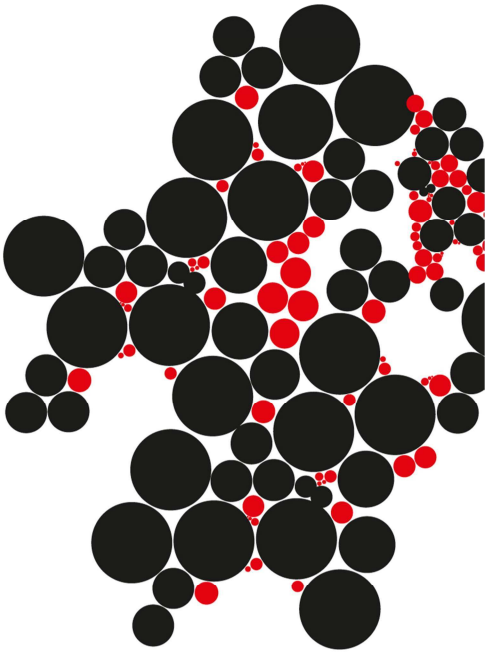
REPORT



THE EFFECT OF TETANIC STIMULATION ON FUNCTIONAL CONNECTIVITY

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Introduction

It is generally believed that cognitive processes like learning and memory highly depend on synaptic connections between neurons. These synaptic connections are plastic, meaning that connections between neurons are continuously altered, new connections are formed and others disappear. The communication between neurons takes place at the synapse, at this synapse the membranes of neurons approach each other closely and are able to communicate with each other using either an electrical current or by the release of neurotransmitters. In Figure 1 a post-synaptic neuron, a pre- synaptic neuron and two synapses are shown. The pre- and postsynaptic neuron are connected through a synapse, this synapse affects the membrane potential of the postsynaptic cell.

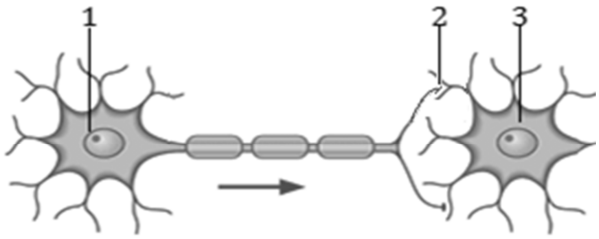

Figure 1 Representation of a synaptic connection with 1: The Presynaptic cell, 2: a synapse and 3: The postsynaptic Cell. The membrane of the postsynaptic cell can be depolarized by the synapse which transmits the state of the presynaptic cell. When the postsynaptic cell is depolarized beyond a certain threshold it will fire an Action Potential (AP).

When the postsynaptic membrane is at rest it has a certain potential, called the rest potential. Depolarization of the postsynaptic membrane is called an excitatory postsynaptic potential (EPSP) and for hyperpolarization we speak of an inhibitory postsynaptic potential (IPSP). Mostly post-synaptic neurons are connected to multiple neurons and all these neurons have an influence on the post-synaptic membrane potential, which is determined by the summation of EPSP's and IPSP's. These are determined by the combination of the pre synaptic membranes and the strength of the corresponding synapses. When the membrane potential exceeds the threshold the postsynaptic cell fires an action potential (AP).

The strengths of these synapses are continuously modified depending on activity, this process is also known as plasticity. Examples of short term forms of plasticity are synaptic facilitation/depression, potentiation augmentation, habituation and sensitization. Long lasting forms of synaptic plasticity (>30min) are long-term potentiation (LTP) and Long-term depression (LTD). LTP is a long lasting increase in synaptic strength and is associated with high frequent activity. LTD is a long lasting decrease in synaptic strength and is associated with long periods of slow stimuli.

A third kind of plasticity also includes the time specific behavior of pre- and post-synaptic neurons. This effect is called Spike timing Plasticity and was investigated by Dan and Poo in [1]. They discovered that the temporal relationship between activity in the presynaptic and postsynaptic cells is a determinant of long term synaptic plasticity. At a given (low) frequency of synaptic activity LTD will occur if presynaptic activity is preceded by a postsynaptic action potential, while LTP occurs if the postsynaptic action potential follows presynaptic activity. The timing of the presynaptic activity is crucial, after a time window of 60ms no changes are visible, see Figure 2. This kind of plasticity is called spike timing dependent plasticity. In [2] Bliss and Lømo investigated the effects of high frequent stimulation on connectivity. They used stimulation called Tetani; repeated trains of high frequent stimuli (20-100Hz). Using single cell recordings they monitored effects of Tetani applied in the dentate area in the hippocampus of a rabbit on synaptic transmission. The reported effect of the applied tetani was a significant increase of EPSP and a decrease in spike latency for stimulated pathways.

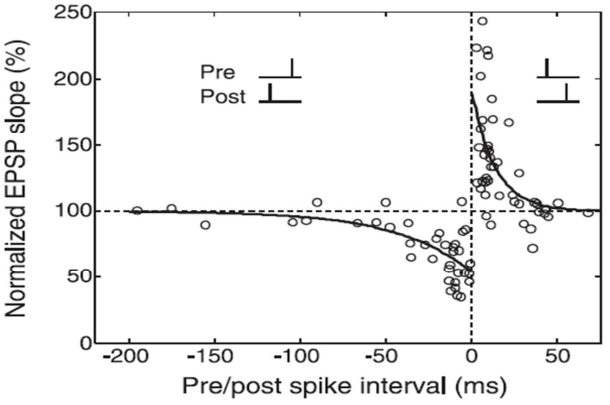

Figure 2 The effect of timing of pre- and postsynaptic spikes on the normalized EPSP. Clearly visible is that firing of the presynaptic neuron after firing of the postsynaptic neuron leads to a decrease in normalized EPSP and vice versa. Results are based on experiments on visual cortical slices from the rats brain, see [2]

Malinow et al. also researched the mechanisms responsible for LTP, caused by applying tetanus stimulation on axons [3]. In Figure 3 the effect of an applied tetanus is clearly visible on the EPSP. In this picture 2 pathways are stimulated, one with a

tetanus and the other without a form of high frequency stimulation. Pathway 1 (with tetanus) is clearly affected and pathway 2 (control) remains unchanged.

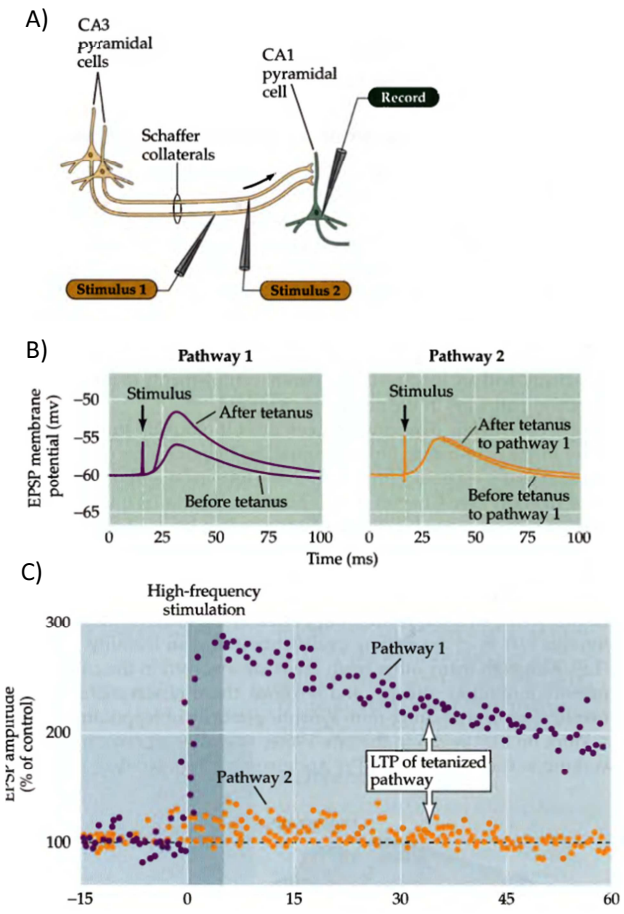

Figure 3 Long-term potentiation of Schaffer collateral-CA1 synapses. (A) Setup for recording synaptic transmission, two pathways each connected with an individual stimulation electrode (1 and 2). Pathway 1 represents the test pathway and pathway 2 is for control (B) Synaptic responses to test stimuli for the tetanized pathway and the control pathway. In the left figure the effect of tetanic stimulation is clearly visible (significant increase of the EPSP) whereas the control pathway has not significantly changed. (C) The change in amplitude of the EPSP slightly decreases over time but for a timescale of 1h the change of EPSP clearly deviates from the control pathway. (Pictures from neuroscience 4th edition p189)

In this project however we are interested in global connectivity changes of a network of neurons caused by tetanic stimulation. A popular method to study cellular and network properties in-vitro is by using cultured neuronal networks. Electrical activity of these cultured neural networks can be measured using a multi electrode array (MEA). Studying these electrical signals provides us a way to study processes, similar to those occurring in our cortex. The MEA's used in this project contained 60 electrodes (8 by 8 grid, without corner electrodes). These electrodes can both be used for measuring AP's of nearby neurons and for stimulation of these neurons. Dimensions of these electrodes are small enough (10µm-30µm) for measuring single cell AP's. Measurement of these AP's can be used to determine relationships between electrode pairs, therefore providing a way to study connectivity of a network. This kind of network connectivity is called functional connectivity. Functional connectivity describes the behavioral relation between electrodes and neurons at these electrodes. Although functional relations between measured neurons are known, nothing can be said about the actual anatomical connection between those neurons. These may consist of multiple pathways between neurons, some examples are shown in Figure 4. These examples suggest a relation between synaptic connectivity and functional connectivity. Recent studies suggest that functional connectivity is directly related to synaptic connectivity. Thus changes in synaptic connections should lead to changes in functional relationships between the recorded neurons.

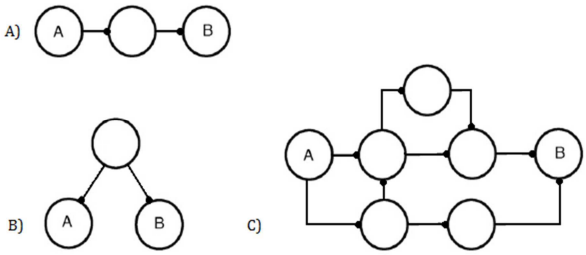

Figure 4 Functional connections exists between neurons A & B. Figure A illustrates a causal pathway between the presynaptic neuron A and the postsynaptic neuron B. Figure B represents an unwanted case, here the neurons A and B share a common input which can result in a false positive functional relation between A and B. Figure C illustrates a complex set of connections, between neuron A and B. This functional connection is based on several pathways which are typically unknown, but still a functional connection between A and B might be measured.

Because functional connectivity and synaptic connectivity are related the reported effects of tetanic stimulation should also have an effect on functional connectivity. This is investigated by Jimbo et al., who did observe changes in functional connections, see [4]. They observed two distinctive changes caused by tetanic stimulation: The first was an increase in the number of AP's in each network burst and also an increase in sensitivity to externally applied test stimuli (higher percentage

of network bursts after test stimuli). The second change Jimbo et al. observed was a decrease of the latency (250.4ms → 225ms) of the response and a reduction of the spread of the latencies (SD 20.2 ms → SD 3.1ms). A much more interesting discovery was published in [5] where they found that tetanic stimulation resulted in homogeneous changes in strengths of pathways. All neurons in the network showed either a homogeneous increased or decreased response (but not both) depending on the test stimulation site, see Figure 5. This figure graphically shows the change in response rate for all identified neurons and stimulation sites. Clearly visible is the homogeneous increase/decrease in response rate for a specific stimulation site, but also that changes are inhomogeneous for a specific neuron. Jimbo et al. concluded from these results that the corresponding pathways between neurons are affected in strength and not the firing rates of the neurons self are affected.

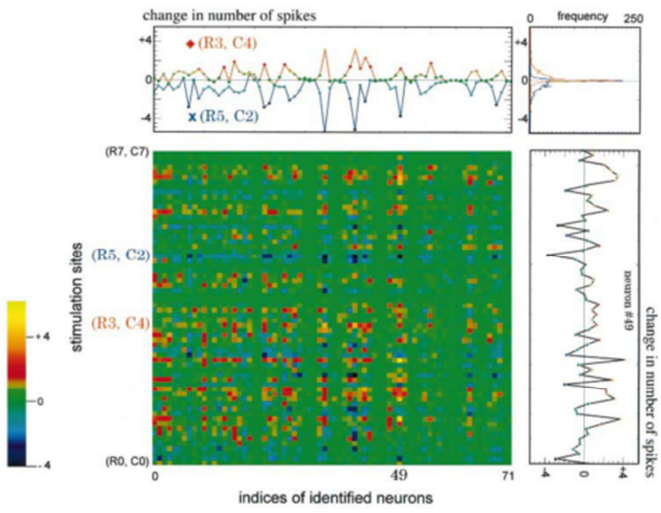

Figure 5 Recordings from shows the transparent electrode array, which consisted of 2 areas of 32 embedded electrode arrays, with a distance of 500 µm electrode dimensions were 30-30 µm and separated 180µm from each other. Neurons were identified at all recording and stimulation sites, to monitor individual neuron firing rates. After applying tetanic stimulation on the electrodes, the effects of stimulation were investigated by test stimuli. Analysis of these test-stimuli had a remarkable result. Either all neurons had an improved excitability or all neurons had a decreased excitability, but not both. Response rates of a neuron for different stimulation site on the other hand didn't show this homogeneous alteration. Jimbo et al. concluded, that synaptic pathways were affected by tetanic stimulation and not the neurons.

Wagenaar et al. attempted to find changes caused by tetanic stimulation protocols, including the same protocol used by Jimbo et al. [6]. They monitored different parameters such as the number of spikes, spike-frequency or other global parameters related to the number of spikes. Out of all experiment setups, only one setup resulted in an observed influence of tetanic stimulation. In this setup a difference in the average number of spikes before and after stimulation was observed. Also network activity was controlled before and after stimulation: electrical stimulation was used to prevent periods of synchronized high frequent spiking. These periods are called bursts and are absent in healthy in-vivo behavior. During a burst the network becomes highly active; neurons exhibit extremely high firing rates. This kind of behavior may trigger plasticity mechanisms and may reverse or mask changes induced by applied stimulation. To prevent this there are methods to suppress bursts, either chemically [7,8] or electrically [9,10]. In [9] Stegenga et al. found changes in functional connectivity using burst suppression by modulated rhythmic background stimulation (mRBS) in combination with applied tetani. Stegenga et al. reports two effects of mRBS: not only are network bursts suppressed by mRBS, but mRBS also modulation of network activity. Instead of neurons exhibiting periods of individual stochastic spiking alternated with bursts, the network exhibits oscillatory behavior within the theta band (4-12Hz). Furthermore he applied tetani at different background stimulation phases; applied tetani on the maximum of background stimulation rates (in-phase) and applied tetani on the minimum background stimulation rate (anti-phase). Stegenga et al. observed the change in post stimulus time histograms (PSTH) for in-phase, anti-phase and control experiments (no stimulation). They found that in-phase stimulation lead to significant increases in PSTH areas compared to control experiments. Anti-phase stimulation on the other hand leads to a slight decrease in PSTH area compared to control experiments, see also Figure 6.

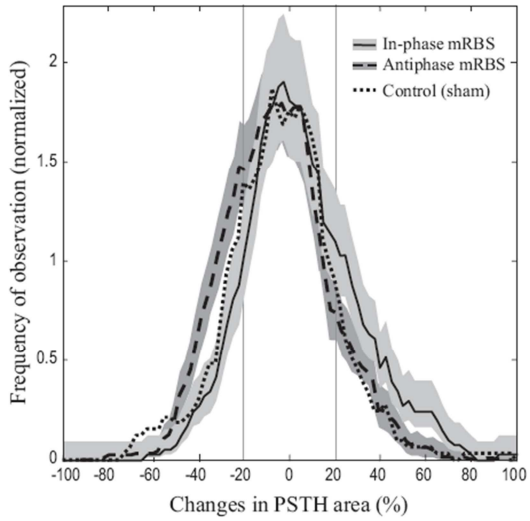

Figure 6 Collected results for tetanic stimulation at different background stimulation phases. This figure contains changes in mean PSTH areas of experiments where tetani were applied at minimum (anti-phase) and maximum (in-phase) background stimulation phases. For comparison the result for experiments without applied tetani (control) are also shown. Visible is a significant increase in PSTH area for in-phase stimulation compared to control experiments. Also visible is a slight decrease for anti-phase stimulation compared to control experiments. (Adapted from [9]*)*

Methods

A. Cultured Networks

This research project uses cultured networks consisting out of cortical cells obtained from the cortex of rats. Activity of these cultures was measured using multiple electrode arrays (MEA). Figure 7 is a picture of a MEA used in this research, combined with a zoomed picture of an electrode with nearby neurons. For creating a culture cortical cells were obtained from newborn Wistar rats or from E18 fetuses. After trypsin treatment cells were dissociated by trituration. About 400 000 dissociated neurons (400 µl suspension) were plated on an MEA in a 10 mm round spot (pre-coated with poly ethylene imide). The ring around this spot was removed after 2–3 h. This procedure resulted in an initial cell density of approximately 5000 cells per mm2, immediately after plating. Neurons were cultured in a circular chamber (inner diameter: 20 mm) glued on top of the MEA. The culture chamber was filled with 700 µl serum-free R12 medium. MEAs were stored in an incubator, under standard conditions of 37^{0}C, 100% humidity, and 5% CO2 in air. Experiments on the cultures began after 20 days in vitro (DIV). After experiment cultures were placed back into the incubator.

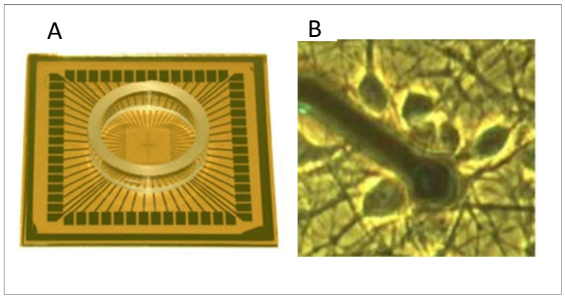

Figure 7 A multi electrode array (MEA), used to record extracellular action potentials of nearby neurons. A represents a global picture of the MEA, containing 60 electrodes with an electrode spacing of 200µm , electrode diameter 30µm and a glass ring to contain the culture. Figure B is a close look at an electrode surrounded by neurons.

B. Measurement Setup

The measurement setup used in this research was based on commercially available MEA recording setup; a standard MEA (200/30-Ti-gr) was used in combination with a 1060BC preamplifier and STG1002 stimulus generator (Multi Channel Systems, Reutlingen, DE). Data was sampled at 16 kHZ using a 6024E DAQ-card (National Instruments, Austin, TX). Stimulation protocols and data acquisition were carried out by custom LabView programs (National Instruments). During measurements, both temperature and CO_2 levels were controlled. The temperature was kept constant by a Peltier element (36^{0}C) to prevent condensation of the medium. To prevent infection of the culture, MEA's were sealed with a water resistant CO_2/O_2-permeable membrane (Multi Channel Systems). Before measurement cultures were left to acclimatize for at least 10 min. After 10 min we started observing noise levels and shapes of the AP's. Electrodes with implausible AP shapes or very high noise levels were grounded and excluded from measurement. All events were stored with an electrode number and a timestamp, including a sample of 6ms.

C. Stimulation Protocols

Probes

Before stimulation the program Probes is run. Probes is a test stimuli program that can be modified to personal preference. The typical settings that were used for the test stimuli are bipolar test pulses of 200µs duration (negative phase first) at a low frequency, usually 0,2-0,4 Hz. Each electrode is randomly stimulated 3-4 times with 3 different amplitudes (8-28µA). Responses to these stimulations are shown graphically in Probes. Electrodes with a high response (high number of

activated electrodes) and natural action potential shapes are considered to be suitable candidates for stimulation.

Plain tetanic stimulation (p-TS)

The stimulation protocol for plain tetanic stimulation (p-TS) is similar to the protocol of Jimbo et al.[5]. Before and after stimulation a period of spontaneous activity is measured. Both these periods lasted 1 hour and were separated in parts containing a fixed number of events. These parts will be referred to as data-blocks (DB). To enable statistical comparison before and after stimulation a minimum of 4 data-blocks per set was required. Each stimulation block (SB) consisted of either p-TS or no stimuli (control experiments). P-TS consisted of pulse trains containing 10 pulses applied at a single electrode, see Figure 8.

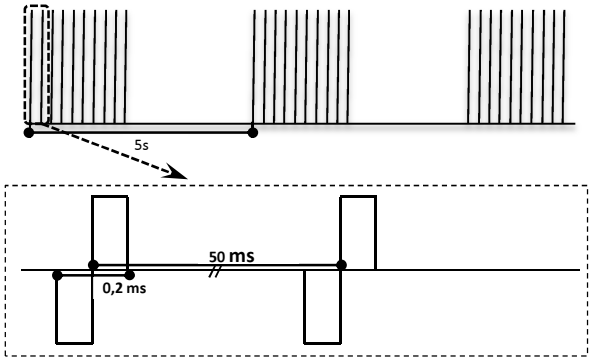

Figure 8 Plain tetanic stimulation (PTS) used in this research. Pulse trains containing 10 pulses, with a inter spike interval of 50ms and a inter train interval of 5s. These tetani were applied at a single electrode with varying stimulation durations (2-15min). The lower figure zooms in on the bi-polar pulses, and shows the actual shape of stimulation applied at an electrode.

Most experiments contained multiple periods of p-TS, where between stimulation periods 1h of spontaneous activity was measured. Also duration of stimulation periods were varied 2-15 min. Control experiments had similar durations of no stimulation compared to p-TS. A schematically representation of a p-TS experiment can be seen in Figure 9.

We investigated 2 possible influences on the effects of p-TS. We analyzed the effect of the duration of p-TS, this was done by varying the stimulation duration; we used p-TS durations of 2 min, 6 min and 15 min. We also investigated the influence of stimulation history; this was done by repeating p-TS. After stimulation a period of 1 hour of spontaneous activity was measured before we repeated stimulation on the same electrode.

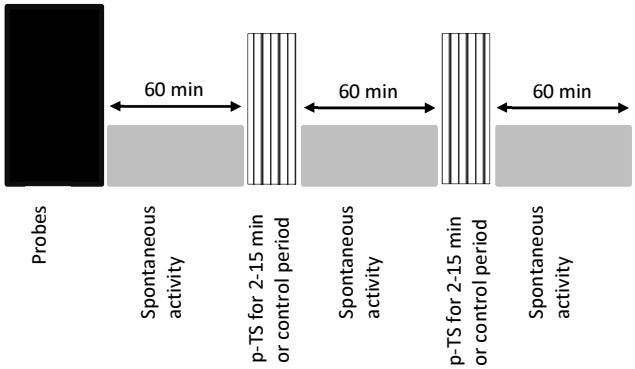

Figure 9 a schematically representation of the measurement/stimulation protocol of p-TS experiments, first a suitable electrode is chosen for tetanic stimulation using probes. Spontaneous behavior is measured before and after stimulation and compared with each other to determine the effect of stimulation. In some cases experiments consisted of multiple stimulation periods, in this example 2 periods of stimulation are shown. For control, periods with no stimulation separated evaluation periods, these had similar durations compared to periods of p-TS.

Theta background stimulation (θ-BS)

Theta background stimulation consists of modulated stimulation on randomly chosen electrodes. At each interval (dt) an electrode was randomly chosen out of a set of 10-13 electrodes. The probability of stimulation at this electrode was stimulation rate dependent. This stimulation rate r(t) is defined by:

$$r(t) = r_0 + r_1 \cos(2\pi f_m t) \quad (1)$$
$$, with\ f_m\ 4\ \text{Hz}.$$

For burst suppression the average rate of stimulation (r_0) was set to 10 Hz. The frequency deviation r_1 was set to 8-10 HZ, resulting in stimulation rates between 0-20 Hz. The decision to stimulated was made by comparing r(t) with a pseudo uniformly distributed random number x, [0 1]

$$Stim = \begin{pmatrix} yes & 1 \le R(t) \cdot dt \\ no & otherwise \end{pmatrix} \quad (2)$$

The effect of this stimulation is oscillatory behavior of the network, comparable to the Theta EEG band (4-12Hz).

Theta locked Tetanic stimulation (θ-TS)

In the stimulation period tetani were applied at a specific phase of the θ-*BS*, 4 pulses a train with a pulse frequency of 200Hz. A Cosine notation was used, such that stimulation at 0^0 corresponds to stimulation at the phase of maximal activity of θ-

BS, this is denoted as θ_0-TS. Applying tetani at the phase of minimal activity was denoted as θ_{180}-TS. The protocol for tetanic stimulation at different background stimulation rates (θ-TS) differed from the plain tetanic stimulation (p-TS) protocol. Before measurement 10-13 electrodes were chosen using probes where θ-BS with fixed amplitude for every electrode was applied. Similar to p-TS an experiment consisted of 2 evaluation periods separated by a stimulation period, see Figure 10. Although the global setup was the same, there were differences. Each evaluation period consists of 2 Probing sequences (15 min) with an additional period measuring spontaneous behavior (30 min). During these probing sessions the electrodes chosen for θ-BS were probed randomly and repeated a number of times with an interval of 5s. The stimulation period consisted of θ-BS in combination with phase-locked tetanic stimulation, where the phase of stimulation was constant in a stimulation block. Each experiment was then repeated with stimulation at the other phase. Similar to the p-TS protocol, evaluation periods were divided into data-blocks, with the difference that not only spontaneous behavior but also responses to test stimuli were measured.

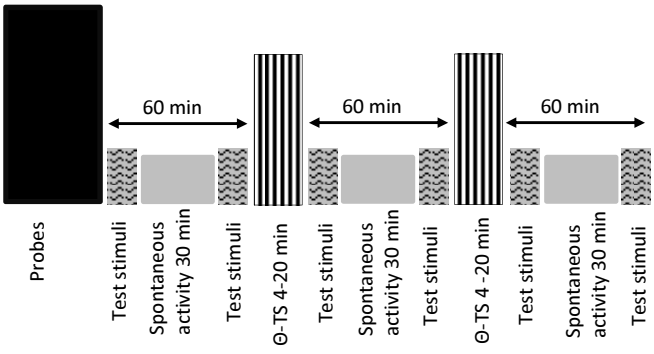

Figure 10 Graphical representation of the measurement/ stimulation protocol of θ-TS experiments, first a set of suitable electrodes are chosen for tetanic stimulation and theta background stimulation (θ-BS) using probes. Evaluation periods consisted out of 2 periods of applied test stimuli (2* 15 min) and 30 minutes of measuring spontaneous behavior. Each experiment consisted at least out of 2 sessions of θ-TS; one period with tetani locked on the peak of θ-BS and the other period with tetani locked at the trough of θ-BS

A complete θ-TS experiment contained both types of stimulation (θ_{180}-TS and θ_0-TS) the order in which they were applied was random. Similar to p-TS we also studied the effect of the duration of θ-TS, again by varying stimulation duration. We also investigated the effect of stimulation history; each experiment contained at least 2 periods of stimulation on different phases of θ-BS. Because Stegenga et al. found phase depend changes we also discriminated on phase. Therefore we compared 4 groups, based on phase and history; 2 with first time stimulation (θ_0-TS_{first} & θ_{180}-TS_{first}), and 2 with sequent stimulation (θ_0-TS_{second} & θ_{180}-TS_{second}). When our analysis showed no significant differences we made larger groups, for example based on stimulation phase.

Test stimuli

We investigated the effect of θ-TS by analyzing experiment data previously used by Stegenga et al. for other research, [9]. They investigated the effect of θ-TS by analyzing post stimulus time histograms (PSTH's) and burst profiles. For the PSTH's they used test stimuli and for burst profiles they analyzed spontaneous behavior. We used both periods for Conditional firing probability (CFP) analysis. Because this analysis is normally done on spontaneous activity we investigated if these test stimuli had an effect on our CFP analysis. This was done by dividing evaluation periods of θ-TS in 2 parts, separated by 10 minutes. This was about equal to the stimulation times of p-TS and θ-TS. We did this to compensate for spontaneous changes, which occur after a certain time. Figure 11 graphically represents the analysis of the effect of test stimuli.

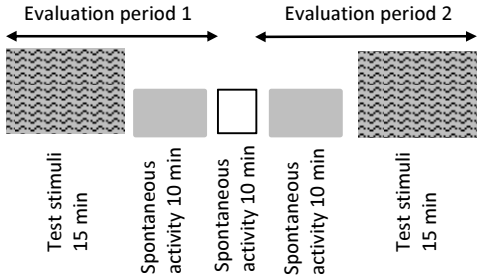

Figure 11 We tested the effect of test stimuli on conditionally firing probabilities by comparing evaluation periods 1 and 2 with each other. We also separated these evaluation periods with 10 minutes of spontaneous activity. This is equal to our analysis of the effects of no stimulation and comparable to the duration of p-TS and θ-TS.

D. Data analysis and structuration

For analyzing connectivity changes, either spontaneous or induced by stimulation, the long term recordings were divided in data-blocks of a fixed number of events. Experiment data from previous research on p-TS and θ-TS was available. Therefore the first step was identifying the useable experiment data. The first criterion that had to be satisfied was that the protocol of the experiment agreed with either the p-TS or θ-TS protocol,

described previously. Secondly the evaluation periods had to comprise a minimum of 2^{15} (~33000) events. The reason for this is that we wanted to have 4 separate data-blocks of at least 2^{13} events for analysis.

Conditional firing probabilities

Evaluation periods before and after stimulation were divided in fixed periods of events called data-blocks. Each data-block was then analyzed by using conditional firing probability analysis [11]. This analysis started by finding the active electrodes, an electrode was active if it contained more than 250 action potentials in a data-block. This number of AP's was necessary for fitting Equation (6), which describes the conditional firing probability of an electrode pair. This was done by placing all AP's at an electrode i in an array $X_i[t]$, containing times at all sample moments n.

$$X_i[t] = \begin{Bmatrix} 1\ for\ \ an\ AP \\ 0\ otherwise \end{Bmatrix} \quad (3)$$

With t the times at all sample moments. This was done for all active electrodes ($i \in [0,59]$). The times of recorded spikes were binned in intervals of 0,5ms. The number of the events on electrode i followed by events on electrode j in intervals of 0,5ms, within a period of 500ms, are calculated by equation (4).

$$N_{follow_{i,j}}[\tau] = \sum_t X_i[t] \cdot X_j[t+\tau] \quad (4)$$
$$with\ 0 \leq \tau \leq 500ms$$

This was done for all possible electrode pairs i,j. The conditional firing probability (CFP) of an electrode pair was then found by dividing N_{follow} by the number of events at electrode i.

$$CFP_{i,j}[\tau] = \frac{\sum_t X_i[t] * X_j[t+\tau]}{\sum_t X_i[t]} \quad (5)$$
$$with\ 0 \leq \tau \leq 500ms$$

These CFPs where calculated for every active electrode pair i,j with i≠j, within a time interval of 500ms. An electrode pair was considered active if both electrodes i&j had more than 250 events in a data-block. Figure 12 represents a calculated CFP curve.

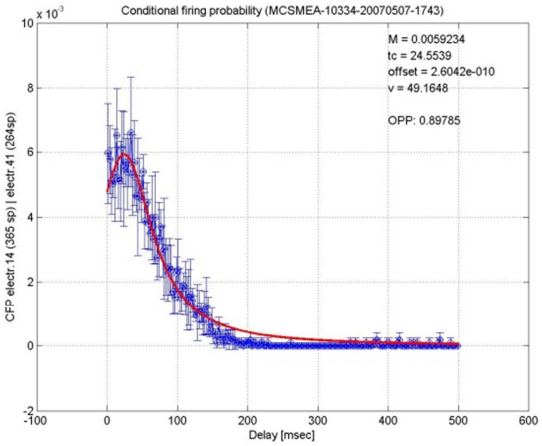

Figure 12 a practical example of a conditional firing probability for the electrode pair 14-41, the red line is the fitted curve of Equation (6). It represents the probability density function that electrode 41 will detect an AP after electrode 14 has detected an AP. Both electrodes must have 250 AP in order to fit Equation (6). The parameters that represent the connection M and T are stated in the upper right corner of the figure

Finally function (6) was tried to fit each calculated CFP curve. This fitted curve is an estimation of the probability density function of recording AP at site I after a recorded AP at site j.

$$CFP_{i,j}^{fit}[\tau] = \frac{M_{i,j}}{1+(\frac{\tau - T_{i,j}}{W_{i,j}})^2} + offset_{i,j} \quad (6)$$

This fitted curve results in 2 useable parameters $M_{i,j}$ and $T_{i,j}$ of every electrode pair i,j. M represents the strength and T the latency of a connection and will be used in this researched. Figure 13 graphically represents these parameters.

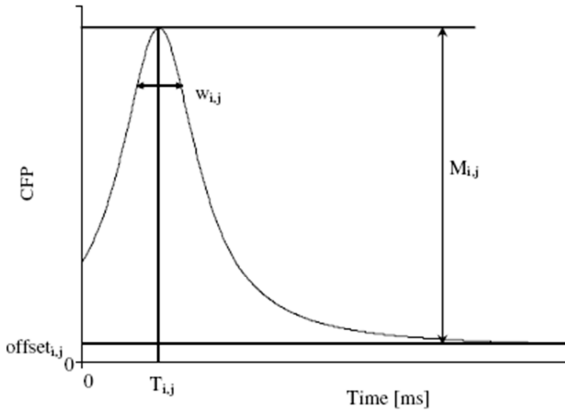

Figure 13 Graphical representation of the fitted CFP describing the estimated probability density function of recording a spike at site j after recording one at j. Important parameters of this CFP are the strength M and the latency T. M will be used in this research for detecting changes between CFP's of electrode pairs. (Adapted from [11]***).***

Evaluation parameters:

The strength parameter M from the $CFP_{i,j}^{fit}$ was used to determine electrode pairs that had a persisting connection, meaning a connection had a given strength in each data-block. For all persisting connections, sets containing the strengths before and after stimulation were made. The first test on these sets was determining stability of the measured network, by calculating the Fano-factor (FF) defined in Equation (7). We calculated the Fano-factor for 2 reasons; first for testing the influence of test stimuli on CFP analysis and secondly to determine if the network was stable enough for analysis. It is not unlikely that the stability of a network has an influence on the effect of tetanic stimulation. Therefore we wanted to have experiments which were equally stable, before comparing them. We also checked if the average firing rate was stable. This is done based on findings of Chiappalone et al, where firing rate stability was a criterion for induced plasticity, see [12].

$$FF = mean(\frac{\sigma^2_{\overrightarrow{M_{i,j}}}}{\mu_{\overrightarrow{M_{i,j}}}}) \quad (7)$$

Where $\overrightarrow{M_{i,j}}$ are the sets of strength before and after stimulation for every persisting connection between electrode i and j. Experiments were the Fano factor exceeded twice the mean Fano-factor of similar experiments were excluded from analysis.

To find (induced) changes, sets of strengths (before and after) were tested for significant deviation using a two-tailed Student's t-test ($p<0.05$). This way changes in both directions were detected. This group of changed connection strengths are placed in $\overrightarrow{M^*_{i,j}}$, containing connection strengths before and after stimulation. The length of $\overrightarrow{M^*_{i,j}}$ corresponds with the number of significantly changed connection strengths. Whereas the length of $\overrightarrow{M_{i,j}}$ corresponds to the number of total connections. According to Equation(8) these sets were used to calculate the fraction of significantly changed connection strengths (FSCS).

$$FSCS = \frac{length(\overrightarrow{M^*_{i,j}})}{length(\overrightarrow{M_{i,j}})} \quad (8)$$

This was subdivided in the increased (↑) and decreased connections strengths (↓). The mean absolute strength change ($|\bar{\Delta}|$) and mean strength change ($\bar{\Delta}$), of sets that were significantly different were calculated as a measure for the relative size of change. See the equations below

$$\overline{|\Delta|} = mean\left(\frac{|\overline{M^*_{before}}(i,j) - \overline{M^*_{after}}(i,j)|}{\overline{M^*_{before}}(i,j)}\right) \quad (9)$$
$$for\ i \neq j$$

$$\bar{\Delta} = mean\left(\frac{\overline{M^*_{before}}(i,j) - \overline{M^*_{after}}(i,j)}{\overline{M^*_{before}}(i,j)}\right) \quad (10)$$
$$for\ i \neq j$$

To express changes in plasticity in a one dimensional parameter the plasticity index (PI) is used, defined as:

$$PI = FSCS * |\bar{\Delta}| \quad (11)$$

PI=0 describing zero changes in connectivity PI=1.0 describing the case that all persisting connections changed with 100% strength.

Results

A. Experiments suitable for study

Using the criteria preciously described, sets of experiments that are suitable for study were selected. This resulted in 18 (p-TS) and 22 (θ-TS) useable experiments. The cultures stimulated according to p-TS protocol usually had a lower activity. To use these experiments for analysis the data-block size was set to 2^{13} events, this was considered the absolute minimum size that still had enough data for statistical analysis. Cultures where θ-TS stimulation was applied were usually more active, therefore the data-block size was adjusted to 2^{14} events. For both the p-TS experiments and the θ-TS experiments the evaluation period before and after stimulation lasted around 30 min. (28 ± 14 min for θ-TS and 33 ± 6 min for p-TS). Meaning on average we only used half of the evaluation periods. This is caused by our decision to keep the number of data-blocks used for analysis constant. We rejected three experiments; one p-TS and two θ-TS experiments, based on criterion of stability. These experiments exceeded the threshold (FF > 2 x mean), see (7). Which gave us 17 p-TS and 20 θ-TS experiments that where further analyzed.

B. Development of connections with time

To investigate the effect of stimulation on possible induced plasticity the strength parameter of the CFP_{fit} was used, see Methods. To check the stability of the strength parameter we plotted sets of connection strengths versus time. Figure 14A is an example of the development of the strength of a connection as a function of time. This particular experiment had a larger fraction of strengthened connections (27%) than weakened (5%), which was not common for p-TS experiments, see Figure 14C. All these decreased connection strengths are shown in the figure together with the first four strengthened connections. Figure 14B is a plot of the latency of these connections as a function of time. This plot supports our choice for using M to detect changes; not only are the latencies of a connection sometimes zero, which makes analysis more difficult, also the stability of the latency of these connections is much lower than that of strengths. We included Figure 14D to show the difference in conditional firing probability (CFP) between connections which weakened and strengthened after stimulation. This figure is a plot of the mean conditional firing probability ($\overline{CFP}$) of individual connections before and after stimulation. These $\overline{CFP's}$ correspond to the same connections plotted in Figure 14A&B. Comparing $\overline{CFP}$'s before and after stimulation, for strengthened and weakened connections, lead to an interesting observation for mean latencies. All connections that were significantly affected in strength after stimulation showed low or negligible differences in mean latencies. Because of the great variation in the latency of connections, we could not determine a significant correlation between changes in strength and latency of a connection.

C. Stability of Strengths

The evaluation periods of the p-TS experiments existed of measuring spontaneous behavior. Θ-TS experiments on the other hand contained 2 periods with responses to test stimuli (2 * 15min) and a period of spontaneous activity (30 min). The stability of sets of connection strengths before and after stimulation was assessed by the Fano-factor (FF), see equation (7). The results can be found in Table 1. We found that FF's for p-TS and θ-TS were significantly different (2-tailed t-test p<0,001). These differences were also present between control protocols, with and without test stimuli (p<0,0015). Visible in Table 1 is that θ -TS experiments had almost a double FF compared to p-TS.

Stim. protocol	Test stimuli	n	FF before ($*10^{-6}$)	FF after ($*10^{-6}$)
p-TS	No	17	36,1±8,8	$40,1±12,1^1$
No Stim.	No	18	36,9±8,6	$35,8±9,9^1$
θ-TS	Yes	20	65±20	$69±24^2$
Test Stim.	Yes	8	64,2±31,2	$57,1±22,4^2$

Table 1 Fano factors of different experiment types, indicating the stability of the strength parameter. Visible is the difference between protocols with applied test stimuli and protocols without test stimuli. We found experiments without test stimuli[1] had comparable Fano-factors, experiments with test stimuli[2] also had comparable Fano-factors. The difference between protocols with test stimuli[2] and protocols without test stimuli[1] were significantly different, (2-tailed t-test p<0,0015).

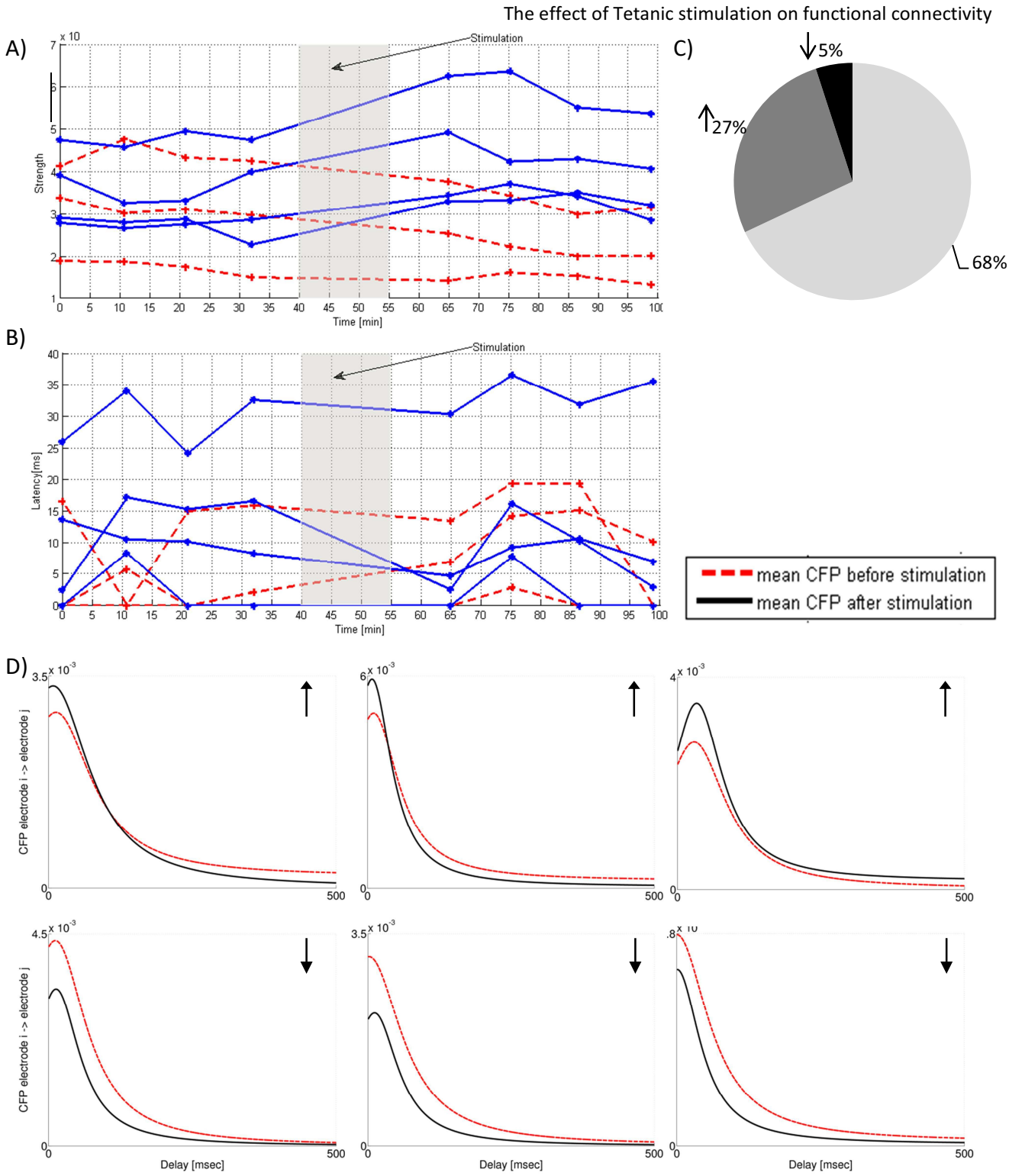

Figure 14 All these figures correspond to the example experiment "20070504". Figure A shows the development of the strength of a connection between electrode pairs as a function of time, The blue continues curve are connections which significantly increased in strength (↑) and the red dashed line are significantly decreased (↓) connection strengths. This experiment is chosen because it's high global change in Plasticity index (a measure for change in plasticity). Stimulation of this network resulted in a significantly higher fraction ↑ compared to the fraction ↓, see also Figure C. Figure B shows the development of the latency of a connection between electrode pairs as a function of time, The blue continues curve correspond to latencies of increased connection strengths after stimulation, displayed in figure A. The red dashed line represents the latencies of decreased connection strengths after stimulation, displayed in figure A. Figure A compared to Figure B shows that the strength parameter is more stable compared to the latency of a connection. This was common for all analyzed experiments and was also mentioned in, [11]. *This is the reason why we used the strength parameter to detect changes. Figure C shows that most persisting connections were not affected by tetanic stimulation, also the fraction of ↑ and ↓ were not equal. This was not a common result for p-TS. Figure D represent mean CFP's of significant increased (↑) connection strengths (upper 3) and 3 significant decreased (↓) connection strengths of experiment "20070504". These correspond to the connections in Figure A&B. Visible is that although mean strengths change, the mean latencies appear stable before and after stimulation. Because the high variation in latency we did not researched latency changes further.*

D. Effects of plain tetanic stimulation

The effect of p-TS duration

We studied the influence of increased stimulation duration. Three different groups where made with 2-3, 6, and 15 minutes tetanus duration. This resulted in no visible changes in PI for different stimulation durations, see Figure 15. All groups of stimulation duration contained both periods of first time p-TS and subsequent p-TS. These differences in stimulation history are equally distributed over different groups of stimulation duration.

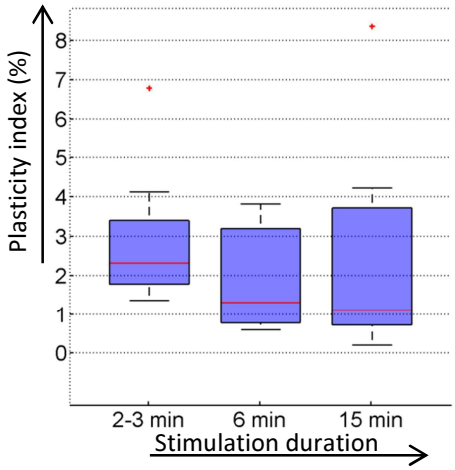

Figure 15 Boxplot of the plasticity index, the product of the fraction of significantly changed connection strengths and the magnitude of change. A slight increase in duration did not lead to different values of plasticity indexes, see also Table 2.

Similar to the PI we also did not detect changes in other parameters such as FSCS, $\bar{\Delta}$ and $|\bar{\Delta}|$ (FSCS), between experiments of different stimulation durations, see Table 2.

Duration (min)	n	FSCS(%)	$\|\bar{\Delta}\|$(%)	$\bar{\Delta}$(%)	PI(%)
2-3	8	15,5±4,7	17,7±5,0	-0,5±13,0	2,88±1,67
6	3	10,6±7,3	17,4±2,2	-3,2±8,2	1,91±1,38
15	7	13,1±9,2	15,4±6,0	2,8±11,6	2,52±2,68

Table 2 A slight increase in stimulation duration does not lead to an increase in the fraction of significantly changed connections (FCSC) and the mean absolute strength change of significant changed connection strengths ($|\bar{\Delta}|$) resulting in no observable changes in plasticity index (product of FCSC and $|\bar{\Delta}|$).

The effect of p-TS history

To investigate the effect of preceding tetani all experiments were grouped based on the number of preceding periods of stimulation (0, 1 and 2 or more). We plotted the PI for groups with an equal number of preceding tetani, see Figure 16. We found that the first period of p-TS had a significantly higher PI, compared to the second and the third period (t-test <0,05). For the fourth period we found changes were not significant. Because the limited sample size (n=2) for third and fourth periods of p-TS, we placed all subsequent periods of p-TS in one group (p-TS subsequent). A one-tailed t-test indicated that the first period of p-TS resulted in a significantly larger PI (p<0,05) than sequent periods of p-TS .

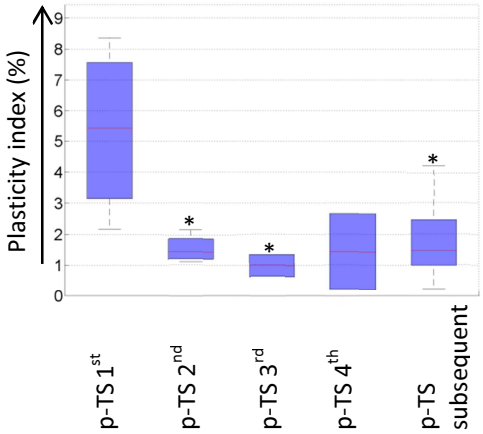

Figure 16 Boxplot showing the effect of previous periods with stimulation on plasticity index; the first period of p-TS yielded a larger change in PI than subsequent periods of p-TS. (one-tailed t-test p<0,05). Only the fourth period of p-TS had no significantly different PI compared to the first period of p-TS.

We also analyzed if these decreasing changes resulted from a lower number of changed connections or from smaller changes in connection strengths. We found that the FSCS differed significantly between first time p-TS and sequent periods of p-TS (p<0,035). We also found that the magnitude of changes, measured by $|\bar{\Delta}|$ did not differ significantly. Indicating that changes were primarly caused by a lower number of significantly changed connections, see Table 3.

History of stimulation	n	FSCS(%)	$\|\bar{\Delta}\|$(%)	PI(%)
Jimbo 1st	4	23,4 ± 6,1[1]	21,7 ± 5,2[2]	9,2 ± 11,4[3]
Jimbo 2nd	4	9,8 ± 2,6	16,3 ± 4,6	-3,4 ± 11,9
Jimbo 3rd	2	9,4 ± 2,4	10,3 ± 1,2	-7,3 ± 1,8
Jimbo 4th	2	8,6 ± 6,8	14,3 ± 2,9	5,5 ± 4,1
Jimbo subsequent	14	11,0 ± 5,1[1]	15,4 ± 4,3[2]	-2,2 ± 10,9[3]

Table 3 After the 1st period of p-TS the PI was significantly larger than after subsequent periods of p-TS ([3] one-tailed t-test p<0,05). Differences in PI were caused by the FSCS and not by the $|\bar{\Delta}|$. FSCS was significantly different between the 1st period of p-TS and subsequent periods of p-TS ([1]t-test p<0,035). The $|\bar{\Delta}|$ did not differ significantly ([2]p>0,11).

Because FSCS between was different between the first period of p-TS and subsequent periods of p-TS, we investigated if connections affected in the first period of stimulation were affected in the same direction in subsequent period of stimulation. For example if a connection strengthened in the first period also strengthened in the second period. The number of experiments that had connections that either further strenthened(1 out of 14) or further weakened(2 out of 14) was small. More common was that connections strengthened in the first period of p-TS and weakened in the second period of p-TS or vice versa, see Table 4.

Reproducibility of induced changes	↑ (%) Sequent period	↓ (%) Sequent period
First period ↑	1,02 ± 3,68	10,0 ± 14,3
First period ↓	6,46 ± 13,58	3,81 ± 9,66

Table 4 Reproducibility of alterations in connection strengths; we found a small fraction of connections affected in the first period were also affected in the sequent period. Even smaller was the fraction of connections that significantly changed in the same direction. For example connections which increased (↑) in the first period and also increased in a sequent period. The fraction of connections affected in the opposite way was also small. Most changed connections in a sequent period were different connections than those affected in a preceding period.

The effect of p-TS on Fractions of ↑ & ↓

On average p-TS tended to yield a more strengthened connections (↑) than weakened ones (↓), see Figure 18. However, this difference was not significant (t-test, p>0,22). It was most common that electrodes showed connections with increased connection strength, as well as connections with decreased strength with other electrodes. In other words we did not find that an electrode had either exclusively increased or decreased connections.

E. Effects of θ-TS stimulation

The effect of θ-TS duration.

For tetanic stimulation with theta background stimulation (θ-TS), the duration of the stimulation was mostly constant (4-6min), although 6 experiments had stimulation durations of > 15 min. Plasticity parameters of these experiments with increased stimulation durations did not significantly differ from experiments with a normal stimulation duration(t-test, p>0,38). Therefore we did not make a distinction between durations in further analysis.

The effect of θ-TS history on $\bar{\Delta}$ and PI.

Also dividing θ-TS experiments into groups by the number of previous tetani and stimulation phase, yielded no significant changes between plasticity indices, see Figure 17. What did seem to differ, were mean changes in strength($\bar{\Delta}$). It seemed that previous stimulation resulted in a decrease in ($\bar{\Delta}$) for both θ_0-TS and θ_{180}-TS stimulation, although differences were not significant (t-test, θ_0-TS p>0,50 & θ_{180}-TS p>0,23). The biggest influence on $\bar{\Delta}$ however was the stimulation phase. Θ_{180}-TS led to a decrease of $\bar{\Delta}$ whereas θ_0-TS yielded an increased $\bar{\Delta}$, see also Figure 20

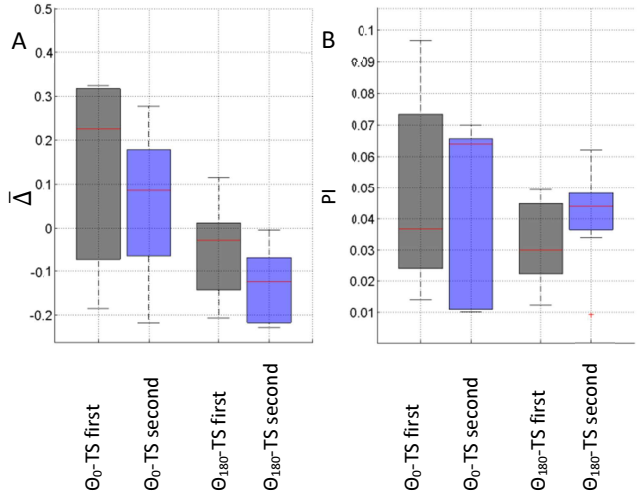

Figure 17 The phase specific effect of pervious θ stimulation stimulation on mean strenght $\bar{\Delta}$ and plasticity index (PI). Visible in in figure B is that previous stimulation did not (significantly) influence the PI. What did differ was the mean strength change which decreased after pervious stimulation, see Figure A and Table 5. Testing changes between first and second stimulation between both phases did not lead to a significant difference (p>0,10)

When we pooled all experiments with various numbers of preceding tetani, for Θ_{180}-TS $\bar{\Delta}$ was significantly smaller than for Θ_0-TS (one-tailed t-test, p<0,005).

Group name	n	FCSC *(%)*	$\bar{\Delta}$*(%)*	PI *(%)*
Θ_0-TS 1st stim.	5	17,8±10,9	13,9±20,0	4,69±2,97
Θ_0-TS 2nd stim.	6	16,1±10,6	5,5±16,5	4,40±2,71
Θ_{180}-TS 1st stim.	5	12,5±4,8	-9,4±10,7	2,79±1,30
Θ_{180}-TS 2nd stim.	7	19,9±9,6	-13,3±7,9	4,11±1,51

Table 5 The phase specific effect of pervious θ-TS stimulation stimulation on mean strenght($\bar{\Delta}$) and plasticity index (PI). We detected no influence of previous stimulation on PI. What we did detect was a slight decrease in $\bar{\Delta}$ for second stimulation experiments. Testing changes for first and second stimulation between both phases did not lead to a significant difference (p>0,10).

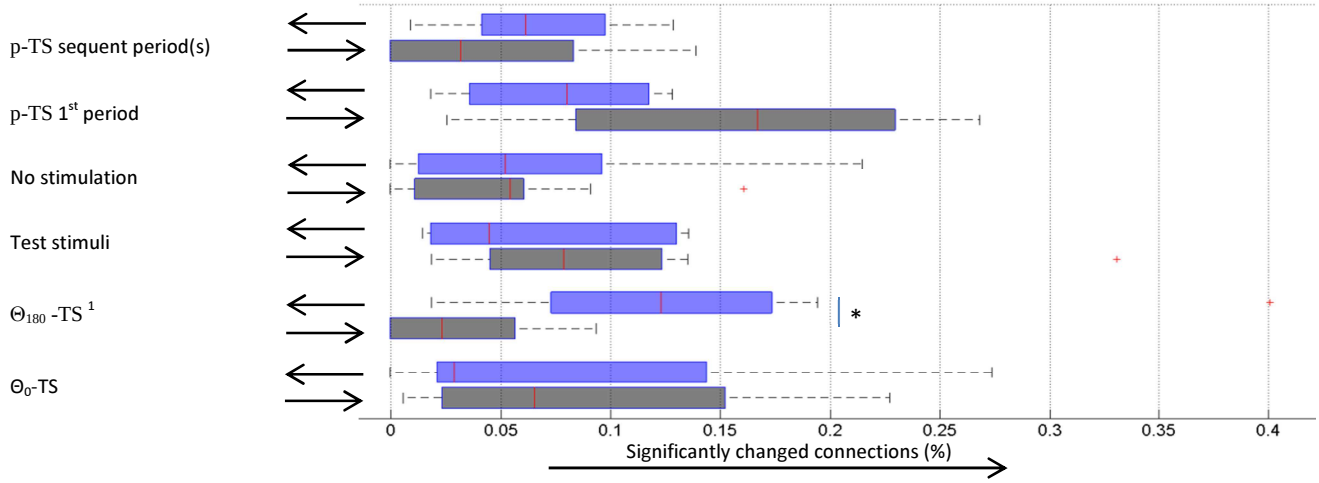

Figure 18 shows the low fraction of strengthened connections (↑) for θ_{180}-TS, analysis showed the fraction of ↓ was significantly larger compared to ↑ (one-tailed t-test p<0,01) . [1]Analysis showed that the fraction of strengthened connections for θ_{180}-TS experiments was significantly smaller compared to θ_0-TS, subsequent p-TS and test stimuli (p<0,05). The difference of θ_{180}-TS compared to no stimulation was not significant (p>0,09). The fraction of weakened connections for θ_{180}-TS experiments was significantly larger compared to no stimulation, first period p-TS and test stimuli (p<0,05). The difference with θ_0-TS was not significantly different (p=0,09). The Fractions of ↑&↓ for θ_0-TS did not differ much from other stimulation types. Also visible in the figure is the high fraction of strengthened connections caused by the first period of p-TS stimulation, but this did not differ significantly differ from no stimulation (p=0,06).*

The effect of θ-TS on Fractions of ↑ & ↓

For further investigation about the effects of θ-TS, the fraction of significant affected connection strengths where divided into increased (↑) and decreased (↓) connection strengths. For θ_{180}-TS experiments the fraction of ↑ was significantly lower compared to the fraction of ↓ (one-tailed t-test, p<0,004). Fractions of ↑&↓ in other experiments showed no significant difference, see Figure 18. We found that the fraction of ↑ for θ_0-TS experiments was significantly higher compared to θ_{180}-TS experiments (one-tailed t-test, p<0,015). We also found that the fractions of ↑&↓for θ_{180}-TS were significantly smaller than fractions for test stimuli (one-tailed t-test, p<0,03). Fractions of ↑&↓ of θ_0-TS were not significantly different compared to test stimuli.

A. Comparison of all protocols

Differences in Plasticity index (PI)

Comparing the effects of different stimulation types we see that only subsequent p-TS, did **not** significantly differ in plasticity index compared to no stimulation, see Figure 19 and Table 8. Other experiment types had a significantly higher PI.

We also found no changes in FSCS, $|\bar{\Delta}|$ and PI between experiments with test stimuli and θ-TS.

Also for the first period of p-TS, we found a significantly higher PI compared to experiments with no stimulation. In one complete θ-TS we discovered a negative outliner in PI; for each phase the PI index was lower than common ($1{,}41*10^{-2}$ and $0{,}94*10^{-2}$ where $4{,}23*10^{-2}$ was common).

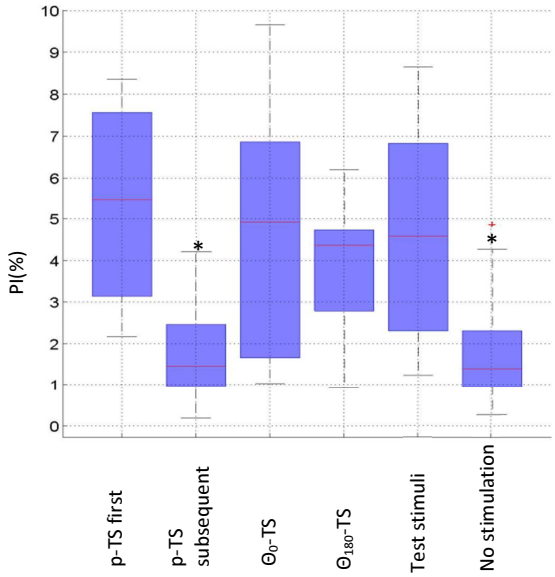

Figure 19 Visible in this figure is that 2 types of experiments had a significantly lower PI than other experiment types (one-tailed t-test <0,05); 1 the p-TS experiments with previous stimulation and 2 the experiments without stimulation. All other experiments had comparable values for PI. This includes the PI found for test stimuli. (* one-tailed t-test <0,05)*

Compared PI of experiment types		p-value
p-TS 1st period	**No stim.**	0,04
p-TS subsequent	**No stim.**	0,48
Theta 0	**No stim.**	0,01
Theta 180	**No stim.**	<0,01
p-TS 1st period	**p-TS subsequent**	0,04
Theta 0	**Theta 180**	0,21

Table 6 the outcome of a one-tailed t-test for PI of different experiment types. We found that all but one (subsequent p-TS) tetanic stimulation protocols had a significantly higher PI, compared to no stimulation. We also found that differences between θ_0-TS and θ_{180}-TS were not significant.

Differences in $\bar{\Delta}$ for different experimental protocols.

We found that $\bar{\Delta}$ for θ_0-TS experiments did significantly differ to θ_{180}-TS experiments. Although $\bar{\Delta}$ had the highest median for θ_0-TS experiments, it did not significantly differ from $\bar{\Delta}$ for no stimulation. $\bar{\Delta}$ after θ_{180}-TS was significantly smaller than $\bar{\Delta}$ after θ_0-TS, test stimuli, or no stimulation. We also compared test stimuli with no stimulation, which resulted in no significant differences. p-TS experiments did not significantly differ from no-stimulation. A boxplot of $\bar{\Delta}$ for different stimulation types is shown in Figure 20. Table 7 contains all p- values, used to investigate the significance of differences. Similar to our findings for PI, we detected some negative outliners for θ_0-TS and θ_{180}-TS. But these did not correspond to a single experiment.

Compared $\bar{\Delta}$ of experiment types		p(value)
Θ_0-TS	**Θ_{180}-TS**	0,009
Θ_0-TS	**No stimulation**	0,132
Θ_{180}-TS	**No stimulation**	0,028
Θ_{180}-TS	**p-TS first**	0,053
Θ_{180}-TS	**p-TS subsequent**	0,082
Θ_{180}-TS	**Test stimuli**	0,003
p-TS first	**No stimulation**	0,245
p-TS subsequent	**No stimulation**	0,673
θ-BS	**No stimulation**	0,078

Table 7 The mean change in connection strengths for different types of experiments, see Figure 20, are tested for significant differences. The compared experiment types can be seen in column 1 and 2, whereas the value for p can be found in column 3. This value corresponds to the outcome of a one-tailed student's t-test.

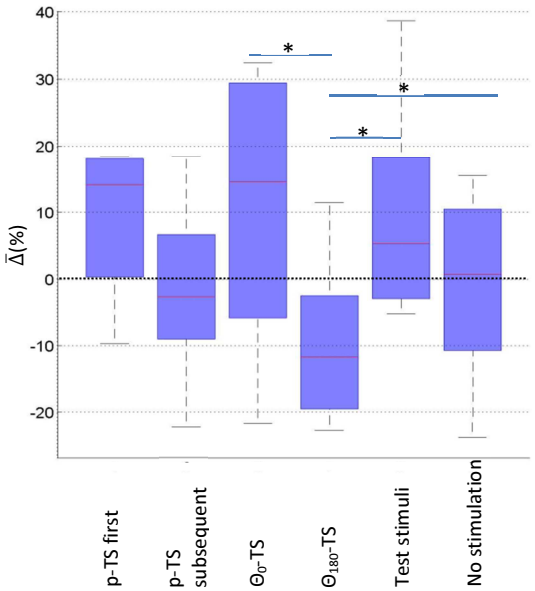

Figure 20 the mean strength of changed connections ($\bar{\Delta}$), comparing phase specific tetanic stimulation on different phases of theta background stimulation (θ-BS). We observed $\bar{\Delta}$ was significantly different between θ_{180}-TS and θ_0-TS. θ_{180}-TS was also significantly different compared to plain tetanic stimulation without previous stimulation (p-TS first) and control experiments with test stimuli. For θ_0-TS experiments we did not find a significant deviation from test stimuli.

Group name	n	Stimulation time	FCSC(%)	$\lvert\bar{\Delta}\rvert$(%)	$\bar{\Delta}(\%)$	PI(%)	Age(DIV)
p-TS first	4	8:56 ± 6:12	23,4±6,1	21,7±5,2	9,2±11,4	5,35±2,38	37±6
p-TS subsequent	14	7:39 ± 5:36	11,0±5,1	15,4±4,3	-2,2±10,9	1,79±1,14[1]	43±7
Θ_0- TS	11	7:42 ± 5:46	17,0±10,8	28,3±8,4	10,1±19,0	4,56±2,85	29±11
Θ_{180}- TS	12	9:12 ± 5:45	16,8±8,7	23,7±5,4	-9,9±10,0[3]	3,74±1,49	29±10
Test stimuli	8	30:00 ± 0:00	17,3±10,1	28,0±7,0	9,4±14,4	4,66±2,50	28±3
No stimulation	18	10:00 ± 0:00	11,6±5,6	14,5±3,4	-0,4±11,3	1,77± 1,17[2]	37± 5

Table 8 The effect of different kinds of stimulation we found that only plain tetanic stimulation (p-TS) with previous stimulation had a lower fraction of significant changed connection strengths and a smaller mean absolute changed connection strength $|\bar{\Delta}|(\%)$. The plasticity index is a product of these 2 parameters and was also significantly different (p value). Theta background stimulation was not only applied in stimulation periods, but also 15 minutes before and after. For this control experiment we found comparable values for PI as in p-TS and θ-TS.

Discussion

We investigated connectivity changes of a cultured neuronal network by applying two stimulation protocols of tetanic stimulation; p-TS and θ-TS. For both protocols we investigated the effect of the duration and history of stimulation. We compared conditional firing probabilities of pre- and post-stimulation periods to spontaneously occurring changes. To quantify the observed changes in functional connectivity, we calculated the fraction of significantly changed connections (FSCS) and the size of absolute changes ($|\bar{\Delta}|$). To express these changes in connectivity in a one dimensional parameter we calculated the plasticity index (PI). We found that both p-TS and θ-TS had a significant influence on functional connectivity. Furthermore for θ-TS we found phase specific changes. We also found that test stimuli used for PSTH's analysis resulted in significant changes in CFP's. We will start the discussion for our results with p-TS.

A. The limited effect of P-TS

We found that applying tetanic stimuli on a single electrode has a limited effect on functional connectivity. Increasing the stimulation duration of the tetanic stimulus did not lead to an increase of both size and number of significantly affected connections. A recent study reported higher values for PI, after increasing the stimulation duration of various stimulation protocols [13]. In their set of stimulation protocols tetanic stimulation was absent, but for no stimulation and random electrode stimulation the PI showed the tendency to increase when the stimulation duration increased. This differs from our findings for tetanic stimulation; a possible reason for this difference is the range of their stimulation duration (1-5h), which is much larger than the range in our experiments (2-15 min). This could indicate that tetanic stimulation duration may have an influence, but our range of stimulation duration is too small to detect these changes. We also found that subsequent periods of tetanic stimulation on the same electrode yielded significantly smaller PIs. Subsequent p-TS resulted in PIs comparable to PIs corresponding to spontaneous occurring changes. Furthermore we found that most connections, affected in the first period of p-TS, were not affected again in subsequent periods of stimulation. This makes it plausible that connections were still affected by the previous stimulation and adapted to p-TS. Another possibility is that tetanic stimulation was not the cause of change in the connection strengths in the first place. This is unlikely, because of the significantly higher PI after the 1st period of p-TS, compared to spontaneous occurring changes, which indicated that tetanic stimulation had an effect on network connectivity. Therefore it's likely that changes induced by our tetanic stimulation lasted for periods > 1 hour, which support the theory that long lasting forms of plasticity such as LTP and LTD took place. In [14], [15] and [2] intracellular experiments were used and these all concluded that tetanic stimulation lead to LTP in the post-synaptic neuron.

We did not found p-TS led to a significant alteration in a mean change of connection strengths. This indicates that changes in connection strengths are balanced, some connections strengthen others weaken but on average they remain constant. Thus p-TS does not lead to a potentiation of the entire network. This was also found by Jimbo et al. [5]. They found tetanic stimulation did not lead to a global increase of neuron firing rates. Instead they found test stimulation site specific changes after stimulation; to some sites the network had an increased response to others a decreased response. Their conclusion was tetanic stimulation led to simultaneous pathway specific potentiation and depression. This is supported by our results; tetanic stimulation induces significant changes in functional connectivity, causing both strengthened and weakened connections.

Ruaro et al. also reported a significant effect of tetanic stimulation on the average firing rate of recording sites in [16]. They used a significantly higher stimulation frequency (250Hz) during a period of ~2min and compared responses of 2 different groups of stimulated electrodes. They could discriminate between these responses, for at least 1 hour. This indicates that long lasting forms of plasticity are present. This found memory for specific stimulation sites could be related to the low PI we found for subsequent stimulations, indicating an adaption of the network to the tetanic stimulation. Therefore our hypothesis is that the network adapted to p-TS. A period of 2-3 min of p-TS could be enough to trigger this adaption. This hypothesis is further supported by our observation that longer tetani did not further change network connectivity. More research has to be done to prove this hypothesis.

B. Test stimuli affected PI

For plain tetanic stimulation p-TS, we found that subsequent tetani had much less effect. For tetanic stimulation applied at different theta background stimulation phases (θ-BS) we did not observe an influence of previous stimulations. This was tested by a period of applying tetani in-phase (θ_0-TS) and anti-phase (θ_{180}-TS) with theta background stimulation (θ-BS). These periods are separated by an evaluation period existing out of test stimuli and spontaneous behavior. Also the stimulation durations varied, but similar to p-TS we found no influence of stimulation duration. This may have had several reasons; the first is that test stimuli have a significant influence on functional connectivity, masking induced changes.

Another reason could be that the effect of tetanic stimulation is limited; longer tetanic stimulation on the same electrode may not result in more changes. We tested the effect of these test stimuli and found a significant influence on connection strengths; test stimuli showed a similar PI as θ_0-TS and θ_{180}-TS, which was significantly larger than PI after no stimulation. Also subsequent stimulation showed a similar PI compared to stimulation in the previous period, which is different compared to p-TS experiments. This could be caused by θ-BS or test stimuli, notice that the duration of applying test stimuli is significantly larger then than the period of tetanic stimulation. This supports the idea that test stimuli either influences functional connectivity or influences the outcome of CFP analysis.

C. Phase dependent changes caused by θ-TS

To find changes we took a closer look at the CFP's and separated connections that weakened and strengthened. When we divided the fraction of significantly changed connections (FSCS) we found θ_{180}-TS resulted in a significantly higher fraction of connections which decreased in strength (↓) compared to connections that increased in strength (↑). Furthermore the mean change in connection strength ($\overline{\Delta}$) was significantly smaller than zero. A decrease in $\overline{\Delta}$ would indicate a global weakening of network connections. This supports the findings by Stegenga et al. [9] who found that θ_{180}-TS had a tendency to cause a decrease in post stimulus time histogram (PSTH) area. These PSTH's, are histograms of the network response to test stimuli. A decrease in PSTH area indicates a possible global weakening of network connections. They also found that θ_0-TS lead to a significant increase in PSTH areas after simulation. We found for θ_0-TS that the fraction of ↑ and ↓ were almost equal, however $\overline{\Delta}$ had a tendency to be positive, the median value was the highest of all protocols tested (see Figure 20). Unlike for θ_{180}-TS this change in $\overline{\Delta}$ was not significant; this was probably caused by 2 experiments which clearly deviated from the rest of the set (n_{total}=11). What was interesting is difference in mean and median of the fraction of ↓ found for θ_0-TS. The median was significantly lower than the mean, indicating that for most experiments the fraction of ↓ was low. This is similar to the median of $\overline{\Delta}$ which was higher than then the mean. This indicates that for most experiments θ_0-TS results in an increase of $\overline{\Delta}$ and a higher fraction of ↑ compared to the fraction of ↓. This agrees with the results of Stegenga et al.

D. θ-TS could have more effect compared to p-TS

Another reason why we did not find significant changes for θ_0-TS could be the presence of test stimuli; these could mask possibly induced changes. We measured that these test stimuli had a significant effect on connection strengths. A reason could be that these test stimuli affect our CFP analysis resulting in larger fluctuations in the connection strengths found across subsequent data blocks. This theory is supported by our results for the variation in connection strengths; this variation was significantly higher for evaluation periods with test stimuli compared to evaluation periods without test stimuli. This higher variation would probably result in less significantly changed connections, because changes have to be larger to

become significant. This could indicate θ-TS had more influence on functional connectivity than p-TS, because results for θ-TS had to exceed a larger threshold to be noted as significant.
Although the possible interference of test stimuli we found that $\bar{\Delta}$ was significantly different between tetani on different theta background phases. This may indicate that θ-BS modulates the excitability of the network, which was also concluded in [9,17,18]. Making it more likely that LTP occurs during stimulation at the peak of θ-BS and making LTD more likely to occur at the trough of θ-BS. Other research also showed that the effect of tetanic stimulation depends on additionally applied stimulation; Chiappalone et al. found in [12] that applying tetanus (20Hz) simultaneously with a low frequency (i.e. 1Hz) train of pulses resulted in an increase of average PSTH area (~40%). Statistical analysis showed this protocol (AT-IN) never resulted in a decreased evoked response and mostly resulted in an increased evoked response (60,68%). They also found that applying a train of pulses between 2 tetani resulted in varying alterations of PSTH area depending on the stimulation site used. This further proves that the effects of tetanic stimulation depend on other stimulation that is applied.

In summary, we showed that tetanic stimulation does affect functional connectivity. We showed that this effect wears off after repeated stimulation, and that prolonged tetani did not have a larger effect on connectivity. We also showed tetanic stimulation induces both strengthening and weakening of connections. These observations support the hypothesis that tetani may induce LTP or LTD within very short periods of stimulation. In all p-TS experiments the number of strengthened connections was in equilibrium with the number of weakened connections. Application of theta background stimulation changed this balance. Tetani locked at the phase of maximum background stimulation tended to increase the average strength of connections, whereas the average strength decreased when tetani were applied at the phase of minimum background stimulation.

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