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Abstract

Variable responses of neuronal networks to repeated sensory or electrical stimuli reflect the interaction of the stimulus' response with ongoing activity in the brain and its modulation by adaptive mechanisms such as cognitive context, network state or cellular excitability and synaptic transmission capability. Here, we focus on reliability, length, delays and variability of evoked responses with respect to their spatial distribution, interaction with spontaneous activity in the networks and the contribution of GABA-ergic inhibition. We identified network-intrinsic principles that underlie the formation and modulation of spontaneous activity and stimulus-response relations using state-dependent stimulation in generic neuronal networks in vitro.

The duration of spontaneously recurring network-wide bursts of spikes was best predicted by the length of the preceding interval. Length, delay and structure of responses to identical stimuli systematically depended on stimulus timing and distance to the stimulation site, which was described by a set of simple functions of spontaneous activity. In addition, the speed of propagation was determined by the overall state of the network at the moment of stimulation. Disinhibition increased the number of spikes per network burst and inter-burst interval length at unchanged gross firing rate, while the response modulation by the duration of pre-stimulus inactivity was preserved. Our data suggest a process of network depression during bursts and subsequent recovery that limit evoked responses following distinct rules. The seemingly unreliable patterns spontaneous activity and stimulus-response relations thus follow a predictable structure determined by the interdependencies of networks structure and activity states.

Keywords: response variability; ongoing activity; MEA; electrical stimulation; network dynamics; closed-loop stimulation; neurotechnology
Introduction

Electrical stimulation of nervous tissue is increasingly used in the treatment of CNS disorders, e.g. by deep brain stimulation, in neuroprosthetic devices aiding sensory perception, as well as in examining the biophysiological properties of single cells and the function of neuronal networks. While the reproducible responses of directly stimulated individual neurons consist of precisely timed single action potentials (APs) or trains of APs under specific conditions (Bryant and Segundo, 1976; Mainen and Sejnowski, 1995; but see Gal et al., 2010), stimulation in recurrent networks elicits multiphasic responses. These typically consist of: i) a fast excitatory component of precise and reliable firing by antidromic or monosynaptic activation of local neurons with delays between $2 \sim 20$ ms, ii) a transition phase with low activity thought to be mediated by inhibitory neurons and iii) a delayed excitatory component driven by recurrent polysynaptic activation (Eccles et al., 1974; Fanselow and Nicolelis, 1999; Butovas and Schwarz, 2003; Wagenaar et al., 2004; Rowland and Jaeger, 2008). More physiological sensory responses induced by, e.g., foot tapping, whisker deflection or air puffs unfold comparable dynamics in various brain regions of awake and anesthetized animals (Cody et al., 1981; Fanselow and Nicolelis, 1999; Rowland and Jaeger, 2005). Across stimulation trials, however, the variability of timing and duration of the response as well as the number and distribution of neurons involved is typically high (Azouz and Gray, 1999; Jones et al., 2007). On short timescales, temporal non-stationarities by modulation of activity and excitability tend to prevail and modify response amplitude, latency and spatial spread (Kisley and Gerstein, 1999; Petersen et al., 2003). In addition, the activity state of the neocortex at stimulus onset may dominate trial-by-trial variability (Arieli et al., 1996; Hasenstaub et al., 2007). Although these influences are known in a general sense, they have not been quantitatively assessed and the prediction of stimulation outcomes for individual stimuli given during autonomous network dynamics is unreliable. Predictable stimulus-response relations, however, become increasingly important to control adequate functionality of neurotechnological devices, which seems incompatible with trial-by-trial variability.

To identify the general rules of the interaction between ongoing activity and stimulus-response
relations, independent of a specific tissue architecture, function or sensory stimulus, we analyzed spontaneous and evoked activity dynamics in generic neuronal networks in vitro. These networks exhibit spontaneous spiking with typical patterns and long-term modulation (Wagenaar et al., 2006) that defines the network state with which different stimuli may interact. Here, we focus on reliability, length, delays and variability of evoked responses with respect to their spatial distribution, interaction with spontaneous activity in the networks and the contribution of GABA-ergic inhibition. We asked, which interactions arise between weak local electrical pulses and network state and how do they influence evoked responses. Specifically, we developed quantitative models how the state of the network at the moment of stimulation determines response length and delay. Closed-loop stimulation relative to ongoing activity significantly reduced trial-by-trial variability and enabled us to examine systematically the influence of network inhibition and short-term plasticity on stimulus-response relations.

**Materials and Methods**

*Cell culture preparation:*

Prefrontal cortical tissue obtained from newborn Wistar rats was enzymatically dissociated and cultured on polyethylene-imine (Sigma-Aldrich) coated microelectrode arrays (MEAs, Multi Channel Systems, Reutlingen, Germany). The culture medium (1 ml) consisted of MEM supplemented with 5% heat-inactivated horse serum, 0.5 mM L-glutamine and 20 mM glucose (all compounds from Gibco Invitrogen). Cultures were stored in a humidified atmosphere at 37°C and 5% CO₂ – 95% air. Medium was partially replaced twice per week. Neuronal density after the first day in vitro (DIV) ranged between 1500–4000 neurons/mm². The final density after > 21 DIV settled at 1500 – 2000 neurons/mm², independent of the initial density (unpublished observation, S. Kandler). At the time of recording network size thus amounted to ≈ 5 – 6*10⁵ neurons. Animal treatment was according to the Freiburg University’s and German guidelines on the use of animals in research.
Neuronal activity was recorded inside a dry incubator with MEAs with 59 TiN electrodes of 30 \(\mu\)m diameter and either 200 \(\mu\)m (square 8 x 8 grid, with corner electrodes omitted) or 500 \(\mu\)m spacing (rectangular 6 x 10 grid). One larger electrode served as internal reference. The primary signal was amplified (gain 1100, 1-3500 Hz) and sampled at 25 kHz/12 bit (MEA 1060-BC, Multi Channel Systems, Reutlingen, Germany). Online spike detection was done with MeaBench (Wagenaar et al., 2005, version 1.0.16) at 6 \(\sim\) 8 fold root mean square noise level for spike threshold. Stimuli were controlled with custom-written C++ applications through a separate stimulus generator (STG2004, Multi Channel Systems). A blanking circuit minimized stimulus artifacts.

**Experimental procedures**

Spontaneous activity was recorded from 22 cultures starting at 12 DIV. Stimulation experiments were conducted at the earliest at 17 DIV. We use the term "culture" to denote a cortical cell tissue preparation and "network" to denote a culture at a specific point in time. An experiment consisted of at least 0.5 hour recording of spontaneous activity, followed by stimulation for a fixed period of time or number of stimuli, and a second recording of 0.5 hour spontaneous activity. To identify electrodes for efficient stimulation, electrodes were first sorted for their rank in the network burst onset dynamics. Stimulation sites were selected from ranks 1-5, i.e. electrodes at which activity on average appeared early in a burst. This procedure identified so called "early-to fire" neurons or "major burst leader" (Eytan and Marom, 2006; Ham et al., 2008). Stimuli were delivered to a single site in almost all experiments; only when it is explicitly mentioned, stimuli were delivered to two sites simultaneously. Monophasic negative voltage pulses of 0.4 ms width and 0.5 \(\sim\) 0.7 Volt amplitude were used to minimize oxidation of TiN electrodes, which would increase their impedance. These stimuli are close to the threshold for responses. Inter stimulus intervals (IstimI) were set to 10 or 20 s to prevent network depression (Eytan et al., 2003) and to avoid interaction between stimuli. For fixed lag stimulation, we continuously monitored spike activity on a selected feedback site. Stimuli were triggered at a defined time without spike on that site (post-burst interval) with a minimal IstimI of 10 s.
Post-burst intervals were randomized in successive trials. At least 1 hour of spontaneous activity was recorded between stimulation sessions at different sites. The recorded spike data was analyzed with Matlab (The MathWorks, Natick, USA).

Disinhibition

To test the influence of inhibition on spontaneous and evoked activity dynamics, 5 µM of the GABA<sub>α</sub>-receptor antagonist picrotoxin (PTX, Biotrend, Cologne, Germany, (bath application of 50 µl stock solution with 100 µM, dissolved in DMSO), were added immediately after a 1 hour spontaneous recording and 2 – 3 hour fixed-delay stimulation period (10 networks, DIV 22-30). The same recording and stimulation protocol, in some cases with re-adjusted post-burst intervals, was run under PTX.

Data analysis

Single-site burst activity was typically defined heuristically based on three criteria. In a series of spikes recorded from one electrode: i) the inter spike interval (ISI) had to be ≤ 100 ms, ii) an interval ≤ 200 ms was allowed at the end of a burst and defined the minimal inter burst interval (IBI), iii) the minimum number of spikes in a burst was set to three. Burst length defined the duration from the first until the last spike of the burst. Further criteria had to be fulfilled for network bursts: i) at least three recording sites had to have ii) burst onsets within 100 ms and iii) only one larger onset interval ≤ 200 ms was allowed. In the following, the term IBI refers to single-site as well as network inter-burst intervals. The values covered the characteristic range of these properties during spontaneous bursting in cortical cell cultures. These default values were fine-tuned for more accurate (network) burst detection as needed. Our main results were robust with respect to variations of burst and network burst detection parameters. Varying these settings within 50% -150% of the values used changed network burst intervals between 96% -101%, the lengths between 78% -111% and the no. of spikes between 91% -103%.

Responses to electrical stimulation were detected from the post-stimulus spike time series for each site individually. Spikes were attributed to the response from the first spike after stimulation to
the first ISI exceeding a standard value of 100 ms. In addition, we included isolated spikes occurring
at an electrode at less than 1s after stimulation. Response length defined the duration from the
stimulus trigger to the last spike of the response. Response delay defined the latency between the
stimulus and the first spike of the response. Response detection was little affected by variations of the
settings. In particular, response delays were robust with respect to the specific values used for
response detection. Response reliability was defined as the fraction of stimulation trials that evoked a
response.

Quantitative models for stimulus-response relations:

With respect to spontaneous activity, the term "inactivity" here identifies periods during which we did
not detect spikes at a given recording site during fixed lstiml stimulation. With fixed lag stimulation,
"pre-stimulus inactivity" is synonymous to the post-burst interval after which the stimulus was
triggered. The F-test for goodness of fit evaluated how well the experimental data from individual
recording sites could be described with the models for response length and delay modulation. To
examine which quantitative models describe the data significantly well, we compared the standard
models, \( y(t) = A(1-e^{-\alpha t}) \) for response length and \( y(t) = Be^{-\beta t} + C \) for response delay against extended
models with more degrees of freedom. We tested whether a non-zero y-intersection in the model for
response length, \( y(t) = A(1-e^{-\alpha t}) + B \), and a biexponential model for response delay, \( y(t) = B_1e^{-\beta_1 t} + B_2e^{-\beta_2 t} + C \), significantly improve the fit to the experimental data. To assess this quantitatively, we
used the adjusted coefficient of determination \( R_{adj}^2 \) that accounts for varying number of parameters
when comparing regression models for their goodness of fit (Zar, 1999).

\[
R_{adj}^2 = 1 - \frac{SS_{resid}}{SS_{tot}} \frac{df_{resid}}{df_{tot}}, \quad \text{with}
\]

\[
SS_{resid} = \sum_i (y_i - \hat{y}_i)^2
\]

\[
SS_{tot} = \sum_i (y_i - \bar{y})^2
\]

\[
df_{resid} = N - p - 1
\]

\[
df_{tot} = N - 1
\]
Where $y_i$ is an experimental data point (i.e. response length or delay), $\hat{y}_i$ is the estimated data point from the model, $\bar{y}$ is the mean over all data points, $N$ is the number of data points and $p$ is the number of model parameters. Whereas the classical $R^2$ increases when a parameter is added to the regression model, $R^2_{\text{adj}}$ will increase only if an added parameter results in an improved fit of the regression to the data (Zar, 1999). Recording sites that fulfilled the goodness of fit criterion for the standard and the extended model were selected when comparing the models for response length and delay modulation. As a result, the extended models did not describe the data significantly better given the higher number of parameters. The mean $R^2_{\text{adj}}$ for recording sites with response length modulation decreased from $0.77 \pm 0.14$ (mean $\pm$ standard deviation (SD) for the standard model to $0.68 \pm 0.37$ for the extended model (median: $0.79$ for both cases, $40$ recording sites, $10$ experiments, $p = 2$ and $p = 3$, respectively). The same result was obtained for the extended model of response delay modulation, for which the mean $R^2_{\text{adj}}$ decreased from $0.81 \pm 0.18$ to $0.74 \pm 0.25$ (median: standard: $0.85$; extended: $0.80$; $116$ recording sites, $23$ experiments, $p = 3$ and $p = 5$).

Cross-correlation (CC) analysis related different measures of pre-stimulus inactivity to evoked response properties (Fig. 4E+F). The zero-lag CC, i.e. the cross-correlation coefficient was calculated between either pre-stimulus inactivity, weighted spike history or number of spikes in the last burst and either response length or delay. For weighted spike history, the pre-stimulus spike trains were convolved with an exponentially decaying kernel with a time constant of $1/3$ of the IBI. The value at the time of stimulation was used in the cross-correlation.

Spatiotemporal correlation between response delay and distance to stimulation site was fit with a linear regression model $y(x) = kx + m$, where $y$ is the response delay, $x$ is the distance to stimulation site and $k, m$ are fit parameters. The inverse slope of the regression model, $1/k$, estimated the speed of propagation.

Statistical significance for changes of distribution means was assessed by t-tests. One-sample Wilcoxon signed rank tests assessed the deviation from a distribution with median 0. Unless noted
otherwise, the significance level for all tests was $\alpha = 1\%$.

Results

*Spontaneous network activity*

When cultured on microelectrode arrays (MEAs), neuronal networks intrinsic spontaneous activity can be monitored non-invasively over long periods of time. During regular baseline activity, neuronal multi-unit activity recorded by MEA electrodes consisted of bursts of variable lengths and intervals, forming a pattern of recurrent activity that follows a stereotypical developmental timeline. In our recordings, the ISI distribution was multimodal for intervals $< 0.1$ s within bursts and $> 1$ s up to tens of seconds between bursts (Fig. 1B). Bursting activity was synchronized across almost all active sites (Fig. 1A). Network burst durations ranged between tens of milliseconds to several seconds. Network burst intervals were approximately lognormal distributed (Fig. 2A). This spike activity can be recorded continuously for periods up to several days under stationary conditions due to the extremely stable recording configuration.

Burst duration and subsequent IBIs reflect an interaction of synaptic depression and recovery mechanisms as well as differential adaptation of excitatory and inhibitory networks (Toib et al. 1998; Eytan et al. 2003). Since these processes modulating spontaneous activity may likewise determine the stability of stimulated responses, we quantified the relation between the intervals between network bursts and their length. We first asked if the preceding interval was predictive for network burst length or if, in turn, network burst length was predictive for the subsequent interval. We calculated the sequence of cross correlations $CC(m)$ between network burst intervals and lengths. The index variable $m$ indicates whether correlation was calculated for preceding ($m < 0$) or following interval ($m \geq 0$). Similarly, the sequence of auto-correlations $AC(m)$ identified temporal correlations between a selected feature of network bursts $n$ and $n+m$. The cross-correlation between network burst length and interval was highest for the preceding interval ($CC(-1) = 0.24$), it decreased for
the following and approached zero-correlation for the one after next interval (CC(0) = 0.13, CC(1) = 0.01; Fig. 2B). The preceding interval thus has higher predictive power for the duration of a network burst than this has for the duration of the following interval. When the intervals were randomly shuffled, the sequence of cross-correlations decays to 0 and is basically a flat line (CC(-2) = -0.0014, CC(-1) = 0.0062, CC(0) = -0.0035, CC(1) = 0.0053). In contrast, in auto-correlation analyses the length of network bursts was not correlated with the length of any of the preceding or following network bursts (Fig. 2C), indicating that there is no serial correlation in the time series of network burst length. These findings suggest that bursts terminate not after a uniform duration but at some reproducible threshold that may be defined by inhibitory activity and/or some limitation of synaptic resources. Network recovery would thus always start at approximately the same level, comparable to mechanisms described by Tabak et al. (2001). A coherent systematic pattern across networks thus emerges. In the following, we quantitatively examined the interaction between spontaneous bursting and stimulus-evoked responses and ask whether similar principles between preceding inactivity and response properties apply.

State-dependent network responses

Stimulation at one site with the same pulse every 10 or 20 s evoked spike activity at many other sites throughout the network. To avoid interaction between stimuli, we chose the stimulation intervals such that spontaneous activity resumed to pre-stimulus asynchronous regime between stimuli; shorter intervals attenuate or suppress spontaneous activity (Eytan et al., 2003). Responses at individual sites consisted of an early (≤ 25 ms post-stimulus) and late component (> 50 ms post-stimulus) or a late component only. Exact transition times between the two response components varied across different sites and networks. A striking difference between these components was the very precise and reliable firing within a narrow window for early responses and, in contrast, seemingly irreproducible firing without any apparent pattern during late responses (Fig. 3A). Recording sites with early and late responses were preferentially located close to the stimulation site, whereas exclusively late components were found at more distant sites (Fig. 3C). This supports local initiation of early
spikes and polysynaptic transfer of activity into distant parts of the network (Butovas and Schwarz, 2003). Early responses are likely a direct and fast activation of neurons close to the stimulation site or bypassing axons (Jimbo et al., 2000; Marom and Shahaf, 2002). These responses could originate from non-synaptic antidromic excitation and/or early post-synaptic spikes with a very low jitter < 2 ms (Wagenaar et al., 2004; Bonifazi et al., 2005). Late responses occurred after a transition period with low firing rates and resembled globally synchronized activity during spontaneous network bursts. The late component is sensitive to glutamate receptor blockers, supporting a polysynaptic origin (Jimbo et al., 2000). It is this component that reflects how a local stimulus will eventually invade the network and that thus should have predictable dynamics (Kermany et al., 2010). Understanding what influences the properties of the polysynaptic response is therefore crucial for a defined interaction with neuronal networks. In the following, we analyze reliability, length, delays and variability of evoked responses with respect to their spatial distribution, interaction with spontaneous activity in the networks and the contribution of GABA-ergic inhibition.

Response length was defined as the time from stimulus onset to the last spike of the detected response. The variability of the response length thus mainly reflected the duration of the late response. Sorting trials for increasing response length showed that high bursting activity immediately preceded short responses, whereas no or weak spontaneous bursting activity preceded long responses (Fig. 4A). The capability to evoke long responses drastically decreased directly after spontaneous bursts, but increased with increasing delay of the stimulus to the last burst. Further analyses revealed that the response length for individual recording sites correlated best with the duration of inactivity before the stimulus, if compared to a weighted spike history or the number of spikes in the preceding burst (Fig. 4E). We thus used this interval as a quantitative indicator of the local network state prior to stimulation. With increasing duration of network inactivity, response lengths increased exponentially and eventually saturated (Fig. 4B), described by a saturating exponential of the form $A(1-e^{-\alpha t})$, where $A$ and $\alpha$ are fit parameters, $t$ is the duration of the inactivity interval preceding the stimulus.
The dependency of response length on pre-stimulus inactivity decreased at recording sites with exclusively late responses (Fig. 4F). Instead, the delays of the responses at these sites were clearly correlated with pre-stimulus activity (Fig. 4C). Recent bursting activity resulted in large delays of the polysynaptic response to stimulation, longer phases of inactivity preceding stimuli led to short delays, indicating progressive recovery of excitability within the network. Response delays exponentially decreased with longer pre-stimulus inactivity and saturated at a low level (≈ 25 ms post stimulus, Fig. 4D). This relation followed the function $Be^{-\beta t} + C$. Overall, the time constant $\beta$ for response delay was shorter than the time constant $\alpha$ for response length (mean: $\alpha$=0.19 1/s, $\beta$=0.41 1/s; median: $\alpha$=0.15 1/s, $\beta$ =0.25 1/s; 26/91 sites, 9/16 exp. 8/13 networks, DIV 22-30/DIV 22-38 for response length respectively delay). Response length and delay were thus modulated by ongoing activity and stimulus efficacy depended on the state of the network at the moment of stimulation. To test if a defined timing of stimulation relative to spontaneous bursting can enhance response reproducibility we developed a closed-loop electrical stimulation paradigm.

**Defined interaction with ongoing activity reduces response variability**

An activity-triggered stimulation paradigm placed stimuli at predefined times relative to spontaneous activity and thus allowed us to successively evaluate the network’s state-dependent input/output relationships. The networks were stimulated with a set of fixed lags after the end of spontaneous bursts during baseline activity (Fig. 5A). Stimulation at fixed lags resulted in smaller coefficients of variation (CV) for response lengths compared to when the timing of stimulation was randomized across trials (Fig. 5B). To generalize this finding, we compared the reproducibility of responses with closed-loop fixed lag stimulation against the standard open-loop stimulation with fixed IstimI (Fig. 5C). Responses to fixed lag stimulation with 0.5, 1 and 2 s post-burst intervals plus minimal IstimI were compared to those from stimulation with fixed IstimI of 10 and 20 s. We analyzed recording sites with combined early and late responses and defined response variability as the ratio between SD of response length and spontaneous burst length. This enabled us to assess response length variability across different networks since it normalized the variability of evoked responses by the intrinsic
variability of spontaneous bursts. Response variability for stimulation with fixed $I_{stim}$ was mainly
distributed around 1, i.e. responses varied as much as the length of spontaneous bursts (Fig. 5C).

**Stimulation with fixed lags reduced response variability to a level below spontaneous burst variability**
with a peak at 0.7. We thus used this protocol in the remainder of this study to examine
the influence of inhibition on spontaneous and evoked activity dynamics.

**The role of inhibition**

Our results so far showed how bursts depress network excitability. Subsequent recovery prepares the
network for the next spontaneous burst or evoked response. Which biological mechanisms govern
the modulation of stimulus-response relations is currently unclear. Synaptic depletion during bursts
followed by replenishment and prevailing widespread inhibition after the end of bursts are two
possibly concurrent processes. In order to expose the role of the inhibitory system more clearly we
performed a set of experiments blocking inhibition with the GABA$_A$-receptor antagonist PTX. We first
asked what is the influence of inhibition on the dynamics of network bursting. Furthermore, **we tested**
whether fading inhibition after network bursts could account for the progressive decrease of
the response delay with increasing time since the last spontaneous burst. Confirming inhibitory
activity in the network, we found an average increase of network burst intervals (88%), network burst
length (163%) and number of spikes (183%; Fig. 6B) after application of PTX (10 networks, DIV 22 -
30). Interestingly, the gross firing rate (average across 0.5 h) remained unchanged. This indicates an
intrinsically-regulated balance between the overall level of activation and inactivation. The seeming
decrease in the number of recording sites with bursts under PTX was due to a subset of recording
sites that contributed with only 1 − 2 spikes per network burst and thus did not fulfill the burst criterion
anymore. Thus, disinhibition leads to a reorganization of activity towards longer and enhanced spiking
during network bursts that needed a longer time to initiate spontaneously. The following analyses
evaluate how disinhibition modulates the structure of the stimulus-response relation.

While some response properties changed significantly from control to PTX **the dependency on**
**the duration of pre-stimulus inactivity persisted** (Fig. 7A-C). Absolute delays between stimulus
and response for individual recording sites typically decreased, resulting in an average decrease by 37.5% (30.7 ± 2.4 ms (mean ± sem) and 19.2 ± 1.4 ms, control and PTX respectively; Fig. 7A). Response length increased by 185.0% (0.17 ± 0.01 s to 0.49 ± 0.02 s) and the number of spikes per response increased by 84.4% (7.8 ± 0.5 to 14.4 ± 1.0 spikes). The modulation of response delays by the duration of pre-stimulus inactivity, however, persisted under disinhibition (Fig. 7B+C).

We thus asked for the temporal dependency on response delay in the time after spontaneous bursting and disinhibition. The change \( \Delta \text{delay} = \text{delay control} - \text{delay PTX} \) estimates this influence for increasing periods of inactivity before stimulation. The most recording sites had a fixed contribution of inhibition (\( \Delta \text{delay} \approx \text{constant}, 128/238 \) sites, 54%). At all other locations the influence of disinhibition decreased with time after a burst, suggesting a decreasing influence of inhibition. Among those, one subset (Fig. 7B, group 1, 12%) started at positive differences and decayed with negative slopes. The remainder (Fig. 7C, group 2, 34%) started at negative differences and decayed with positive slopes. Interestingly, the recording sites in group 1 tended to be closer to the stimulation site than those in group 2 (Fig. 7D). These analyses suggest that the effect of inhibition on response delay for each recording site depends both on the timing of stimulation relative to the last burst and its distance to the stimulation site, which raises the question how the spatiotemporal dynamics of evoked activity is affected by disinhibition.

To this end, we quantitatively assessed changes of response delay under control and PTX with respect to the distance to stimulation site and the timing of stimulation. Linear regression of the form \( y(x) = kx + m \) was fit to the data under control and PTX for experiments with different post-burst intervals. The squared correlation coefficient \( R^2 \) estimated the correlation between response delay and distance to stimulation site and the inverse of the regression slope estimated the speed of propagation. Response delays were positively correlated with the distance to stimulation site in the majority of experiments under control conditions (Fig. 8A+B, \( p < 0.05 \) for 55/108 post-burst intervals). This effect was enhanced under disinhibition as the distribution of \( R^2 \) clearly shifted to larger values with PTX (Fig. 8B, \( p < 0.05 \) for 96/104 post-burst intervals). These data indicate a propagation of
evoked activity from the site of stimulation that is further facilitated by blocking GABA-ergic inhibition.

A possible mechanism could be a more reliable activation of post-synaptic neurons due to reduced inhibitory inputs in polysynaptic responses, e.g. because of on average more long-range projections of excitatory neurons. In support for this is that the average response reliability for the upper quintile of recording sites with largest delays to the first spike in the response increased more strongly from control to PTX (58% vs.73%) than that for all other sites with smaller delays (70% vs. 73%). The slope of the regression was extracted when the linear regression model provided a significant good fit (F-test for goodness of fit, $\alpha = 5\%$, for three experiments, $\alpha$ was set to 9%). The goodness of fit criterion was reached for 65/108 post-burst intervals under control and 85/104 with PTX. Propagation speed increased with longer post-burst intervals and was typically higher within the same network under disinhibition (Fig. 8C). Disinhibition thus increased the correlation between response delay and distance to stimulation site and pronounced the dependence of activity propagation on the timing of stimulation.

Discussion

The variability of networks responses to identical stimuli is a key issue in many neurobiological and neurotechnological paradigms and has been attributed to interactions between ongoing and evoked neuronal activity. We reduced this interaction to simple electrical stimuli applied to generic neuronal networks in vitro that were spiking spontaneously with bursts of variable lengths and IBIs. The effect and efficacy of these stimuli depended on the time-delay between previous network burst and the stimulus, the distance to the stimulation site and the efficacy of GABA-ergic inhibition. We derived analytical stimulus-response relations and find that response length modulation is best described by a saturating exponential function $y(t) = A(1-e^{-\alpha t})$. Response delay modulation followed a single exponential decay function $y(t) = Be^{-\beta t} + C$. The modulation of stimulus-response relations persisted under the blockage of inhibition, suggesting modulatory mechanisms such as synaptic depletion during the burst and subsequent replenishment in the period of inactivity (Cohen and Segal, 2011).
Our findings suggest that trial-by-trial variability in neuronal networks arises to a significant extent from state-dependent input-output relationships. Alternating periods of bursting and inactivity modulate neuronal excitability such that it is low directly after bursts and gradually recovers during inactivity.

**Mechanisms for network-state dependent response dynamics**

Single electrical stimuli induced early responses locally and a polysynaptic transfer of activity to more distant areas, showing as late responses (Fig. 3C). The length of responses with both early and late components increased with increasing delays of the stimulus to the preceding burst; concomitantly, polysynaptic delays decreased. Comparing response delays with pre-stimulus activity revealed a remarkable relation: variations of pre-stimulus inactivity in the range of seconds correlated with changes of the delay of late responses of tens of milliseconds (Fig. 4C-D). How can stimulus timing modulate response length and delay? Inhibition shortened the responses at most sites and increased response delays. Response modulation persisted, however, when the networks were disinhibited (Fig. 7B+C), suggesting that inhibition interacts with additional mechanisms modulating stimulus-response relations. We therefore propose that short-term synaptic depression by spontaneous bursts due to depletion of readily releasable neurotransmitter vesicles (STD) limits response length and delay in conjunction with GABA-ergic inhibition. STD modulates synaptic transmission depending on the temporal characteristics of presynaptic input (Tsodyks and Markram, 1997; Tsodyks et al., 1998). Sustained synaptic vesicle exocytosis during a burst depletes the readily releasable pool until firing ceases. Subsequent replenishment increases resource availability with increasing time after a burst. Placing a stimulus in the late recovery phase yields longer responses for nearby sites because more synapses have recovered. Correspondingly, delays for distant sites decrease because synaptic input to these neurons increases as more synapses recover and thus integration times shorten. Although we could not directly validate STD and the depletion of presynaptic resources, the rate of replenishment and release probability that controlled population burst duration and timing in CA3 pyramidal cells support it (Staley et al., 1998). STD has
been reported for synapses in culture (Opitz et al., 2002; Vogt et al., 2005) and the rate of replenishment is in good agreement with spontaneous burst intervals (3-16 s, Stevens and Wesseling, 1998). Synaptic vesicle depletion in hippocampal microcultures terminated spike-triggered network bursts (Cohen and Segal, 2011). Furthermore, when compared to cellular excitability modulation, a model of activity-dependent synaptic depression better replicated the dynamics of oscillatory discharges and increasing response lengths with longer duration of preceding inactivity in the chick spinal cord (Tabak et al., 2000, 2001). A close link between single-neuron threshold dynamics and magnitude of synchronized network activity has recently been demonstrated in cortical neuronal networks in vitro, too (Wallach and Marom 2012). In addition, spike-frequency adaptation in single neurons during high bursting activity could further affect stimulus-response dynamics. Giugliano et al. (2004) have previously described comparable collective dynamics for cultured cortical neurons and for leaky integrate-and-fire neurons with spike-frequency adaptation. A build-up of the adaptation during the transition from silence to bursting could mediate the termination of bursts.

Although it is largely unknown which biological mechanisms generate and modulate synchronous network-wide bursting, a variety of functional roles have been suggested (Meister et al., 1991; Lisman, 1997; Mongillo et al., 2008). What can be understood, though, is the causal relation between bursting and inactivity through the correlation between preceding or following interval and burst duration (Tabak et al., 2001). Our findings suggest that network bursts may depress synaptic transmission to a fixed low threshold (Fig. 2B) and that this in conjunction with inhibition (Fig. 6) terminates a burst. Synaptic efficacy would then recover in the following period of inactivity until spontaneous fluctuations, e.g. of the activity of small populations of neurons (Gritsun et al., 2010), trigger the next burst. Removing inhibition allowed bursts to become longer and thus deepen the synaptic depression. Consistently, the minimal periods of recovery to a level that enables the next spontaneous burst became longer, too (Fig. 6). Recovery exceeding this minimum in the period until the next burst is initiated builds up resources that extend the duration of the upcoming burst.
Disinhibition facilitates spatial-temporal propagation

Evoked responses propagate from the site of stimulation. Recording sites with exclusively late responses were more distant to the stimulation site than those with early and late responses (Fig. 3C), suggesting that early responses reflect direct stimulation or monosynaptic connections. In addition, we found response delays generally increasing with increasing distance to stimulation site (Fig. 8A). The connectivity in these cultured networks is not spatially homogeneous but, because of density and developmental effects, tends to be clustered and includes long-range connections (Kriegstein and Dichter, 1983; Soriano et al., 2008). This likely contributes to a rich set of connection motives, resulting in a broad range of response delays and directions of propagation. In addition, propagation speeds would vary locally. We found that propagation speeds largely depended on the individual network and to a smaller degree on the timing of stimulation (Fig. 8C). Our findings are in good agreement with the properties of the propagation activity upon electrical or sensory stimulation of cortical tissue in vitro and in vivo. In vivo, direct anti- or orthodromic activation of the most excitable elements, namely the axons, (Nowak and Bullier, 1998; Tehovnik et al., 2006) in the vicinity of the stimulation site is conveyed polysynaptically into distant areas (Biella et al., 2002; Butovas and Schwarz, 2003). In our study, disinhibition by blockage of GABA_A-receptors increased the correlation between response delay and distance to stimulation site and thus facilitated the propagation of excitation (Fig. 8B). The median propagation speed increased and the SD across all networks decreased. The latter was mainly due to a smaller variability of propagation speeds across different networks and therefore suggests a strong heterogeneity in the strength and spatial distribution of inhibition across different networks. For individual networks, however, we saw an increased modulation of propagation speed by the timing of stimulation. This could be explained by stronger synaptic depression after longer bursts with more spikes under PTX, followed by full recovery during periods of inactivity.

The observation that the contribution of inhibition to the modulation of the responses changed with distance to the stimulation site suggests that while local inhibition strongly shapes the properties of local responses, it appears to show with a greater effect on response reliability during polysynaptic
transmission towards distant network locations. This would be in agreement with the assumption of mainly local output of inhibitory neurons and long-range excitatory connectivity (Eytan et al. 2003). Network responses at some distance would thus decreasingly depend directly on the specific design of the stimulus, but indirectly through its shaping influence on local responses and recruitment.

**Defined interaction with ongoing activity**

Stimulation at fixed lags relative to bursts at a selected feedback site decreased response length variability below the level of variability for spontaneous burst length (Fig. 5C). Responses under fixed $I_{stim}$, and thus varying lags to preceding bursts, varied approximately as much as spontaneous bursts. These findings confirm that the state of the network at the moment of stimulation critically determines neuronal responsiveness (Arieli et al., 1996; Kisley and Gerstein, 1999; Curto et al., 2009; Hasenstaub et al., 2007). Our goal was to obtain analytical models for state-dependent stimulus-response relations and we found nonlinear interactions between the duration of pre-stimulus inactivity and response properties. Current models for sensory-evoked responses in functional cortices incorporated the dynamics of ongoing activity and a longer history of pre-stimulus activity (Hasenstaub et al., 2007; Curto et al., 2009). For example, late responses upon click-stimuli showed stronger modulation with the network state than early components (Curto et al., 2009) and increasing response spikes correlated with increasing time after termination of an UP state and transition to a DOWN state (Hasenstaub et al., 2007). The functional relation closely resembled that for our model of exponentially saturating response length.

Since the underlying principles of state-dependent neuronal responsiveness are similarly observed in neuronal networks in vivo and in vitro such observations may result from low-level network properties. This could be independent of the specific architecture of neuronal networks, except for the spatial relation of inhibitory and excitatory projections. Defined interaction based on local ongoing spiking activity may need to follow only a few rules and could become a useful approach towards improved stimulus-response predictability and efficacy in neuroprosthetic devices (Venkatraman et al., 2009; Marzullo et al., 2010) and for improved efficiency of deep brain stimulation devices in neurological
disorders (Li and Mogul, 2007; Shah et al., 2010, Rosin et al. 2011).

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Disclosure

The authors declare that no competing interests exist.

Author contribution

OW planned and performed the experiments, did the data analysis and wrote the manuscript. SO and JEM contributed to the design of the study and helped interpret the results. UE wrote the manuscript and supervised the work.
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Figure legends

Figure 1 - Spontaneous activity in cortical networks in vitro

A. Raster plot for 60 channels in a DIV 34 network for 200 s (left), and zoom in on 30 s (right, gray box in the left plot). Epochs of synchronous, network-wide bursting and inactivity are clearly distinguishable. Network burst length and interval varied considerably. B. ISI probability distribution during 1 h recording for 4 channels marked green in (A). The distribution with a peak around 0.01 s reflects intervals within bursts, the peak around 5 s intervals between bursts. inset: no. of spikes/burst histograms.

Figure 2 - Network burst interval and length statistics

A. Probability distribution for network burst intervals and lengths (27 networks, DIV 12 – 63). Logarithmic scale on both x-axes. B. The length of all network bursts was compared to all preceding \((m \leq 1)\) and following \((m \geq 0)\) intervals. Main panel: Cross-correlation coefficients for inter-burst-intervals and network burst lengths (gray: individual datasets, black: average). CC was highest between preceding interval and network burst length for 19/27 networks. This correlation was lost when the intervals were shuffled (not shown). Inset: schematic of the analysis for cross-correlation. C. Auto-correlation of network burst length. There was no significant correlation for lags \(m > 0\) (one-
sample t-test for zero mean, \( p = 0.16, 31 \) networks, DIV 22-63). Qualitatively the same was found for correlation between bursts from individual sites. The results in (B) and (C) were robust with respect to variations of burst and network burst detection parameter settings.

**Figure 3 - Bimodal stimulus-response dynamics**

A. 250 stimuli delivered at 20 s intervals, 0.5 Volt amplitude. Response raster for a single recording site. The early response had a characteristic precise and reliable spike-timing in the first 25 ms post-stimulus. bottom: Variable spike-timing in the late response. The number of spikes induced and response duration varied across trials. B. 8 x 8 MEA-grid (0.2 mm pitch) with stimulation and recording sites (0.45 mm distance). C. Distributions of linear distance between stimulation and recording site for responses with early and late component and with exclusively late component. Recording sites with early components were observed at smaller distances to the stimulation site. Average distances: early and late: 0.98 ± 0.48 mm (mean ± SD., 26 sites, 7 experiments, 5 networks); exclusively late: 2.59 ± 1.14 mm (56 sites, 9 experiments, 5 networks).

**Figure 4 - Pre-stimulus activity modulated response length and delay**

A. Raster plot for one recording site with early and late responses during 247 stimuli applied at two sites (mean distance 2.1 mm). Each dot represents a spike. Trials were aligned at time 0 and sorted for increasing response length from bottom to top. High activity preceded short responses, low or no activity preceded long responses. B. Examples from different networks, lower panel represents data from (A). Response length was a function of pre-stimulus inactivity. Each dot represents one trial. The data followed a saturating response length of the form \( A(1-e^{-\alpha t}) \). (top: \( R^2 = 0.31 \); middle: \( A = 0.56, \ \alpha = 0.36, R^2 = 0.71 \); lower: \( A = 1.18, \ \alpha = 0.26, R^2 = 0.43 \)). C. left: raster plot for one site with exclusively late responses (471 stimuli, 20 s Istim). Stimulation and recording site were 3.2 mm apart. right: Zoom in on stimulation time with trials sorted for increasing response delays. Response delays correlated inversely with the duration of pre-stimulus inactivity. D. Response delay as a function of
pre-stimulus inactivity (data from (C)). Solid line: Exponential decay function \( Be^{-\beta t} + C \) \( (R^2 = 0.30) \). In (B) and (D), only trials without spikes in a period of 250 ms before stimulation were used to avoid analysis of responses that overlap with spontaneous bursts. E+F. Cross-correlation between different measures of pre-stimulus activity and response properties. Bars represent the average CC (mean ± 95% confidence interval) over all single-site analyses. Statistical significance was tested for differences of the absolute value of the mean CC for response length in (E) and response delay in (F) (one-sample t-test, **p < 0.01). E. Recording sites with early and late component. Correlation was highest between the duration of preceding inactivity and response length (26 recording sites, 7 experiments, 5 networks). F. Recording sites with exclusively late component. Correlation was highest between the duration of preceding inactivity and response delay (56 recording sites, 9 experiments, 5 networks).

**Figure 5** - Stimulation with pre-defined timing relative to spontaneous activity increased response reproducibility

A. Exemplary case of fixed lag stimulation. Raster plot of responses for one site with early and late component during 2063 stimuli applied at 0.2 mm distance. This site was evaluated for the trigger criterion. During the experiment, the lag of the stimulus was randomly chosen from 8 values in every trial (0.5, 1, 1.5, 2, 2.5, 3, 4 or 5 s post-burst interval), but trials were sorted for stimulus lag for the plot. Groups of trials with the same stimulus timing are colored for clarity. Responses are marked red. Response length increased with increasing duration of pre-stimulus inactivity. B. CV for the length of responses in (A). X-axis refers to the groups of sorted trials and an equal number of randomized trials (shuffled 100 times). CV was significantly decreased for stimulation with fixed lag for 5/8 groups. CV for 0.5 s stimulus delay was significantly increased and response reliability was decreased; one-sided, two-sample test for equal CV (Miller, 1991), *p < 0.05, **p < 0.01) C. Histogram of response variability (SD of response length divided by the SD of spontaneous burst length) for recording sites with fixed lag and fixed IstimI stimulation. Variability was lower for fixed lag stimulation and even
...decreased below the level of spontaneous burst length variability. A few outliers > 4 for fixed Istim are clipped in the histogram. (fixed Istim: mean 2.4, median 1.3, 119 recording sites, 11 experiments, 9 networks; fixed lag: mean 0.84, median 0.74, 185 recording sites, 13 experiments, 4 networks).

Figure 6 - The influence of inhibition on spontaneous network burst dynamics
A. Network burst length and interval probability distribution for control and 5 µM PTX. Network bursts became longer but also occurred at longer intervals (10 networks, DIV 22-30). B. Relative change of various activity parameters. Each bar represents the mean ± sem of the medians from all networks in (A). Values were normalized to control condition. Network burst interval, length and number of spikes significantly increased (Wilcoxon signed rank test, *p < 0.05, **p < 0.01). Average long-term global firing rate, and thus the total number of spikes recorded, did not change significantly. The small decrease of the number of sites with bursts was due to a subset of recording sites that had only 1 or 2 spikes per burst under PTX and thus did not fulfill the burst criterion. Absolute values: interval: 17.1 ± 1.8 s and 30.3 ± 3.7 s; length: 0.41 ± 0.04 s and 0.83 ± 0.14 s; no. of spikes: 109 ± 16 and 260 ± 49; firing rate: 9.8 ± 1.6 Hz and 12.3 ± 1.4 Hz; no. of sites with bursts: 25.3 ± 2.4 and 22.6 ± 2.7.

Figure 7 - The effect of GABA\textsubscript{A}-receptor blockage on stimulus-response relations
A. Change of response characteristics from control (black) to PTX (gray). Each bar represents the mean ± sem of the medians from 160 recording sites (14 experiments, responses with at least 3 spikes, minimal reliability of 20% in both conditions). Responses changed significantly with respect to delay, length and no. of spikes (one-sided t-test for equal mean, **p < 0.01). B+C. Response delay (median ± semi inter-quartile range) vs. pre-stimulus inactivity during control and PTX for two recording sites ($R_{\text{cont}}^2 = 0.94$, $R_{\text{PTX}}^2 = 0.99$ in (B) and $R_{\text{cont}}^2 = 0.90$, $R_{\text{PTX}}^2 = 0.96$ in (C)). The differences between PTX and control ($\Delta$delay = control - PTX) are plotted as dashed lines. Negative
slopes of $\Delta$delay were found for 29/238 recording sites in 10/14 experiments (group 1; B), positive slopes for 81/238 recording sites in 12/14 experiments (group 2, C). For the remaining recording sites, the differences were flat, i.e. $\Delta$delay $\approx$ const. D. Distribution of distance to stimulation site for different subsets of recording sites. Recording sites with negative slopes for $\Delta$delay had smaller distances to the stimulation site (medians: negative slope: 1.06 mm; positive slope: 2.09 mm; constant contribution: 1.50 mm).

Figure 8 - Disinhibition strengthened the correlation between response delay and distance to stimulation site

A. Response delay plotted against distance to stimulation site under control (black) and PTX (gray) for 7 s (top) and 13 s (bottom) post-burst intervals in two different networks. Linear regression (solid lines) was used when the F-test confirmed the significance of the fit. Asterisks indicate statistical outliers to the fit. The correlation between response delay and distance to stimulation site increased with PTX. (top: $R^2_{\text{cont}} = 0.01$, $R^2_{\text{PTX}} = 0.53$; bottom: $R^2_{\text{cont}} = 0.55$, $R^2_{\text{PTX}} = 0.82$). B. Distribution of $R^2$ for 108 post-burst intervals in control and 104 under disinhibition (11 experiments on MEAs with 500 µm spacing, 9 networks). $R^2$ clearly increased with PTX. C. Propagation speed in dependence of the post-burst interval. Different markers indicate data from 6 experiments with a modulation of propagation speed in both control and PTX. Propagation speed under disinhibition was overall more strongly modulated by the post-burst interval. Mean ± SD and median propagation speed across all post-burst intervals and experiments: control 0.23 ± 0.2 mm/ms and 0.20 mm/ms PTX: 0.26 ± 0.12 mm/ms and 0.26 mm/ms.