Latency-Related Development of Functional Connections in Cultured Cortical Networks

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ABSTRACT   To study plasticity, we cultured cortical networks on multielectrode arrays, enabling simultaneous recording from multiple neurons. We used conditional firing probabilities to describe functional network connections by their strength and latency. These are abstract representations of neuronal pathways and may arise from direct pathways between two neurons or from a common input. Functional connections based on direct pathways should reflect synaptic properties. Therefore, we searched for long-term potentiation (this mechanism occurs in vivo when presynaptic action potentials precede postsynaptic ones with interspike intervals up to ~20 ms) in vitro. To investigate if the strength of functional connections showed a similar latency-related development, we selected periods of monotonously increasing or decreasing strength. We observed increased incidence of short latencies (5–30 ms) during strengthening, whereas these rarely occurred during weakening. Furthermore, we saw an increased incidence of 40–65 ms latencies during weakening. Conversely, functional connections tended to strengthen in periods with short latency, whereas strengthening was significantly less than average during long latency. Our data suggest that functional connections contain information about synaptic connections, that conditional firing probability analysis is sensitive enough to detect it and that a substantial fraction of all functional connections is based on direct pathways.

INTRODUCTION

In the brain, neurons form networks through a multitude of synaptic connections. Whereas the formation and development of connections is assumed to be crucial in the process of learning, their conservation is probably essential for memory. Assuming that network connections are reflected in the patterns of electrical activity, connectivity studies often entail simultaneous measurement of activity in a large number of neurons. This is yet not feasible in vivo, and several groups now use preparations of cultured neurons grown over a multi-electrode array (MEA, see Fig. 1). This enables simultaneous measurement from multiple electrodes as well as network manipulation using selective electrical stimulation.

Many studies investigated the development of neuronal connections using various methods to induce plasticity (1–4). All of these methods were based on the hypothesis that certain patterns of activity may change synaptic efficacy. Although some results appeared successful, other experiments yielded ambiguous results or were difficult to reproduce (5). An important complicating factor is the high variability in spontaneous activity patterns in cultured cortical networks, which may mask induced alterations.

Spontaneous activity shows alternating periods of seemingly uncorrelated firing at some electrodes and of short synchronized firing at many electrodes, usually referred to as network bursts (6,7). As a consequence, the network experiences varying influences that may change its connectivity. Thus, induced connectivity changes may go undetected among the massive spontaneous fluctuations or may disappear again, due to spontaneous activity, hampering detection of changes in a selected connection. Therefore, it seems more feasible to detect connectivity changes on a larger, network-wide scale of monitoring.

To study connectivity in a larger part of the network, we used conditional firing probability (CFP) analysis (8). CFP analysis reveals relationships in the action potential firing activity between pairs of electrodes, which are characterized by two parameters: strength and latency. Fig. 2 shows an example of a CFP curve and the calculated strength and latency. CFP analysis is related to cross-correlation and yields descriptions of functional connections, abstract representations of neuronal pathways between neuron pairs (9). Temporal correlations in the firing activity between neuron pairs characterize the activity dynamics in the neuronal network, structured by its neuronal properties and their underlying synaptic connectivity patterns. Temporal correlations in firing will be interpreted as functional connections, arising either from a direct synaptic pathway between two neurons through one or more synapses, or from correlations in the network activity dynamics via common inputs to both neurons. The question addressed in this study is: to what degree do functional connections represent direct synaptic pathways?

To answer this question, we focused on a remarkable phenomenon observed in structural synapses: the ability to change strength depending on the exact timing of pre- and postsynaptic action potentials (spike-timing-dependent plasticity (10–12)). Intracellular measurements have shown that structural synaptic connections may be strengthened when
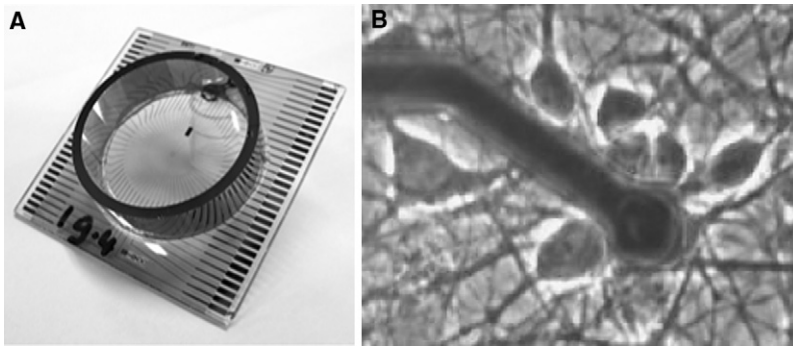
FIGURE 1 (*A*) MEA, used to record neuronal activity in cultured networks of cortical neurons. It is based on a glass substrate (5×5 cm) with 61 embedded electrodes in the center of the chamber, hexagonally spaced at a distance of 70 μm of each other. The glass ring (diameter 30 mm) glued on top was filled with glia-conditioned growth medium and firmly sealed. (*B*) Close up of one of the electrodes and several neurons. Electrode diameter, 12 μm. Most electrodes did not pick up signals from more than one neuron.

postsynaptic action potentials occur within a time window of ~20 ms after presynaptic firing (13–15). This phenomenon, known as long-term potentiation (LTP), is considered one of the fundamental mechanisms underlying memory and learning (16–19).

If functional connections represent underlying structural synaptic connections, they should exhibit a similar latency-dependent strengthening as in the aforementioned LTP studies. Therefore, we investigated if and how the development of strength was related to latency in functional connections at network level.

METHODS

Cell culturing and data recording

Data were obtained from four cultures; for details about the preparation of the cultures see van Pelt et al. (7). In short, cortical cells obtained from Wistar rat fetuses (E18) were dissociated by trituration after trypsin treatment. The dissociated neurons were plated on a MEA (see Fig. 1) precoated with poly ethylene imine, and kept in culture in a glia-conditioned medium. Initial cell densities were ~5000 cells/mm^2. MEAs were stored in an incubator under standard conditions of 37°C, 100% humidity, and 5% CO_2 in air. For recording, we covered the culture chambers with a glass lid, firmly sealed it with parafilm, and placed the cultures in a measurement setup

outside the incubator. Once a week, ~200 μl of medium was replaced by 300 μl of fresh medium. Spontaneous activity was recorded almost continuously, only interrupted by medium change and an occasional technical problem, starting in the second week after plating.

We recorded data for 5–6 weeks from four cultures using MEAs that contained 61 electrodes (diameter 12 μm, 70 μm apart). An action potential was detected if the signal at an electrode crossed a preset, regularly updated, discriminator level. Time stamps were stored with 100 μs precision, corresponding to an effective sample frequency of 10 kHz (technical details have been described in (7)). The shape of action potentials was not taken into account. The sequence of time stamps was divided into data blocks (on average ~30 data blocks per day).

It was probable that the followed procedure resulted in recorded spike trains originating predominantly from single units (neurons) (7). Yet, in this work, we will refer to electrodes rather than to neurons.

Data analysis

For analysis, we divided long-term recordings into data blocks of 2^{15} events each. An event consisted of one or more simultaneous action potentials. An electrode was considered active if we recorded more than 250 action potentials at this site within the duration of a data block. In each data block, we calculated the CFP for all pairs of active electrodes $(i,j)|\ I \neq j$ as the probability of an action potential at electrode j at $t = \tau$, given that one was detected at electrode i at $t = 0$. Pairs with peaked CFP were considered related. A first-order approximation of a Gaussian distribution function (Eq. 1; see le Feber et al. (8)) was fitted to all CFP curves to obtain values for strength and latency:

$$CFP_{i,j}^{fit}[\tau] = \frac{M_{i,j}}{1 + \left(\frac{\tau - T_{i,j}}{w_{i,j}}\right)^2} + offset_{i,j}. \qquad (1)$$

Relational strength ($M_{i,j}$) was defined as the maximum CFP above offset, latency ($T_{i,j}$) as the delay at $CFP^{fit} = M_{i,j}$. Fig. 2 shows an example of measured data and the fitted curve, used to calculate $M_{i,j}$ and $T_{i,j}$ (8). In each data block we constructed two matrices, M and T, containing all strengths and latencies of related pairs of active electrodes. Thus, we obtained values for strength and latency of individual functional connections at various time points. $M_{i,j}$-graphs were smoothed using a moving average filter to highlight longer-term trends. The filter averaged each point of the curves with its five neighbors on both sides. Fig. 3 shows an example of the development of strength and latency of a functional connection during more than 2 weeks.

First, we focused on periods of at least 1 day of monotonous decrease of $M_{i,j}$ (weakening of a functional connection) or increase (strengthening). M-graphs were smoothed using a moving average filter to highlight longer-term trends. The filter averaged each point of the curves with its five neighbors on both sides. To exclude analysis of functional connections that were only found in few data blocks (e.g., if it existed in only two data blocks, it would increase or decrease monotonously by definition), selected episodes had to comprise at least 10 data blocks. This criterion ensured that the length of all selected
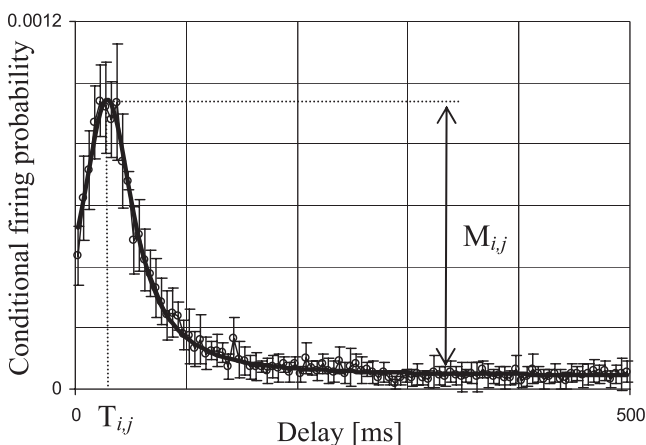

FIGURE 2 Example of estimated CFP ($\bigcirc$, mean $\pm$ SD of five consecutive bins of 0.5 ms each). Solid line represents least mean square fit of Eq. 1. This fit is used to obtain values for strength ($M_{i,j}$) and latency ($T_{i,j}$) of the functional connection between a pair of electrodes (i,j).
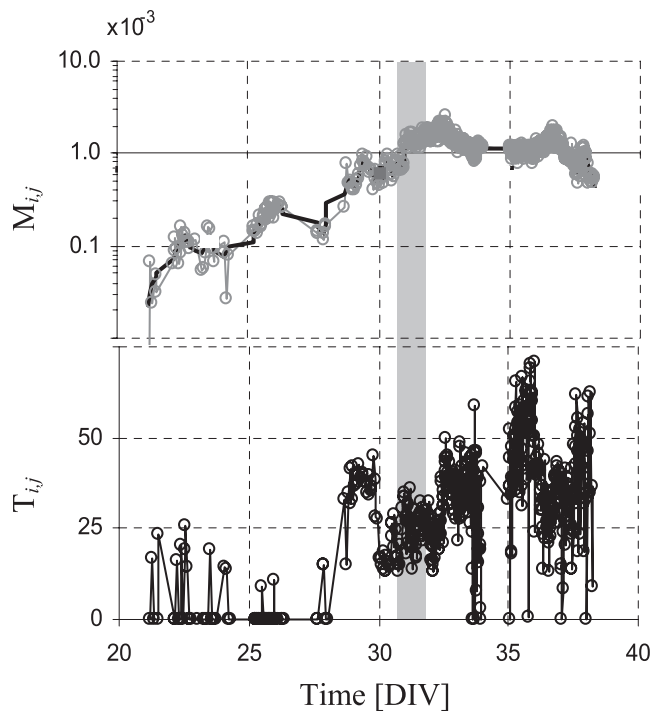
FIGURE 3 Example of the development of strength ($M_{i,j}$) and latency ($T_{i,j}$) of a functional connection. A long-term recording was divided into data blocks. In each data block, strengths and latencies of functional connections between all pairs of active electrodes were determined. If a pair was not related in a data block, strength was set to zero. Only electrode pairs that were related in at least 100 data blocks were included for analysis. M-graphs were smoothed using a moving average filter to highlight longer-term trends (*solid line*). The filter averaged each point of the curves with its five neighbors on both sides. Thus, we observed the development of functional connections between pairs of electrodes. Shaded area at 31 DIV indicates a period >1 day of monotonous strengthening. Note also the period of monotonous weakening between 32 and 34 DIV.

episodes exceeded certain threshold, in duration (time) and activity. An example of such a period is indicated by the gray bar in Fig. 3. We examined the latencies of functional connections in these periods of monotonous weakening or strengthening. These long periods gave a first impression of latencies that occurred during strengthening. However, selection of such long periods only, excluded all data from 50% of the cultures. Therefore, we reduced the minimum required period length to 10 h, which included all cultures.

To include more data, we also investigated periods with latency in certain ranges. These ranges were determined from the latency distributions in the 10 h of weakening or strengthening. Because the natural fluctuations in latencies were much higher than those in strength (8), we started with short periods (1 h). Obviously, possible changes in strength were less noticeable in such small periods, but a higher threshold reduced the number of periods for analysis. To find an adequate compromise we varied the threshold from 1 to 48 h. To assess the average strengthening (or weakening) in such periods we used a linear fit to $M_{i,j}$.

Finally, we investigated the influence of the initial strength on the development of functional connections. Strengthening or weakening was determined as explained above, but now it was related to the initial strength of the functional connection. To obtain a stable estimate of the initial strength, this was also calculated from a linear fit. Initial strengths were grouped into 16 bins of width $5 \cdot 10^{-4}$. If a bin contained <2 values, it was combined with the next bin. On the x axis we plotted the average initial strength in a (combined) bin.

RESULTS

We used long-term recordings from four cultures. All cultures were spontaneously active and showed regular bursts. Using the >=250 spikes/data block criterion from le Feber et al. (8) to obtain the active electrodes for CFP analysis, we found 9.1 ± 2.7 active electrodes on average (range: 1–29). This includes early measurements, which often showed few active electrodes. Furthermore, the applied criterion was stringent, a milder criterion as applied in (6,7) yielded 29 ± 9 active electrodes. On average ~2/3 of all possible combinations of active electrodes $((i,j) \mid i \neq j)$ were related (i.e., the CFP curve was not flat).

Because of the high variability between and within cultures we can only roughly indicate values of the parameters M_{ij} and T_{ij}. In the second week in vitro M_{ij} averaged $(0.95 \pm 2.4) \cdot 10^{-3}$, and T_{ij}: 26 ± 49 ms. With time, functional connections tended to become faster and stronger, with M_{ij} at 7 week in vitro averaging $(3 \pm 7) \cdot 10^{-3}$, and T_{ij}: 9 ± 25 ms. However, the spread per week was so large that these differences were not significant.

Although our analysis did not discriminate between spikes inside or outside bursts, we routinely used a burst criterion adapted from Stegenga et al. (20) to detect and describe bursts ("normalcy check"). Bursts were detected whenever the summed activity of all electrodes exceeded a threshold, which was set at one spike for each active electrode in 10 ms bins. Thus, we found 17,174 bursts with an average width of 391 ± 146 ms, and a peak firing rate of 0.58 ± 0.88 spikes/ms. Setting the threshold to half this value yielded many more bursts (78,801) with similar average width (416 ± 131 ms) and peak firing rate (0.57 ± 0.49 spikes/ms).

Two cultures showed functional connections with periods ≥ 1 day of monotonous strengthening (47 electrode pairs) or weakening (41 pairs). These periods consisted of 2267 data blocks in total. Fig. 4 A shows their normalized latency distributions, compared to the overall latency distribution in these cultures. The latency distribution during strengthening slightly exceeded the average distribution by ~20 ms. More convincingly, the latency distribution during weakening dropped below average at latencies between 10 and 25 ms. Furthermore, latencies around 40 ms tended to coincide with weakening functional connections. To increase the size of the dataset we dropped the minimum required duration to 10 h. Now, all cultures contributed to a dataset that consisted of 211 periods of strengthening and 274 periods of weakening in total. The results, which are shown in Fig. 4, B, were similar to Fig. 4, A, but the differences were less pronounced. Latencies between 5 and 30 ms occurred predominantly during strengthening, whereas latencies of 40–65 ms more often coincided with weakening.

Next, we investigated the periods of strengthening and weakening in more detail. In our experiments, we found a few periods ≥ 10 h in which both functional connections $i \rightarrow j$ and $j \rightarrow i$ monotonically changed strength (increase or
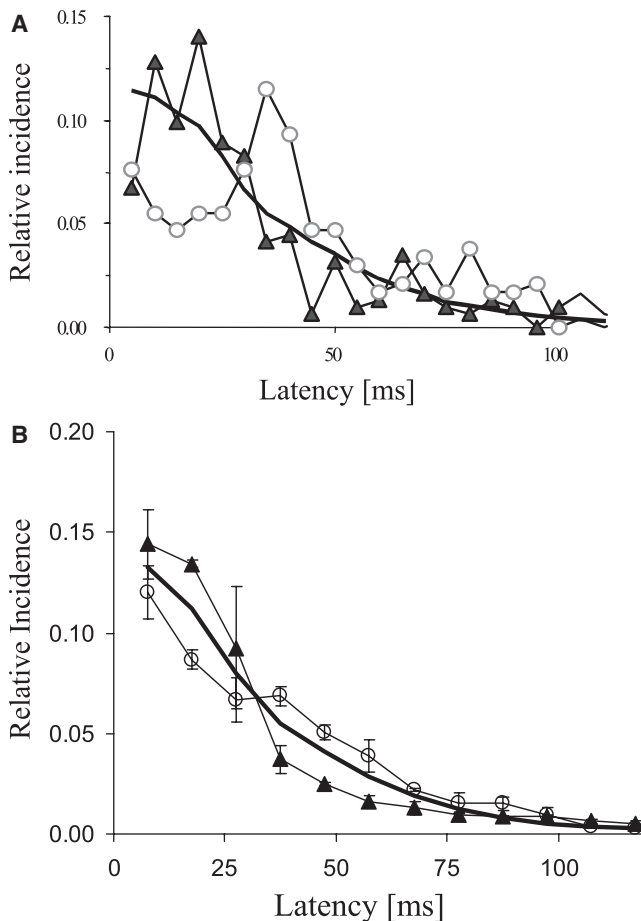
FIGURE 4 Latency distribution of functional connections in periods of monotonous strengthening (▲) or weakening (○) with a minimum duration of one day (A), or 10 h (B). In graph A, latencies of 5 $n \pm$ 2.5 ms were pooled ($n = 1…20$). Graph B: similar, but now mean $\pm$ SD of two consecutive bins is shown. Data from all cultures were pooled for these curves. The figure shows that the effects are comparable, but less pronounced using the shorter threshold period length of monotonous strengthening or weakening.

of the long-term recordings that fell before $T = 21$ DIV. At all ages, periods of strengthening and weakening did not coincide among pairs; all pairs had their individual patterns. Thus, strengthening or weakening were not global properties of the networks.

To extend the dataset, we also followed the "reverse" approach, select periods with latency ($T_{i,j}$) in the range 5–30 ms or 40–65 ms. Usually, $T_{i,j}$ varied more, and more rapidly, than $M_{i,j}$ (8). Therefore, it was more difficult to find a fair compromise between the chosen threshold period length (and thus the expected magnitude of changes) and the size of the included dataset. The number of such periods decreased with the chosen threshold as shown in Fig. 5. Again all included episodes had to contain at least 10 data blocks. Therefore, lowering the required minimum length below 3 h did not yield any additional periods. We counted the number of included periods with latency 5–30 ms ($N_{5–30}$), with latency 40–65 ms ($N_{40–65}$), and all periods with unrestricted latency (N_{all}) at all minimum period lengths. We always found $N_{40–65} < N_{5–30} < N_{all}$, regardless of the chosen minimum length (see Fig. 5). At a chosen threshold of 24 h, $N_{40–65}$ was 35; at 48 h, it equaled 14. All cultures contributed to the number of included periods shown in Fig. 5.

Fig. 6 A shows that strengthening per hour for short latency periods tended to be higher than average, but this difference was not significant (Wilcoxon signed rank, $p = 0.18$). Strengthening during long latency was significantly lower than average ($p < 0.01$) or during short latency ($p < 0.01$).

We also calculated the percentage of functional connections that increased in strength during long or short latency. The fraction of functional connections with increasing strength for short latency periods was significantly higher than average, whereas this fraction was significantly lower than average for long latency periods (Wilcoxon signed rank, $p < 0.01$). Results are shown in Fig. 6 B.
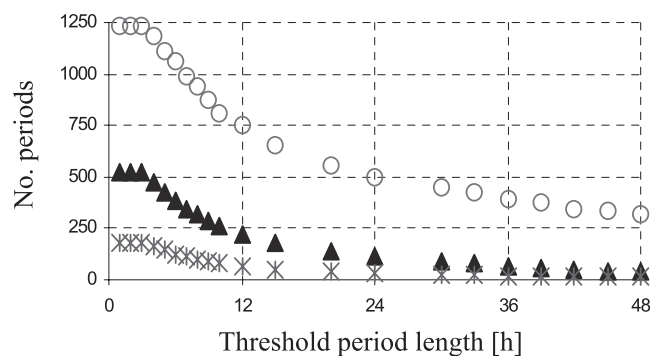
decrease). We observed 28 of such cochanges, usually with only partial overlap in time (6.3 $\pm$ 2.7 h on average). Most frequently, $i \rightarrow j$ strengthened if $j \rightarrow i$ weakened (12 pairs), but it also occurred that $i \rightarrow j$ and $j \rightarrow i$ weakened (10 pairs) or strengthened (6 pairs) simultaneously.

There were pairs that only showed one or more periods (>10) of monotonous strengthening (31%), only periods of weakening (47%), and pairs that showed both (22%). Thirty-three pairs showed more than one period of strengthening, and 41 pairs had more than one period of weakening (we hardly found more than two such periods for one pair).

Twenty-four percent of all periods of monotonous increase or decrease were recorded in younger cultures (<21 days in vitro (DIV)). In these cultures, we found slightly more periods of weakening (13.2%) than strengthening (10.3%). In older cultures (>21 DIV), periods of strengthening and weakening were almost perfectly balanced. The fraction of periods found in young cultures corresponds to the fraction

FIGURE 5 Number of periods during which functional connections had a latency in the range 5–30 ms ($N_{5–30}$, ▲) or 40–65 ms ($N_{40–65}$, ж) as a function of the chosen threshold period length. For comparison the number of periods with the required length but without latency restrictions is also shown (N_{all}, ○). All periods had to contain at least 10 data blocks. At all minimum lengths we found $N_{40–65} < N_{5–30} < N_{all}$. At 24 h, $N_{40–65}$ was 35; at 48 h, it equaled 14.
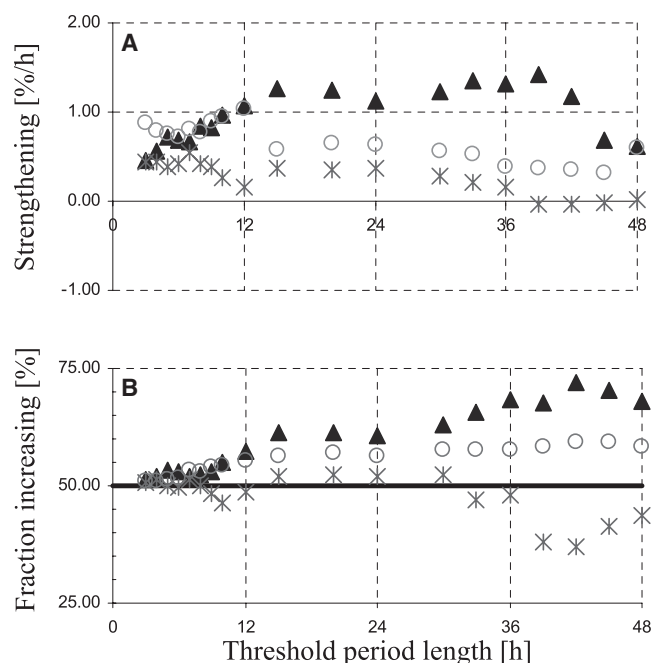
FIGURE 6 Differences in development of the strength of functional connections between periods with latencies of 5–30 ms (▲), 40–65 ms (✳), or without latency restrictions (◯). *A* shows the relative strengthening per hour, as a function of the chosen threshold period length. *B* shows the percentage of functional connections with increasing strength. Bold line: equilibrium between increasing and decreasing functional connections.
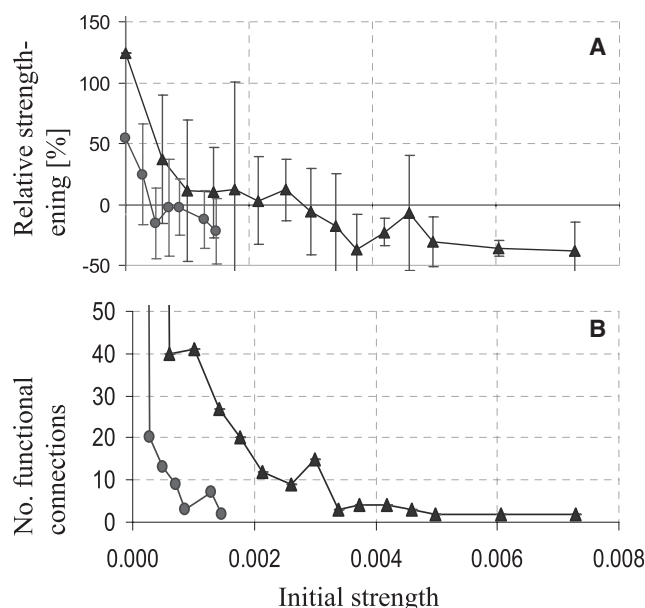

FIGURE 7 Strengthening during periods of low latency (5–30ms, ▲) or high latency (40–65 ms, ●), as a function of the initial strength of functional connections. *A* depicts strengthening as a percentage of the initial strength. The curves show that strengthening decreased with increasing initial strength and eventually even changed into weakening. However, *B* shows that the number of functional connections decreased rapidly with increasing initial strength. Weakening during short latency occurred infrequently as there were only 20 functional connections (1.3%) with initial strength >0.003.

Finally, we investigated the influence of initial strength on the development of functional connections.

It appeared that functional connections with low initial strength grew more than those with high initial strength. In fact, Fig. 7 suggests that only functional connections with small initial strength tended to strengthen during short latency, whereas initially stronger functional connections tend to weaken in such periods. However, the impression that functional connections with high initial strength tended to weaken was based on 20 observations only (~1%) and might therefore not be generalized.

During long latency, when the dominant development was weakening, there was still some strengthening if the initial strength was low, as shown in Fig. 7. The general shapes of the curves for long and short latency are comparable: functional connections with low initial strength tend to strengthen, which changes into weakening beyond a certain initial strength (the crossing point). However, Fig. 7 shows that this crossing point is much lower (shifted to the left) for long latency (~0.0004) than for short latency (~0.0028).

DISCUSSION

We used CFPs to investigate whether the development of the strength of functional connections in cultured cortical networks was related to their latency. In most cell research, signals are recorded from a single cell to directly measure

latency and height of excitatory postsynaptic potentials in response to presynaptic action potentials. In our study, we used 61 electrodes to probe cells extracellularly. An important difference between intracellular measurements in single-cell studies and our observations from (cross-correlation based) CFPs is that we looked at functional connections rather than structural synaptic connections (9). Therefore, it cannot be excluded on forehand that processes like long-term potentiation, which take place at the cellular level, might go undetected using CFP analysis. This study aimed to relate functional connections to cellular processes like LTP, focusing on excitatory connections.

Approximately 10–20% of all neurons in cortical cultures are inhibitory (21–23), and it is probable that we recorded from a comparable fraction of inhibitory neurons. Thus, a vast majority (80–90% of all analyzed pairs) described excitatory connections. Furthermore, it has been shown that cross-correlation-based techniques are far more sensitive to excitatory than to inhibitory connections (9,24,25). Thus, we expect to have described mainly excitatory connections, with little influence of the inhibitory system.

CFP analysis is related to cross-correlation, but uses a different normalization factor to enable interpretation of the relationships between neurons, similar to the approach followed by Marom and Eytan (26,27). They referred to this measure as functional association strength, an

abstraction of the biological situation, in which several pathways may be present between each pair of neurons. CFP analysis yielded functional connections between electrode pairs. Correlated activity may arise from direct synaptic connections between pairs of active electrodes or from correlations in the network firing dynamics via common input to both neurons.

Strengthening mechanisms such as LTP critically depend on the timing relationships between pre- and postsynaptic firing and, obviously, require direct synaptic connections between the neurons. The observed relationship between changing strength and latency in firing between neuron pairs may thus reflect mechanisms such as LTP, supporting the plausibility of direct synaptic connections between these neurons pairs.

Intracellular measurements in the visual cortex showed induction of LTP upon activation of the postsynaptic neuron within a ~20 ms time window after presynaptic firing (13). Similar observations were obtained in hippocampal cultures (14); see Bi and Poo (15) for a review. Experimental evidence from several different preparations showed that long-term strengthening of synapses occurs if presynaptic action potentials precede postsynaptic firing by no more than ~50 ms (10). Our results agree with these findings and suggest latency-dependent strengthening of functional connections in cortical cultures.

Our analysis, which is related to cross-correlation analysis (8), describes the strength or ''effectiveness'' of functional connections (24) as well as the latency between a presynaptic action potential and a postsynaptic one. The exact timing of the postsynaptic action potential depends considerably on input from the surrounding network. This network influence might be strong enough to mask the effects of LTP in an individual synapse between the two neurons. However, our study shows that the development of this effectiveness, or strength of functional connections, is related to their latency, comparable to the development of synaptic strength during LTP (15). Therefore, it is probable that structural synaptic connections substantially contribute to the functional connectivity estimated by CFP analysis.

Whereas the latency range associated with strengthening may be well explained by the time between pre- and postsynaptic action potentials that induces LTP, weakening during periods of longer latency (40–65ms) may possibly be attributed to LTD. Traditionally, LTD is induced when a postsynaptic spike precedes a presynaptic one, so at negative latencies, which would exclude LTD as a possible explanation for the observed long (positive) latency weakening. However, Nishiyama et al. (28) found two distinct windows for the induction of LTD in hippocampal slices. Besides the usual potentiation and depression windows, they observed an additional depression window at longer (positive) intervals between pre- and postsynaptic firing than the potentiation window.

One explanation for the observed weakening might be the (lost) competition with other synapses. Hebbian learning is based not only on activity-dependent synaptic modifications, but also on a mechanism that forces different synapses to compete with one another so that when some synapses to a given postsynaptic neuron are strengthened, others are weakened (10). This mechanism might cause weakening of functional connections with latencies outside the interval associated with strengthening. However, it fails to explain why weakening does not occur more clearly during long latency (>65 ms). A high initial strength might also be a factor leading to weakening. However, long latency functional connections usually had a low initial strength.

Thus, we don't have a clear explanation yet for weakening of functional connections with long latency. Still, it may be an important phenomenon in the processes of learning and memory, open to further investigation.

Obviously, functional connections resulting from a common input, or from other unknown factors, may have latencies in the same ranges that have been associated with strengthening or weakening for direct synaptic pathways. For such connections, no mechanism has been shown yet to relate strengthening or weakening to their latency. However, these connections do contribute to Fig. 4, and because their relationship between latency and strength development remains unclear, they may function as a source of the ''noise'' as shown in that figure.

Occasionally we found CFP curves with two separate peaks, indicating that the functional connection between the two electrodes was based on more than one pathway. Fitting Eq. 1 resulted in an average of both peaks, and thus to an abstract description of the average effects of multiple pathways, just like it did in relationships that showed a single peak. Such approximations probably also contributed to the noisiness of Fig. 4. However, even in the presence of these masking factors, the relationship between latency and strengthening appeared significant.

In this study, we found that strength development was related to latency. Of course, it is possible that this was not a causal relationship. We suggest that this finding originates from spike-time-dependent synaptic plasticity mechanisms at the synapses in the mono- or polysynaptic pathways between the neurons. The latency range related to strengthening agrees well to that determined from intracellular measurements, and also the range associated with weakening has been found before (28).

Further support for this interpretation could be obtained by calculation of the cross-correlation between latency and the derivative of strength. If maximum correlation would be reached at a certain time lag, we could determine whether strength changes were usually preceded by latencies in a certain range, which would be a strong indication for direct synaptic effects in the functional connection. Unfortunately, noisy factors in our data as addressed above hampered such

analysis. Our selection of monotonously stable periods emphasizes extreme situations, in which it appeared possible to detect a significant correlation, even though there were many sources of noise.

To investigate a possible relationship between latency and strength development of functional connections, we first examined periods ≥ 24 h of monotonous strengthening or weakening. This gave us not more than a first impression, because there were only a few such periods. This low number of periods (88) was limited by the extremely high threshold duration of the period rather than by the available number of pairs. To increase our dataset, we dropped the threshold duration to 10 h, which yielded 485 appropriate periods. Furthermore, we also investigated periods with latency in the short or long range. This further extended our dataset as shown in Fig. 5. We needed these extreme periods because the latency $\leftrightarrow$ strength development relationship was prone to several noise factors, as described above.

In our experiments, we found a few periods ≥ 10 h in which both functional connections $i \rightarrow j$ and $j \rightarrow i$ monotonically strengthened or weakened. This illustrates that the functional connections $i \rightarrow j$ and $j \rightarrow i$ are (at least to some extent) independent. Strengthening of a functional connection $i \rightarrow j$ means that j will fire more frequently in the analysis interval (0–500 ms after the spike in i). However, these extra-action potentials, in principle, do not influence the strength of the functional connection $j \rightarrow i$, because this is analyzed in the 500 ms interval after an action potential in j. If we focus on functional connections that arise from direct (causal) activation, $i \rightarrow j$ is a representation of all pathways from i to j, whereas $j \rightarrow i$ represents all pathways form j to i. These are different pathways, using other synapses, which may, in principle, strengthen or weaken independently.

Besides latency-related development of relational strength we also found a relationship with the initial strength, in agreement with a study using whole-cell perforated patch recordings by Bi and Poo (14). In the short- and long-latency data sets, functional connections with low initial strength tended to strengthen, whereas initially strong connections tended to weaken. Although both curves shared a similar shape, the maximum initial strength that could still be associated with growth was much lower during long latency than during short latency (see Fig. 7). The low number of functional connections with high initial strength in our study, however, hampered a firm conclusion about dependency on the initial strength.

Although latency-dependent strengthening has been shown before in intracellular measurements, to our knowledge this study is the first to demonstrate latency-related strengthening of functional connections between neurons on a large scale in vitro. It shows the sensitivity of functional connections to LTP-like changes in synaptic coupling, which makes CFP analysis a powerful tool to study network connectivity. Furthermore, it provides insight into the mech-

anisms that play a role in spontaneously occurring plasticity in developing cultured cortical networks, which may be important to study learning and memory.

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