopes of 1.061 and 1.078, and a coefficient of determination $R^2=0.94$. This result confirms that significant changes in the firing and bursting rate is indeed attributable to electrical stimulation rather than to spontaneously occurring activity drift.

The number of network bursts increases after stimulation over development

To evaluate the effects of chronic low-frequency stimulation on network dynamics over development, the percentage changes in the NB parameters introduced before (cf. *Experimental procedures*) were evaluated. Interestingly, the number of network bursts almost invariably increased after stimulation over weeks. The percent changes in the number of network bursts observed in all stable experiments (open circles averaged through the grey line) or in the subgroup made of selected experiments with a significant variation of the MFR after the stimulation (i.e., $|mrd_{\text{prepost}}|>1$, filled circles averaged through the black line) are shown in Fig. 5A. The vast majority of recordings showed a clear-cut increase in the number of network bursts throughout the entire development, with few oscillations during the first DIV. Although this trend was more evident in the selected experiments, all cultures displayed a progressive increase in the number of network bursts after stimulation over time *in vitro*. This effect levelled off once maturation was achieved (after the 4th week) and stabilized at a relatively constant increase of 30–40%.

The other network burst parameters did not show clear changes. Particularly, the NBPV of the network bursts after stimulation remained constant (see Fig. 5B), the NBRP displayed only a slight increase at later stages while NBFP and NBD showed a tendency to decrease after the 3rd–4th week (see Fig. 5C.2, C.3, C.1 respectively). Moreover, for the sake of comparison with other studies, we reported the absolute values of the network burst parameters in Fig. S3 (cf. *Supplementary material*, section SM.3).

Finally, the quantitative analysis of the data showed that in about 92% of the recordings (i.e., 44 out of 48 showing network bursts) an increase of the number of network burst was observed after the stimulation. Only one recording did not change its number of network burst while three recordings showed a decrease.

At the same time, 54.2% (i.e., 26 out of 48) of recordings increases it NBPV and 62.5% (i.e., 30 out of 48) showed a decrease of NBD. More specifically, 60.4% (i.e. 29 out of 48) of recordings increased its NBRP and 62.5% (i.e., 30 out of 48) decreased it NBFP.

As further analysis (cf. *Experimental procedures*), in order to exhaustively characterize all the experiments performed, we sought for network bursts also in the recordings with $|mrd_{\text{prepost}}|<1$ whose stability criteria $|mrd_{\text{pre}}|<1$ and $|mrd_{\text{post}}|<1$ were fulfilled (i.e., the entire set of 98 stable recordings). Our choice was dictated by the aim of giving a complete overview of the results, included the ones which could have been hidden by the restrictive criterion adopted (i.e., $|mrd_{\text{prepost}}|>1$). We found that network bursts were present in 93 recordings out of the 98 stable ones and in 81.72% of these (i.e., 76 out of 93) an increase of the number of network bursts after the stimulation was observed. Only 1.1% of the recording (i.e., 1 out of 93) did not change the latter number while 17.2% of the recordings (i.e., 16 out of 93) showed a decrease. Furthermore, when the analysis was restricted to recordings after

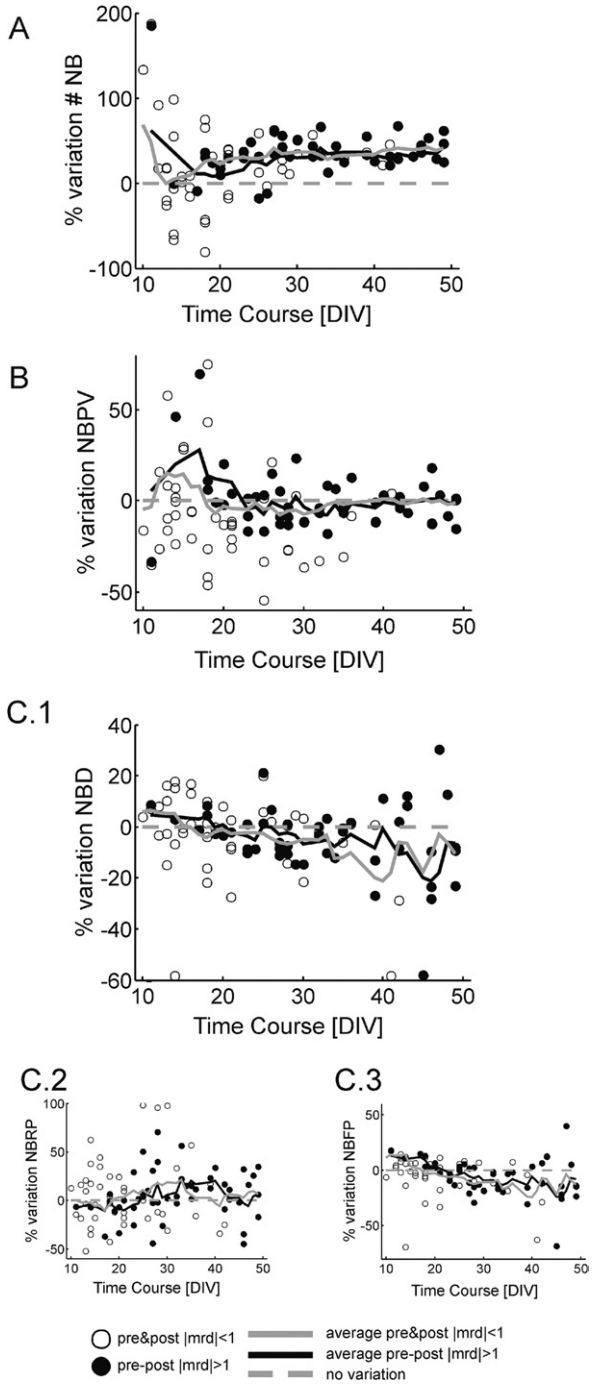

Fig. 5. Percentage variation of network burst parameters of all cultures over development. Open symbols refer to all stable recordings according to the adopted stability criterion, while filled symbols indicate the subset of the previous group showing a significant difference in the firing rate after stimulation. Average lines obtained through a 10 DIV wide sliding window are plotted for open and filled symbols (grey and black respectively). (A) Percentage variation of the number of network bursts after stimulation over development. The number of network bursts stably increased in both sets of experiments after the 4th wk *in vitro*. Major oscillations were present during the first DIV, while at later stages both groups showed the same trend as indicated by the average lines. (B) Percentage variation of the network burst peak values after stimulation. The peak values tended to increase during the first DIV and to slightly decrease during the 3rd–5th wk of development, while the late phase showed no variation. (C1–3) Percentage variation of the network burst duration (computed as the summation of the rising and the falling phase) after stimulation. The rising phase of the network burst showed a slight trend to increase over development (a more clear increase is visible during the 5th wk), while the falling phase shows a more remarkable trend to decrease since the 25th–28th DIV. As a consequence, a decreasing trend in burst duration was observed, in accordance with the increase in the number of network bursts.

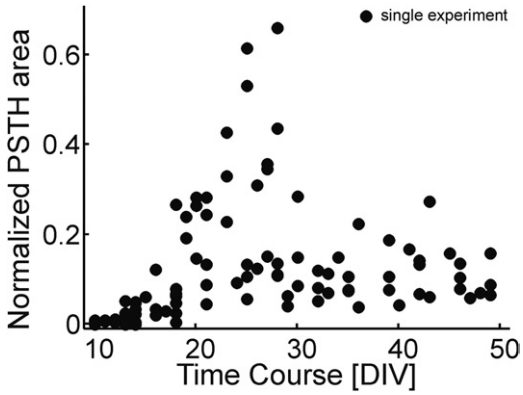

Fig. 6. Post Stimulus Time Histogram (PSTH) areas of all recordings showing stability before and after stimulation are shown. The areas were normalized with respect to the overall PSTH area of every single culture during development. A clear peak was present around the 3rd–4th wk *in vitro*, indicating that the responsiveness of the cultures reached its maximum during the central stage of maturation. After DIV 30, stabilization was observed which paralleled the stable increase in the number of network bursts shown in Fig. 5A.

DIV 21, 94.4% (i.e., 51 out of 54) showed the aforementioned increase while only in 5.6% of them (i.e., 3 out of 54) a decrease was reported. The latter result confirms that after the maturation period, almost the entirety of the recordings showed a clear-cut increasing trend.

Finally, for the sake of comparison with non-stimulated cultures, we counted the number of network bursts in the latter group in recording periods *lep4* and *lep7* and computed the percentage variation. We found that 33 (64.7%) out of the 51 stable recordings increased the number of network bursts in the *lep7* period. Moreover, if the analysis is performed on data collected after the third week (i.e., DIV 21), 19 (59.3%) cultures showed such an increase while the remaining 13 (40.7%) produced a smaller number of Network Bursts. These results strongly contrast with the ones observed for stimulated cultures in which the afore mentioned percentage were respectively 81.7% and 94.4%, indicating a much stronger increase due to the stimulus delivery. In Fig. S5 of the Supplementary Materials (section SM.4) we report the percentage change of the number of Network Bursts for all stable long spontaneous experiments.

Network responsiveness to stimuli stabilizes with the increase in the number of network bursts

The responsiveness of the cultures to electrical stimuli during development was evaluated by means of a PSTH analysis. The normalized PSTH areas for all stable experiments (i.e., 98) are reported in Fig. 6. First of all, an almost

flat response (i.e., few evoked spikes) was observed during the first 2 weeks *in vitro*. This could, to some extent, justify the lack of a net variation in the number of network bursts after the stimulation during the same period. In fact, it is expected that a very weak short-term response to stimuli can hardly elicit a long-lasting change in activity (i.e., for the 30 min following the stimulation). A clear peak is present around the 4th–5th week *in vitro*, suggesting that the responsiveness of neuronal cultures to stimuli reaches its maximum during the central period of network development and stabilizes afterwards. Interestingly, by comparing Fig. 6 with Fig. 5A, it is possible to observe that the stabilization of the PSTH area after DIV 30 coincides with the stabilization of the percentage change in the number of network bursts.

IBI distribution changes after stimulation and comparison with spontaneous activity

The IBI distribution of three sample cultures over development is reported in Fig. 7. The profile of the distribution strongly depended on the age of the culture and was rather variable among different cultures. However, the stimulation always induced a change in the pattern of bursting activity as revealed by a marked change in the IBI distribution profile. For example, after the stimuli delivery, culture 2 at DIV 46 showed a shifted IBI profile which kept the same shape as the one preceding the stimulus, while its pre-stimulus profile at DIV 49 was completely disrupted after the stimulus itself. Finally, the inter-burst intervals of stimulated cultures when compared with those evaluated in non-stimulated cultures showed no significant differences after 30 DIV ($P\sim0.4$, Wilcoxon rank sum test) indicating that no significant change was induced by the stimulation with respect to the temporal distribution of network bursts.

Stimulated and non-stimulated cultures develop in a similar way

A comparison between the development of the electrical activity in the chronically stimulated cultures and their non-stimulated mates was performed. We computed the II (cf.

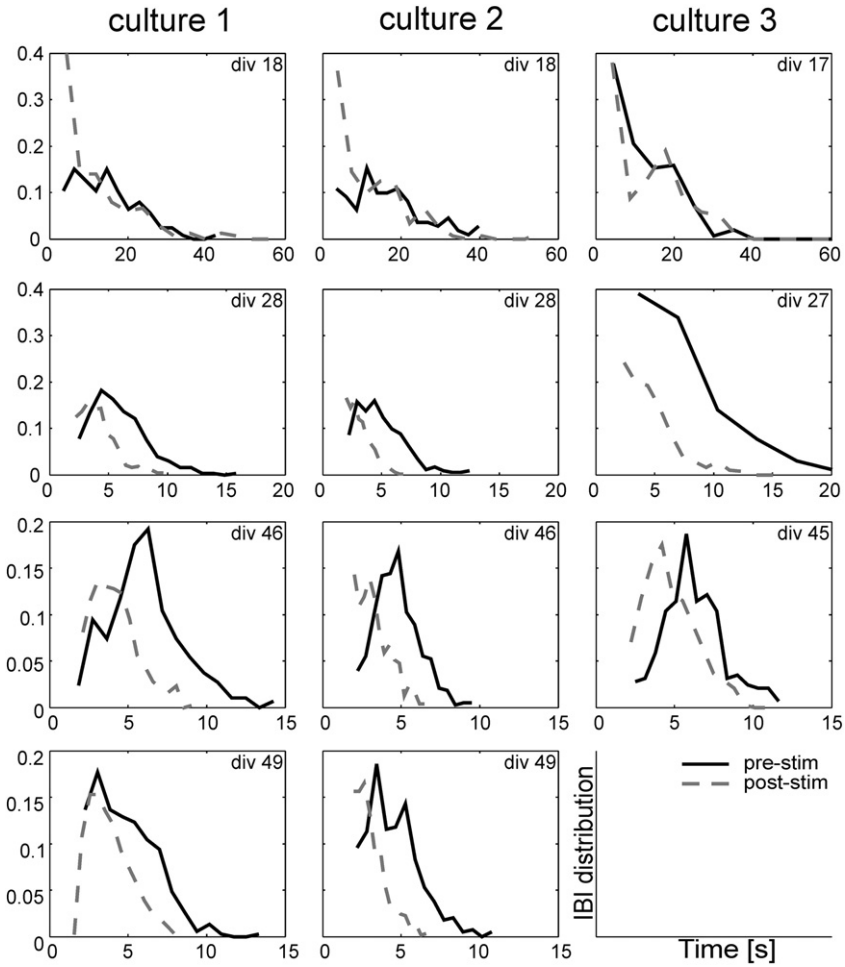

Fig. 7. Inter Burst Interval (IBI) analysis. IBI distribution of 11 recordings from three different cultures, before and after stimulation. A clear change in the IBI distribution was observed over development, indicating that each developmental stage has its own burst activity pattern. In many cases, the shape of the distribution was maintained, albeit it was shifted towards lower frequencies, indicating the stimulation induces an increase of activity without modifying its temporal pattern. In few other cases, the shape of the IBI distribution completely changed, suggesting a possible reorganization of the activation path involved in the generation of the network bursts.

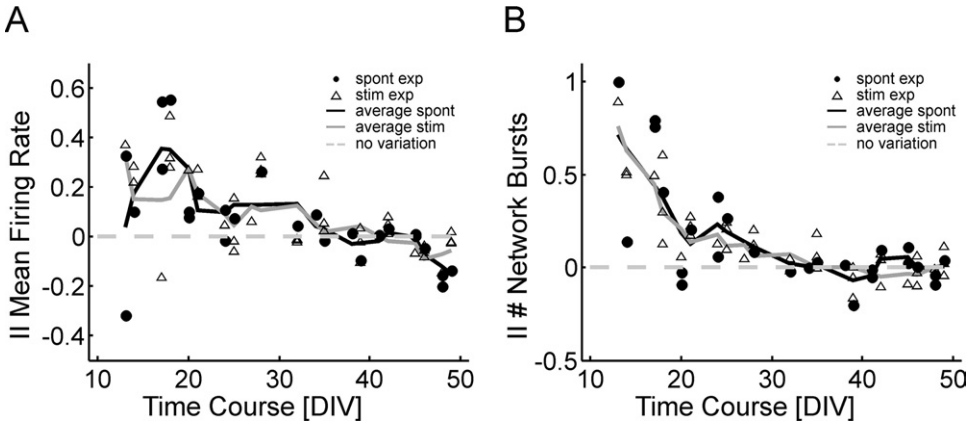

Fig. 8. The Increment Index (II) is reported for both non-stimulated and stimulated cultures (single batch) with respect to their MFR (A) and number of NB (B). For the stimulated cultures only the 30 min preceding the stimulation were taken into consideration. Average lines obtained through a 3 DIV wide sliding window are plotted for circles and triangles (black and grey respectively). The index is computed by comparing the values the parameters assume in two consecutive DIV. The closer the parameter is to 1 (−1), the higher its value is at DIV $n+1$ (n) with respect to DIV n ($n+1$). We can observe that both the spontaneous and stimulus driven activity showed a coefficient whose value approached zero during time *in vitro*, indicating that the networks achieve a stable pattern of activity after the maturation phase.

Experimental procedures) with respect to the MFR, NB number, NBPV, NBRP, NBFP and NBD. Statistical analysis showed no significant changes for the considered parameters between the two groups of recordings (MFR: $P\sim0.42$, NB number: $P\sim0.15$, NBRP: $P\sim0.74$, NBFP: $P\sim0.56$, NFPV: $P\sim0.47$ NBD: $P>0.9$, Wilcoxon rank sum test), indicating that the low-frequency stimulation delivered twice a week did not significantly change the development of the stimulated cultures. The II values for MFR and the NB number of a single batch, as representative examples of dynamics changes over weeks, are reported in Fig. 8A, B respectively. One can clearly observe a strong decrease of the II during the first DIV. Such a trend indicates that the activity of the cultures enriches on a daily basis, leading to both higher MFR and NB number. After the 4th week, once the complete network maturation has been achieved, the descending trend ceases, driving the networks to activity stabilization.

DISCUSSION

The impact of chronic low-frequency stimulation on the spontaneous and evoked activity of developing primary cortical cultures was monitored during development from DIV 10 to DIV 49. The stimulation protocol was applied from eight distinct sites twice a week all over the development and the spontaneous activity of each culture was evaluated before and after the stimulation in terms of spiking and network bursting behavior. The analysis led to the following conclusions. The cultures showed the proper developmental course and dynamics, strictly related to the age of observation, and displayed a stable behavior after 4 weeks *in vitro*. The chronically applied low frequency stimulation increased, acutely, the spiking and bursting activities without changing the network burst features. These changes, however, did not affect the developmental profile of the cultures, suggesting that no major long-term plastic phenomena had occurred.

Cultures of primary neurons represent a widely used experimental model for investigating the effect of environmental stimuli on neural activity under highly controlled conditions. Moreover, the use of neuronal networks coupled to MEAs allows for non-invasive (Gross et al., 1977; Pine, 1980) and long-term measurements (Kamioka et al., 1996), electrical stimulation from specific sites of the network (Wagenaar et al., 2004), and repeatability of the same protocol over time. The experimental protocols proposed in the literature usually range from simple observation of the spontaneous activity of the cultures (Chiappalone et al., 2006; Wagenaar et al., 2006), to more complex repeated high-frequency stimulations applied to induce plasticity changes (Maeda et al., 1998; Tateno and Jimbo, 1999; Madhavan et al., 2007; Chiappalone et al., 2008). However, only a very few studies on the application of low-frequency stimulation to the cultures (Eytan et al., 2003) and on its effect on a long-term basis have been performed thus far (Vajda et al., 2008) (Brewer et al., 2009).

In this study, we delivered a rare and low-frequency stimulation since the very early stages of the network morphological and functional formation. The main finding of our work is the observation that, regardless the age of the culture, and provided that the latter showed stability before and after the stimulation (see Fig. 3), chronic stimulation enhanced bursting activity after the stimulus delivery. This result differs from a previous study in which a change in the activity, but no changes in bursting was observed (Vajda et al., 2008). While in both studies the changes in activity affected the whole culture, thus validating the dynamics modification as a network effect, the different effects observed in our experiments can be explained by clearly distinct stimulation protocols. In fact, while the previous study was performed on cultures of different ages which were not systematically stimulated over their development, our

approach was specifically aimed at applying an early stimulation of the networks that was maintained during the entire period of observation.

The observed enhancement in the number of bursts was not accompanied by a strong difference in the network burst peak and/or duration, suggesting that the stimulation did not disrupted the shape of the bursts themselves, but mostly reorganized their temporal dynamics. Such a possibility is supported by the change in the inter burst interval profile after stimulation with respect to the one observed before stimulation. Furthermore, the analysis of the PSTH areas in stimulated cultures shows the changes in responsiveness the cultures undergo during their developmental process.

However, the absence of significant differences during development between stimulated and non-stimulated cultures belonging to the same preparations indicates that the continuously delivered low-frequency stimulation may influence the way cultures of dissociated neurons react to electrical conditioning rather than the unconditioned activity they exhibit at various stages of maturation. We argue whether a low-frequency stimulation delivered since the very first stages of network development could enhance the response capability of the cultures rather affecting its development related activity.

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APPENDIX

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.neuroscience.2009.11.018.

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