Network Burst Activity in Hippocampal Neuronal Cultures:

The Role of Synaptic and Intrinsic Currents

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Abstract

The goal of this work is to define the contributions of intrinsic and synaptic mechanisms towards spontaneous network-wide bursting activity, observed in dissociated rat hippocampal cell cultures. This network behavior is typically characterized by short-duration bursts, separated by order of magnitude longer interburst intervals. We hypothesize that while short-timescale synaptic processes modulate spectro-temporal intraburst properties and network-wide burst propagation, much longer-timescales of intrinsic membrane properties such as persistent-sodium (Na_p) currents, govern burst onset during interburst intervals. To test this, we used synaptic receptor antagonists PTX, CNQX and CPP to selectively block GABA_A, AMPA and NMDA receptors and riluzole to selectively block Na_p channels. We systematically compared intracellular activity (recorded using patch-clamp) and network-activity (recorded using multi-electrode arrays), in eight different synaptic-connectivity conditions: GABA_A+NMDA+AMPA, NMDA+AMPA, GABA_A+AMPA, GABA_A+NMDA, AMPA, NMDA, GABA_A, and all receptors blocked. Furthermore, we used mixed-effects modeling to quantify aforementioned independent and interactive synaptic-receptor contributions towards spectro-temporal burst properties including intraburst spike-rate, burst-activity index, burst duration, power in the local field potential, network connectivity and transmission delays. We found that blocking intrinsic Na_p currents completely abolished bursting activity, demonstrating their critical role in burst onset within the network. On the other hand, blocking different combinations of synaptic receptors revealed that spectro-temporal burst properties are uniquely associated with synaptic functionality and that excitatory connectivity is necessary for presence of network-wide bursting. In addition to confirming the critical contribution of direct excitatory effects, mixed-effects
modeling also revealed distinct combined (nonlinear) contributions of excitatory and inhibitory synaptic activity to network-bursting properties.
1. Introduction

Spontaneous network-wide synchronized bursting activity appears to play an important role in several aspects of the nervous system including development (Shatz 1990; Meister et al. 1991; Yuste et al. 1992; Gu and Spitzer 1995), integration in the sensory system (Engel et al. 1992), but also in the initiation of pathological activity such as epileptic seizures (Gutnick et al. 1982; Miles and Wong 1983). Therefore, clarifying how the underlying synaptic and intrinsic neuronal properties modulate and cause this network behavior is likely to provide valuable understanding about overall network function and even macroscopic scale neurological phenomena. Despite having a relatively simpler network organization, *in-vitro* cell cultures also display similar network-wide synchronous bursting activity and are hence attractive experimental models for studying the interplay between individual neuronal activity, synaptic connectivity and network activity (Pine 1980, Eckmann et al. 2007). It has been widely reported that during development and maturation of dissociated hippocampal cell cultures as well as brain slice cultures, multiple network patterns emerge as intrinsic neuronal properties develop and synaptic connections are being made (e.g. Mody and Staley 1994; Potter and DeMarse 2001; Karpiak and Plenz 2002; Gonzalez-Sulser et al. 2012; Staley et al. 2011). These network patterns range from sparsely and randomly occurring spikes to synchronous and intense network-wide bursts, often resembling epileptiform activity (van Pelt et al. 2004; Wagenaar et al. 2006; Chiappalone et al. 2007; Fuchs et al. 2007;).

The stereotypical spontaneous activity patterns in unperturbed networks can be manipulated by changing the balance between inhibition and excitation in the network (Chen and Dzakpasu 2010), applying electrical stimulation (Wagenaar et al. 2005; Durand 2009;), neuromodulators
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(Hasselmo 1995; Whalley et al. 2005; Hammond et al. 2013), pharmacological synaptic blockers (Li et al. 2007; Niedringhaus et al. 2013; Verstraelen et al. 2014) or intrinsic membrane current blockers (van Drongelen et al. 2006; Diao et al. 2013). Such experimental manipulations provide powerful venues to unravel the diverse mechanisms and critical factors involved in the generation of physiological and pathological network behavior.

Although much is known about the synaptic effects of NMDA, AMPA and GABA<sub>A</sub> receptors on neural activity, a systematic assessment and quantification of both individual and combined-interactive effects of all these three synaptic receptors in a single preparation is, to the best of our knowledge, not available. Furthermore, while multiple studies have investigated the role of these synaptic receptors in spike train generation (Wagenaar et al. 2006; Madhavan et al. 2007; Chiappalone et al. 2007; Eisenman et al. 2015; Slomowitz et al. 2015), the relationship between the lower frequency component of the local field potential (LFP) and spike generation, under different synaptic connectivity conditions, has not been well understood. Ultimately, such detailed knowledge of the role of synaptic transmission and the low frequency components of the LFP may help us to better interpret activity recorded at a larger scale, such as the electroencephalogram (EEG).

This work helps define and quantify the specific contributions of intrinsic neuronal voltage-sensitive, excitatory and inhibitory synaptic currents in shaping synchronized network-wide burst activity in mature dissociated rat hippocampal cell cultures. Previous reports (e.g., Wagenaar et al. 2006; Chiappalone et al. 2007) and our own observations strongly suggest that spontaneously occurring network bursts are an order of magnitude shorter in duration, compared to interburst intervals. To explain these two time scales, we hypothesized that the fast dynamics of synaptic transmission play a crucial role in shaping spatiotemporal and spectral aspects within the burst as
well as its network-wide propagation; while much slower intrinsic neuronal properties, such as
the depolarizing persistent-sodium (Na_p) current, control the burst onset process during interburst
intervals. To separate these effects, we used pharmacological agents to systematically block
persistent-sodium channels, fast inhibitory (GABA_A) and excitatory (NMDA, AMPA) synaptic
receptors, and observed their effects on network bursting activity. We used mixed-effects models
to quantify how different synaptic receptor combinations contribute to network level burst
properties, power in the LFP signal, network connectivity and transmission delays. Our findings
clarify the functional importance of intrinsic membrane currents and synaptic receptors in
shaping network burst initiation, intra-burst properties and network-wide burst propagation. We
discuss how these findings may ultimately contribute to a better understanding of overall
network dynamics with special emphasis on the use of network cultures as a model for epilepsy.

2. Methods

All experimental procedures involving animals were approved by and were in
compliance with the Institutional Animal Care and Use Committee (IACUC) at The University
of Chicago.

2.1 Preparation and selection of the cultures

Embryonic rat hippocampal neurons were isolated and maintained in-vitro according to
previously published methods (Shelat et al. 2013). Briefly, hippocampi from embryonic day 18,
Sprague Dawley fetuses were dissociated into single cells with trypsin (0.25%) and mechanical
trituration. Cells were seeded onto polyethylenimine-coated multi-electrode arrays (MEAs) to
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record multi-unit network level activity and onto poly-lysine-coated coverslips to record single
cell activity. The seeded neurons on MEAs and coverslips were maintained in neurobasal
medium containing B27 supplement and GlutaMax (all from Life Technologies) in a humidified
atmosphere (5% CO2, 95% atmospheric air at 37°C). Cultures were maintained by replacing
half of the media volume with freshly made culture media, every 4-5 days. The neurobasal
medium contained (in mM): 51.7 NaCl, 26 NaHCO3, 0.91 NaH2PO4, 0.81 MgCl2, 5.33 KCl, 25
D-glucose, and 1.8 CaCl2.

Prior studies and our own experience suggest that the neuronal network activity patterns
observed in this type of hippocampal culture preparations display large and not well-understood
variability (e.g., Wagenaar et al. 2006). In order to increase reproducibility, we have taken
special steps to render the cultures included in our study as similar as possible. At the level of the
experiments, we confined our analysis to cultures that were 14-17 days in-vitro. We plated the
MEAs and coverslips at similar densities of 500-1000 cells/mm² - as judged by counting the
number of neurons immediately around the MEA and the patch clamp electrodes. Furthermore,
we restricted our study to cultures with network behavior consisting of mixed single spike
discharges and bursting patterns. In addition, for our MEA data, we selected cultures showing
spontaneous and artifact-free activity in at least 55 channels including at least 20 spontaneously
bursting channels. Channels with artifacts in any of the experimental conditions were excluded
from the analysis across all conditions. Recordings were never done within 24 hours after
feeding. All cultures in the study displayed spontaneous bursting in neurobasal medium at 37⁰C.
To avoid underestimation of the remaining variability in our data, we used a hierarchical
bootstrapping strategy that employs re-sampled data sets to approximate the distributions of
population statistics instead of the standard determination of mean and standard error of the
mean (SEM) (further details in Section 2.8). Finally, we employed a mixed-effects model that
takes into account both inter-culture and intra-culture repeated measurement variability and
included both linear and interaction terms among excitatory and inhibitory synaptic receptors
(see Section 2.8 for details).

2.2 Recordings

Multichannel extracellular recordings

Multichannel recordings were performed with multi-electrode arrays (MEAs, Fig. 1-A1)
and a MEA 2100 device (Multichannel Systems, Reutlingen, Germany). The MEAs contain 60
titanium nitride electrodes, laid out in a square grid: electrode diameter was 30 µm and inter-
electrode distance was 200 µm. Experiments were performed in a controlled environment (5%
CO₂, 95% atmospheric air, and temperature 36 - 37°C). Each recording corresponding to a given
pharmacological condition was done over a 15 min time period (Fig. 1-A2), at a sample rate of
25 kHz/channel and a bandwidth of 1 Hz – 3 kHz. All further data processing was performed
offline. All recordings from the MEA were performed in neurobasal medium.

Intracellular recordings

Standard electrophysiological recordings were obtained from the coverslips using whole-
cell current-clamp technique under the visual guidance of a Axioskop 2 plus microscope (Carl
Zeiss, Inc., Thornwood, NY, USA), connected to a Nikon CoolSnap HQ2 camera (Nikon
Corporation, Tokyo, Japan) and imaged using Nikon Imaging Software (NIS Elements AR,
Nikon Inc., USA). Patch electrode pipettes were fabricated from filamented borosilicate glass
capillaries (Warner Instruments LLC, Hamden, CT, USA) using a P-97 micropipette puller
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(Sutter Instrument Company, Novato, CA, USA). The electrodes were filled with pipette solution containing (in mM): 140 K-gluconate, 10 HEPES, 2 MgCl₂*6H₂O, 4 Na₂ATP, 1 CaCl₂*6H₂O and 10 EGTA (pH 7.3-7.4) with a resistance between 3 and 5 MΩ and all recordings were performed in extracellular artificial cerebrospinal fluid (ACSF) solution containing (in mM): 118 NaCl, 25 NaHCO₃, 1 NaH₂PO₄, 1 MgCl₂*6H₂O, 3 KCl, 30 D-glucose, and 1.5 CaCl₂. Neuronal activity was recorded using a MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA), and digitized and acquired at 25 kHz using a Digidata 1440A interface (Molecular Devices).

2.3 Experiments

MEA experiments

To investigate the role of slow intrinsic neuronal processes, we focused on the role of the voltage-sensitive persistent sodium current (Naₚ) towards network burst onset. Riluzole (20 µM) is known to selectively block voltage-gated Naₚ currents (Urbani and Belluzi 2000; Del Negro et al. 2002; Spadoni et al. 2002; van Drongelen et al. 2006). We bath-applied this drug to an independent experimental group of cultures (n=5) grown on MEAs, that displayed spontaneous network-wide bursting in neurobasal medium at 37⁰C. We recorded and quantified network bursting activity before and after adding riluzole to test the effects of the drug.

To investigate the role of synaptic activity on network-burst behavior, we recorded network activity from another independent experimental group of cultures (n=17) grown (in neurobasal medium) on MEAs. To the culture medium, we added three different selective antagonists to synaptic receptors: 1) 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, 20 µM) was used to block the α-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptors, 2)
3-(2-carboxypiperazine-4-yl) propyl-1-phosphate (CPP, 20 µM) was applied to block N-methyl-D-aspartic acid (NMDA) receptors and 3) picrotoxin (PTX, 50 µM) was applied to block γ-aminobutyric acid (GABA$_{	ext{A}}$) receptors. All three drugs were obtained from Sigma-Aldrich (St Louis, MO, USA). For a given culture, we obtained four consecutive 15 min recordings: 1) without drugs (control); 2) after first drug was applied; 3) after second drug was applied; and finally, 4) after the third drug was applied. There was a 5 min pause between the bath-application of each drug and start of the recording (Fig 1-A2). We thus used 17 different cultures to evaluate eight possible pharmacological conditions that arise out of using one, two or three drugs at a time: 1) control (without drugs), 2) PTX, 3) CPP, 4) CNQX, 5) PTX+CPP, 6) PTX+CNQX, 7) CPP+CNQX, 8) CPP+CNQX+PTX (Table 1).

Whole-cell patch clamp experiments

The cells that were cultured on coverslips and maintained in neurobasal medium at 37°C in an incubator, were transferred to a holding chamber filled with recording ACSF solution. This solution was equilibrated with carbogen (95% O2 and 5% CO2), and maintained at 36-37°C using an in-line heating element (Warner Instruments), temperature controller (TC-344B) and temperature controlled bath. The same eight drug combinations used for recording multi-unit activity in the MEA experiments, were used to record single cell activity. Neurons were identified and selected for patching based upon pyramidal morphology as visualized using DIC microscopy, detected via infrared imaging, using a 40× water-immersion objective. The resting membrane potentials of neurons were corrected for the liquid junction potential arising between the ACSF bath medium and the intracellular pipette solution. The liquid junction potential value, calculated using Clampex10.4 software (Molecular Devices) was -15 mV and this value was
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added to the observed resting membrane potential. Only neurons that appeared to have regular spiking action potential phenotypes, upon depolarizing current injection, were included (PTX (n=5), CPP (n=4), CNQX (n=5), PTX+CPP (n=4), PTX+CNQX (n=5), CPP+CNQX (n=5), CPP+PTX+CNQX (n=8)). Neurons that exhibited qualitatively identified fast-spiking behavior were excluded under the suspicion of being interneurons.

In a separate set of patch-clamp experiments (n=5), we recorded intracellular activity upon depolarizing current injection, before and after the addition of riluzole, to test that the drug only selectively blocks Na\textsubscript{p} currents and not the fast sodium currents.

2.4 Filters and spike detection

Extracellular recordings were filtered off-line by two digital filters (a Butterworth filter, first order low pass <4 Hz and a second order band pass 300 Hz - 1.5 kHz). The filters were defined in Matlab (MathWorks, Natick, MA, USA) using the butter command and signals were filtered using the filter command. The high frequency output (300 Hz - 1.5 kHz) was used to detect spikes, defined as negative deflections that exceeded five standard deviations of the filtered signal (first trace, Fig. 1-B2). The multi-unit spike trains were saved in rasters as arrays of 0’s (no spike) and 1’s (second trace, Fig. 1-B2). As the interest of this study was to evaluate the overall activity across the network, we did not attempt to sort the detected spikes. The output of the low-pass filter (<4 Hz) was evaluated as the low frequency component of the local field potential (L-LFP, also referred to as delta frequency band) (fourth trace, Fig. 1-B2).

2.5 Burst detection and characterization
An index of burst activity was obtained as described by van Drongelen et al. (2006). Briefly, the spike raster was used as input to a leaky integrator with a time constant of 50 ms, a value close to the time constant of a hippocampal pyramidal cell (Staff et al. 2000). The leaky integrator’s output which represents the integrated spike activity was used as an instantaneous index of spike activity (third trace, Fig. 1-B2). The area under the curve of the integrated signal during a burst, divided by the time interval between start and end of burst was used as a burst index.

The integrated signal was also used to quantify network bursting activity before and after the addition of riluzole, to test the effect of attenuating persistent sodium currents on interburst interval durations. Within these integrated traces, bursts and ongoing activity were clearly visible and the burst detection threshold was set at four standard deviations of the integrated spike activity amplitude to identify the individual bursts (Fig. 3-A2). We thus quantified the burst frequency before and after the application of riluzole (Fig. 3B).

2.6 Spectro-Temporal Burst Metrics

Spike rasters were used to detect bursts and to quantify correlation across channels in the MEA. Each pharmacological condition tested within a culture was recorded for 15 min (Fig. 1-A2), out of which only a 5 min period was used to detect and analyze bursts (Fig. 1-B1). To ensure stable bursting activity, the 5 min periods were selected >5 min after onset of each 15 min recording session. Finally, for every burst within the 5 min period, a 10-sec epoch surrounding the burst, was the basis of burst analysis. Our burst detection algorithm was calibrated using a baseline activity consisting of a 1-sec quiet (non-spiking) period preceding the first burst. For each 10-sec burst epoch, we determined the following metrics used in our analysis: (1) the
overall spike rate within the burst, (2) a bursting index (as described in Section 2.5), (3) burst duration, (4) interburst interval, calculated as the inverse of the number of bursts, recorded over a 5 min period (Table 2), and (5) an inter-channel correlation parameter (normalized sum of maximal correlation, detailed in Section 2.7). To represent the entire network, metrics (1)-(4) were determined using the overall (averaged) activity across all electrodes in the array. We would like to point out that while computing the interburst interval metric, although information on variability is lost by taking an inverse of number of bursts within the 5-min period, our main focus was to understand the average timescales of interburst intervals in comparison to timescales of synaptic transmission. Our goal is not to report or compare the absolute values across different synaptic connectivity conditions. In the frequency domain, the broadband signal was examined for power in the EEG bands (Fig. 1C), which added another set of seven metrics: delta (<4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-20 Hz), gamma (20-80 Hz) and high gamma (80-150 Hz). In this paper we also use the term non-delta to lump frequencies (4-150Hz) above the delta band (<4Hz). To compute the individual power spectra, we employed the multitaper approach, which improves the spectral estimate by significantly reducing both leakage and variance in the estimated power (Thomson 1982). Briefly, Thomson’s multitaper approach averages a pre-determined number of periodograms, each computed using different windows (tapers). The selected windows have two mathematical properties –1) they are orthogonal, hence the periodograms are uncorrelated and averaging them gives an estimate with a lower variance than using just one taper, 2) the tapers have low energy in the side lobes and best possible concentration in the frequency domain for a fixed signal length, thus they minimize leakage of energy across frequency bands. Our goal was to quantify and compare the spectral content of bursts across all pharmacological (i.e., synaptic connectivity) conditions. To quantify this metric,
we analyzed every instance of network-wide burst that occurred within the 5-min analysis period. Network-wide bursting is typically captured as synchronous activity across multiple MEA electrodes and spanning across not more than 10 seconds. Therefore, for every electrode that captured the burst, we computed the power spectrum in a 10-sec epoch surrounding the burst and averaged it across all such MEA electrodes. We repeated this for every instance of network-wide burst within the 5-min analysis period and averaged across all instances, to quantify the spectral content of bursts.

2.7 Cross-Correlation

Imaging and electrophysiological studies have shown that (due to low impedance of the medium surrounding the neurons) the electrodes of the MEA can only capture activity of neurons that lie in very close vicinity to the electrodes (e.g., Herzog et al. 2011). We have observed the same phenomena by patch-clamping single cells at different distances from the electrodes: only when we patch-clamped neurons in direct contact with MEA electrodes did we observe synchronized MEA and intracellular neuronal activity (data not shown). This observation therefore suggests that correlations observed in these multi-electrode array recordings must come from direct or indirect biological connection (i.e., network connectivity) and not by volume conduction. Therefore cross-correlation of spike activity between all electrode channels in the MEA, gives us an idea of the network connectivity.

We used both spike rasters and the integrated signal from the rasters to determine cross-correlation across channels in the MEA (Figs. 1-D2, 1D3). Employing the stationarity and ergodicity assumptions, commonly applied in analysis of neuronal activity, we computed the cross-correlation between signals $S_i$ and $S_j$ for a given lag $\Delta$, as the time average of $[S_i(t + \Delta) S_j]$...
(t)], where \( t \) is time (van Drongelen, 2007). We implemented this using the Matlab `xcorr` command with the `coeff` option to normalize correlation between 0-1. For an \( n \)-channel recording this generated \((n^2-n)/2\) correlograms for each burst (we excluded autocorrelations for obvious reasons). The cross-correlations across the MEA can be arranged in symmetric pairs: one cross-correlation between channel \( i \) and \( j \) and one between \( j \) and \( i \). Because of this symmetry, we computed only one, for each pair of \( i \) and \( j \). In each cross-correlogram we determined the maximum correlation and associated lag. Since the correlograms of the spike rasters and of the integrated signals showed a similar position of the maxima, we used the integrated signal correlation, which has been shown to be reliable for peak detection and for the purpose of finding the lag associated with maximal correlation. For this reason, the correlation values we report are higher than those that are typically reported for spike trains.

For all channel combinations, the maximum correlation was plotted against its associated lag, thereby generating one scatter plot per burst (Figs. 1D1-1D4). We computed these cross-correlations for all bursts within the 5-min selection period. We determined the sum of all maximal correlations across all channel combinations and normalized this using the factor \(100/((n^2-n)/2)\) to obtain our NSMC (normalized sum of maximal correlations) parameter, an estimate of the percentage of connectivity in the network (Fig. 9B). Subsequently, we estimated the channel interconnections by counting the number of strong fast correlations \((r \geq 0.5\) with absolute value of lags between 2 and 10 ms) and strong slow correlations \((r \geq 0.5\), with absolute value of lags between 20 and 150 ms). The rationale for determining these fast and slow correlated electrode pairs was that these lags may represent synaptic delays of neurons that are coupled via one or few connections. The fast 2-10 ms delays and the slow 20-250 ms delays were used to investigate putative monosynaptic delays associated with synaptic transmission via
the AMPA and NMDA receptors, respectively. Although these delays were based on data from
cortex (e.g., Destexhe et al. 1995; Myme et al. 2003), their precise lower and upper limits were
chosen arbitrarily without attempts to optimize them for the results presented in Figure 9. For
each burst, a short and a long delay graph was constructed in which the channels were
represented by the nodes and the channel interconnections by the edges (Fig. 9A). The
connectivity in each of these graphs was quantified by the mean number of edges per node. For
each burst, the ratios between the mean numbers of short- and long-delay connections as well as
the inverse ratios were used for further analysis (Fig. 9C).

2.8 Statistical analysis

In our MEA experiments, we recorded multi-unit activity and analyzed bursts during
eight combinations of three drugs from 17 cultures. In total, we recorded 514 bursts from all 17
cultures (Table 1). We used SPSS (IBM, Armonk, NY, USA), Matlab (MathWorks, Natick, MA,
USA) and R (RDC Team, 2012) to compute basic statistics, perform correlation analysis and to
fit linear regression and mixed-effects models.

Hierarchical bootstrapping

Our goal is to estimate the spectral (power in 0-150 Hz frequencies) and temporal burst
parameters (intraburst spike rate, burst duration and burst index (Fig. 6)) across different
pharmacological (i.e., synaptic connectivity) conditions. This estimation is based on analysis of
individual burst measurements (enclosed within 10-sec epochs) that are nested within multiple
cultures (for e.g., we had n=17 cultures, for the control condition, see Table 1). However, each
culture has different baseline activity to start with, by virtue of inherent network properties.
Therefore, while burst measurements from within a single culture can be considered independent if sufficiently separated, they are clustered within different cultures, each with possibly different stochastic properties. Therefore all burst measurements put together, from across multiple cultures, cannot be considered independent (values from the same culture will be related to each other and therefore display correlations when statistical properties are computed). Thus a direct assessment of variability, e.g., by simply calculating the standard error of the mean (SEM) of a burst measurement, from all cultures, would most likely misrepresent the variability in the sampling distribution of the statistic. Moreover, if we were to ignore the intraculture grouping of the burst measurements, the distribution of these measurements across all groups would most likely be skewed (non-normal), the variances will be unequal and the mean will no longer be representative of the true population (which appears to be closer to multimodal than normal). We confirmed this using standard tests, Shapiro-Wilk and Kolmogorov-Smirnov tests, both of which had extremely significant p-values, <.001, implying a non-normal sample (these are negative tests of normality, thus significance implies lack of normality). Since our sample data consisting of burst measurements across multiple cultures, are not independent and their distribution is not normal we used a non-parametric method called hierarchical bootstrapping (Efron and Tibshirani, 1994; Gallas et al. 2009) to create an empirical sampling distribution, based on which the burst statistic can be estimated. Briefly, the bootstrapping method involves sampling with replacement from the sample data (essentially treating it as the population) to produce random samples. Each of these random samples provides an estimate of the burst statistic of interest. Next, we repeat the sampling a large number of times, which provides information on the variability of the estimator.
Since our data are present within a 2-level nested structure – level 1 comprising of cultures and level 2 comprising of burst measurements under each culture, we first sampled with replacement from cultures at level 1 and then for each culture picked, we sampled burst measurements with replacement, at level 2. We repeated this 5000 times which generated the sampling distribution of the burst metric of interest. This distribution was used to estimate the mean and SEMs for a given burst metric. This procedure was used for all spectral and temporal burst metrics (e.g., Figs. 6, 9) in order to investigate how they are affected by synaptic connectivity. Our overall interest was to understanding the general tendencies of network behavior resulting from different combinations of functional excitatory and inhibitory receptors. We therefore report the p-values only when the difference in drug-response is statistically significant compared to the control condition i.e., when no drug is added (all receptors are functional). In these evaluations we used Bonferroni family-wise p-value cutoff at 0.01 = 0.05/5 (for each burst metric, we compared only the five conditions that showed bursting activity out of a total of eight drug conditions. The other three conditions were devoid of any bursting activity (Fig. 2), and are therefore not included in the tests).

**Mixed-effects models**

We utilized mixed-effects models to understand which predictors (functional synaptic receptor status) likely play a significant role in network activity, and how the receptor activities collectively underlie a given metric of network activity within a highly variable nested sampling scheme. These models formally express the relationship between functional receptors and burst parameters under each drug condition, while taking into account both the inter-culture and intra-culture variability of the burst measurements. We employed the model to fit 11 burst parameters:
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spike rate, burst index, burst duration, inter-channel correlations (Normalized Sum of Maximal Correlations, Section 2.7) and power in delta, non-delta, theta, alpha, beta, gamma and high-gamma frequency bands. The fixed covariates in the models are indicators of whether each receptor type was functional (coded as 1) or blocked (coded as 0). For example, in the case involving the addition of PTX alone, the functional receptors are NMDA and AMPA and the blocked receptor is GABA_A; therefore the covariates NMDA and AMPA would both be coded as 1, while GABA_A would be 0. Table 2 summarizes functional and blocked receptors across drug conditions.

More formally, the mixed-effects model was fit using the R package nlme (version 3.1-117), based on the following data model:

$$Y_{ij} = \beta_0 + (\beta_1 GABA_A + \beta_2 NMDA + \beta_3 AMPA) + (\beta_4 GABA_A NMDA + \beta_5 GABA_A AMPA + \beta_6 NMDA AMPA) + (\beta_7 GABA_A NMDA AMPA) + b_j + e_{ij}$$

where $Y_{ij}$ is the burst measurement of burst $i$ in culture $j$; NMDA, AMPA, GABA_A are the indicator variables for activity of the corresponding receptors; $\beta$s represent the coefficients; $b_j$ is the random effect for culture $j$; and $e_{ij}$ is random error. $b_j$ and $e_{ij}$ are each assumed to be normally distributed with mean 0 and are assumed to be independent of one another. Culture and random measurement error were modeled as nested random factors as was done in the bootstrap model. This is because we considered our 17 cultures as a random sample from a potentially large number of diverse network cultures. Likewise, since we randomly sampled our 5-minute period containing the burst measurements from a potentially large period of activity, the burst measurement was also considered a random effect. Given the nested nature of the model,
normality is much less of an issue here. We used the likelihood-ratio test as implemented by the
R function, \textit{anova}, to compare the following three models: (1) a model with first order terms
\((\beta_1*GABA_A + \beta_2*NMDA + \beta_3*AMPA)\) (2) an extension also including second order terms
\((\beta_4*GABA_A*NMDA + \beta_5*GABA_A*AMPA + \beta_6*NMDA*AMPA)\), and (3) a full model with first,
second, and third order terms (i.e., the terms in the full model shown above). Here, we report
results obtained with the third type of model since we found that its model fit was consistently
better. Overall, the likelihood ratio was significant, and the AIC (Akaike Information Criterion)
and BIC (Bayesian Information Criterion) both decreased for all metrics that were modeled, as
more terms were added. The one exception was the power in the alpha frequency band (8-12
Hz), where, after a large drop between the first and second model, the third produced a small
increase in both AIC and BIC values. We examined combinations of random coefficient models
as well, which produced a reduced variance in the residuals, but did not affect the outcome of the
analysis. In our evaluations we used Bonferroni family-wise p-value cutoff at 0.0006 = 0.05/88
(we tested 11 outcome variables and each complex model has eight terms that arise from first,
second and third order combinations of the three receptors: AMPA, NMDA and GABA_A).

3. Results

We examined the contributions of intrinsic neuronal properties and synaptic interactions
to the spontaneous rhythmic network-wide bursting behavior, observed in mature dissociated
hippocampal neuronal cultures aged between 14-17 days \textit{in-vitro}. The network behavior
typically consist of episodes of short duration (<2.5 sec) intense bursting activity, separated by
much longer interburst intervals (25-83 sec), including occasional spikes. We hypothesized that
slow intrinsic membrane currents, particularly from depolarizing persistent sodium channels charge the neurons, determine burst onset and interburst intervals, whereas the much faster dynamics of synaptic transmissions drive the intraburst properties and network-wide burst-propagation at faster timescales. In the following, we describe how we investigated these hypotheses by pharmacologically disabling the persistent-sodium current and combinations of inhibitory (GABA_A) and excitatory (NMDA, AMPA) synaptic receptors.

Intrinsic current is required for burst onset

In the presence of excitatory synaptic connectivity (a necessary condition for the presence of network-wide bursting activity (Fig. 2)), it was striking to observe that interburst intervals (25-83 sec, Table 2) were always at least an order of magnitude larger than burst durations (<2.5 sec, Fig. 6D). Given that the average interburst interval durations were quite similar (about half a minute) across most of the synaptic transmission conditions, we hypothesized that slow intrinsic neuronal processes, such as those governed by the persistent sodium current (Na_p), depolarize the neuronal membrane during the interburst interval, and cause a subset of the neurons to trigger burst onset. We found that inhibiting persistent sodium currents using riluzole (20 µM) abolished bursting behavior after application of the drug (Fig. 3A), confirming previously reported findings in cortical networks (van Drongelen et al. 2006). Although this result does not exclude the role of other intrinsic currents (e.g., voltage gated calcium currents), it supports the hypothesis that slow intrinsic currents from depolarizing persistent sodium channels, play a decisive role in mechanisms leading to burst onset. Furthermore, using intracellular recordings, we found that while spontaneous neuronal burst activity was abolished on addition of riluzole, spike activity
could be re-evoked on depolarizing current injection (Fig. 3C), showing that that riluzole at this concentration does not significantly block fast sodium currents.

Excitatory synaptic connectivity is required for network-wide burst propagation

We found that without the presence of excitatory synaptic functionality, there was no burst propagation across the network (Fig. 2, Table 2). The only exception to this statement is condition GABA_A+NMDA, where even though excitatory connectivity from NMDA receptors is functional, there is no bursting triggered in the network. Interestingly, during network-wide bursting, the spatial structure of bursts, reflecting the sequence of electrode channels leading burst onset, varied considerably across bursts. Figure 4A depicts four MEA channels with four successive synchronized burst sequences recorded under the control condition. Figure 4B shows the details of these burst sequences during the first few seconds of burst onset. In this typical example, it can be seen that spike sequences vary across the channels from one burst to the next. In spite of this stochastic property, we found that a number of electrode channels were involved in burst onset more frequently as compared to others, suggesting that these might represent primary hubs triggering network-wide burst onset (data not shown).

Intra-cellular recordings show paroxysmal depolarization shifts in disinhibited networks

Figure 5, panels A1-A5, depict intracellular recordings across the different synaptic connectivity conditions that exhibited bursting activity: GABA_A+NMDA+AMPA, NMDA+AMPA, GABA_A+AMPA, AMPA, NMDA. Since no burst activity was observed in the remaining three conditions (GABA_A+NMDA, GABA_A, none of the three receptors functional), they are not included in the figure. Intracellular recordings of spontaneous activity in the control
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condition, GABA$_A$+NMDA+AMPA, show regular bursting behavior (Fig. 5-A1) which was also observed in the MEA recordings (Fig. 5-B1). During disinhibited activity in condition: NMDA+AMPA, an interesting observation that was not apparent in the extracellular multi-electrode array recordings (Fig. 5-B2) was seen in intracellular recordings (Fig. 5-A2). Absence of inhibitory synaptic receptors leads to paroxysmal depolarization shifts (PDS) approximately 30mV above resting potential, accompanied by a reduction in spike amplitudes (Matsumoto and Marsan, 1964a, b). Disabling NMDA receptors alone strongly reduces burst activity (Fig. 5-A3). In a purely AMPA driven network, intracellular recordings display short burst durations with spikes in very quick succession (Fig. 5-A4) while a purely NMDA receptor-driven network exhibits longer burst durations (Fig. 5-A5). Overall, we observed that intracellular activity patterns (Fig. 5, panels A1-A5) match the extracellular spiking patterns (Fig. 5, panels B1-B5) across the different synaptic connectivity conditions.

Synaptic transmission determines spectro-temporal properties of individual bursts

We analyzed individual bursts from extracellular MEA recordings and found that different synaptic connectivity conditions are associated with unique spectro-temporal properties. Fig. 5, panels B1-B5, show representative spike-filtered signals from individual 10-sec burst epochs, from a single channel in the MEA. We found that networks driven by AMPA or NMDA receptors only, displayed fast-brief and slow-prolonged bursting (Figs. 5-B4, B5) respectively. When GABA$_A$ receptors were also functional, bursting activity was either reduced (GABA$_A$+AMPA, Fig. 5-B3) or abolished (GABA$_A$+NMDA, not shown). Disinhibited networks with functional NMDA+AMPA receptors showed prolonged bursts that started with intense firing followed by a tail of smaller bursts (Fig. 5-B2).
Fig. 5, panels C1-C5, show the low frequency local field potential (L-LFP, also referred to as delta frequency: < 4 Hz), averaged across all MEA electrodes, corresponding to the 10-sec burst epoch shown in the row above. The L-LFP signal is of lower amplitude in conditions with GABA\textsubscript{A}+NMDA+AMPA (Fig. 5-C1) and GABA\textsubscript{A}+AMPA (Fig. 5-C3) as compared to the other conditions.

The bottom plots in Fig. 5 (panels D1-D5) show the frequency characteristic of the MEA activity, i.e. the power spectrum within the same 10-sec burst epoch indicated above, averaged across all electrodes. The grey area in the spectral plots highlights the power in the delta frequency band. In the presence of GABA\textsubscript{A}+NMDA+AMPA receptors, it is remarkable that the averaged time domain L-LFP did not show high amplitude fluctuations (Fig. 5-C1) while the averaged power spectral plot shows a large delta rhythm component (Fig. 5-D1). Since phase differences present in the temporal domain are ignored in the average of power spectra, the observation that the time domain amplitude of delta rhythm in the overall averaged activity was relatively low, indicates a low level of synchrony in the delta rhythms across channels. This overt low L-LFP synchrony distinguished the control condition (GABA\textsubscript{A}+NMDA+AMPA) from all others where at least one synaptic receptor was disabled. For example, in condition GABA\textsubscript{A}+AMPA, both the L-LFP (Fig. 5-C3) and the delta component in the spectral average (Fig. 5-D3) were weak. Interestingly, in the disinhibited conditions NMDA+AMPA, AMPA and NMDA, we observed high amplitude fluctuations in the L-LFP as well as strong delta component in the spectral plots, indicating a high level of delta rhythm synchrony across the array.

A summary of spectral burst properties across the synaptic connectivity conditions is depicted in Figs. 6A1-A3. Disinhibited networks have been implicated in the generation of
epileptiform activity, with the EEG power spectrum (0-150 Hz) characterized by high power in
delta (<4Hz), theta (4-8Hz), and high gamma (80-150Hz) frequency bands. We therefore
quantified the power of the LFP in the 0-150 Hz frequency range across the different
pharmacological conditions and were particularly interested in disinhibited conditions where
GABA_A receptors were blocked. Figure 6-A1 depicts the power in the delta and the higher (non-
delta) frequencies across all synaptic transmission conditions. The non-delta component included
theta (4-8Hz), alpha (8-12Hz), beta (12-20Hz), gamma (20-80Hz), and high-gamma (80-150Hz)
frequency bands. The amount of delta significantly dominated the LFP spectral composition
when GABA_A receptors were disabled in conditions NMDA+AMPA and NMDA as compared to
control condition, GABA_A+NMDA+AMPA (Fig. 6-A2). The theta power significantly increased
across all conditions with disabled GABA_A receptors (NMDA+AMPA, AMPA, NMDA) as
compared to control condition (Fig. 6-A3). Higher (non-delta) frequencies dominate the spectral
composition of the LFP in conditions NMDA+AMPA+GABA_A and GABA_A+AMPA (Fig. 6-
A1).

Comparison of temporal burst properties in terms of intra-burst spike rates, burst index
and burst duration (Fig. 6B, C, D) also revealed significant differences under different
combinations of the three receptors, NMDA, AMPA and GABA_A: a) Disinhibited networks
(NMDA+AMPA) showed a significant increase in both intra-burst spike rate and burst duration,
as compared to the control condition; b) NMDA-blocked networks in the presence of inhibition
(GABA_A+AMPA, GABA_A) showed a significant decrease in activity metrics or no activity at all;
c) AMPA-blocked networks in the presence of inhibition (GABA_A+NMDA, GABA_A) are
completely devoid of bursting activity. On the other hand, purely AMPA-driven networks
showed the highest intra-burst spike rate and burst index.
Mixed-effects modeling reveals critical role of linear and non-linear synaptic interactions in shaping spectro-temporal burst parameters

The data depicted in Figure 6 quantifies the overall synaptic effects on the different spectro-temporal burst parameters but it does not show how individual synaptic effects may interrelate. Therefore, we employed mixed-effects models to quantify linear as well as interaction effects of GABA_A, NMDA and AMPA receptors (fixed factors) on the time- and frequency-domain bursting and network activity metrics during network-wide burst propagation. The complete mixed-effects model included linear terms for each factor (i.e., functional receptor), an intercept (i.e. the level spontaneous activity in the absence of synaptic function), as well as the nonlinear 2nd and 3rd order cross-terms of these factors (i.e. interactions between receptor function):

\[ Y = \beta_0 + (\beta_1 \cdot GABA_A + \beta_2 \cdot NMDA + \beta_3 \cdot AMPA) + (\beta_4 \cdot GABA_A \cdot AMPA + \beta_5 \cdot GABA_A \cdot NMDA + \beta_6 \cdot NMDA \cdot AMPA) + (\beta_7 \cdot GABA_A \cdot NMDA \cdot AMPA). \]

Here, \( Y \) is the burst property to be fitted, while GABA_A, NMDA and AMPA are the variables indicating functional absence or presence of the corresponding synaptic receptors (0 or 1). The \( \beta \)s represent the model’s coefficients. The model was used to fit 11 burst measurements: spike rate, burst index, burst duration, inter-channel correlations, delta (<4 Hz), theta (4-8 Hz), alpha (8-12 Hz), beta (12-20 Hz), gamma (20-80 Hz), high gamma (80-150 Hz) and non-delta (0-150 Hz). In Table 3 and Figure 7, the values for coefficients (along x-axis) as well as their p-values are shown. Since the outcome for the frequencies higher than 4 Hz was similar, we lumped these
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into a non-delta band (4-150 Hz) and report results for six parameters: spike rate, burst index, burst duration, normalized sum of maximal correlations, delta and non-delta.

The model demonstrated that the linear effects of functional NMDA and AMPA receptors were significant and contributed towards increasing the modeled network metric. The interaction term between NMDA and AMPA was significant in most cases, except for burst duration. One of the salient results of this modeling is that the overall AIC (Akaike Information Criterion) and BIC (Bayesian Information Criterion) were best for this complete model, for all quantified burst and frequency parameters, when compared to first and second order models (see Methods, Section 2.8). This indicates that in addition to significant linear effects, the second and third order (nonlinear) interactions between the receptors play a critical role in explaining network burst properties. The only exception was the model for power in the alpha frequency band (8-12 Hz), where, after a large drop between the first and second model, the third produced a small increase in both AIC and BIC values. Since bursting was only observed when synaptic excitation was present, as expected, the model’s intercept and the linear effect of GABA_A (quantifying the effect of synaptic inhibition alone) did not significantly contribute in any case. However, the significant effect of GABA_A was apparent in its 2nd and 3rd order interaction terms with the excitatory receptors (NMDA, AMPA). Finally, the third order interaction term between all three receptors is a significant contributor towards all the quantified network parameters, with the exception of burst duration.

Unique correlations between LFP and spike and burst parameters depending on synaptic transmission
Since the lower frequency components strongly relate to overall membrane potential fluctuations, it is reasonable to assume they will be correlated with spike and burst generation. As shown in Fig. 8, we indeed found significant correlations between LFP rhythms and spike rate and burst index. However, the frequency band of the LFP that correlated most with spike and burst properties depended on the synaptic connectivity condition. In cases where the NMDA receptors were functional and the GABA_\text{A} receptors disabled (NMDA+AMPA, NMDA), the correlations were significantly determined by the higher frequencies in the LFP (non-Delta). In contrast, network activity governed purely by AMPA receptors showed significant correlations with the delta component of the LFP.

**Unique network connectivity and transmission delays depending on synaptic transmission**

To quantify functional connectivity in the network we computed cross correlations of spike trains across all the electrodes in the MEA (Fig. 1D) and summarized this information using connectivity graphs and histograms (Fig. 9). The connectivity graphs in Fig. 9A depict two representative examples of intra-burst, inter-channel correlations with short (2-10ms) and long (20-150ms) delays in networks purely driven by AMPA or NMDA receptors, respectively. In this example it can be seen that the fast AMPA receptor driven network is dominated by short delay inter-channel correlations, while slower NMDA receptor driven network is dominated by longer inter-channel transmission delays. We show these two examples of AMPA and NMDA driven networks to illustrate the connectivity graphs because they represent two extreme cases of fast and slow network delays. However, although qualitatively informative, the connectivity graphs are difficult to interpret because they do not provide quantitative information. Therefore, we present this information in the form of the histograms shown in Figs. 9B and 9C. Fig. 9B
shows a comparison of the overall correlation across all synaptic connectivity conditions in terms of weighted sum of maximum correlation values (see methods section 2.7). It shows that, with exception of condition GABA\textsubscript{A}+NMDA, presence of excitatory connections (AMPA and NMDA receptors) is associated with overall strong correlations across the network’s activities. Fig. 9C depicts how the timing of the maximal cross-correlation relates to condition, i.e. synaptic function. For clarity we present the result as a ratio between the slow and fast components; the grey bars represent the mean ratio between maximum correlations with slow and fast delays and the black bars represent the mean ratio between fast and slow delays. Note that the result for conditions for AMPA only (Fig. 9C, condition 5) and NMDA only (Fig. 9C, condition 6) quantify the connectivity shown in Fig. 9A. Interestingly, we also found that slow correlations are dominant in conditions GABA\textsubscript{A}+NMDA+AMPA and GABA\textsubscript{A}+AMPA, while fast correlations are more important in conditions NMDA+AMPA, suggesting that inhibition slows down burst propagation.

4. Discussion

Here, we used mature dissociated hippocampal neuronal cultures to investigate the role of intrinsic neuronal properties and synaptic receptors in shaping network-wide bursting behavior. This network-wide pattern was characterized by brief burst activity of the order of a second (Fig. 6D), separated by much longer interburst intervals of the order of tens of seconds (Table 2). We used riluzole to block the persistent sodium (Na\textsubscript{p}) current, an important intrinsic voltage-sensitive slow-depolarizing component, and synaptic antagonists CPP, CNQX and PTX to selectivity block glutamatergic (NMDA, AMPA) and gaba-ergic (GABA\textsubscript{A}) synaptic receptors.
respectively. We found that the slow intrinsic membrane dynamics of persistent-sodium (Naₚ) currents are critically important for the burst onset (Fig. 3), while shorter-timescale synaptic processes modulate spectro-temporal intraburst properties, functional network connectivity and transmission delays within the network (e.g. Figs. 6, 7, 9). Furthermore, excitatory synaptic connectivity is a necessary condition for network-wide burst propagation (Fig. 2). Statistical analysis using mixed-effects models revealed that the linear effects of NMDA and AMPA were significant contributors towards the quantified network burst metrics (Fig. 7). Moreover, the model revealed that nonlinear contributions (cross-terms) of the receptors were capable of explaining most of the network properties. Since the GABA_A receptor mediates inhibitory effects, it isn’t surprising that it only has an effect in the cross terms, i.e., when excitation is present.

Because the timescale of synaptic receptor dynamics is fast as compared to the timescale observed for interburst intervals (25.6-83s, Table 2), we hypothesized that intrinsic slow-depolarizing membrane currents are likely candidates for causing burst onset at the end of a long interburst epoch. Although this burst onset process depends on the overall level of excitation that is also affected by synaptic function (e.g. AMPA driven networks have interburst intervals of ~30 sec, while NMDA driven networks have interburst durations of ~80 sec), synaptic function can only transmit activity that is present. Therefore, the slow driving current responsible for burst onset would have to be intrinsic. We specifically investigated one such candidate, the persistent sodium current and indeed found that disabling this current in the presence of functional synaptic receptors, destroys burst activity across the network (Fig. 3). Although this finding indicates the critical role of Naₚ in burst onset, it does not exclude contributions from other intrinsic currents including the voltage-sensitive component of the NMDA receptor.
Next, we found that network-wide burst propagation takes place only in the presence of excitatory synaptic connectivity (Fig. 2, Table 2). One exception to this rule is the condition $\text{GABA}_A+\text{NMDA}$, where no bursting is found in spite of the presence of excitatory functionality from NMDA receptors. This could be related to the presence of the magnesium block on the receptor due to insufficient functional depolarizing currents. Consequently, this restricted dynamics of NMDA receptor combined with the inhibitory effects from $\text{GABA}_A$ could be responsible for complete shutdown of burst propagation in this case.

The observations that both excitatory synaptic connectivity and intrinsic currents are necessary conditions for synchronized network-wide bursting, has lead us to posit the following three step process. First, during the interburst interval, slow depolarizing intrinsic currents charge the neuronal population, and eventually a first well-connected neuron or a small well-connected subnetwork reaches the threshold for spike generation and starts firing. The area of initial activation can vary across subsequent bursts (Fig. 4), similar to burst initiation reported in slice preparations of respiratory neurons (Carroll and Ramirez 2013). Second, the initial activation provides a source of activity that is then propagated across the network, resulting in the onset of a network-wide burst. Unsurprisingly, the excitatory receptors, AMPA and NMDA, play an important role in this function (Fig. 7). Third, a combination of mechanisms causes burst cessation. Once a burst is evoked, its intensity is reduced by inhibitory synapses (columns 3 and 4, Fig. 7). However, termination of bursts even in disinhibited networks (Fig. 2, panel 2) demonstrated that synaptic inhibition is not a requirement for burst offset. The mechanisms underlying burst offset cannot be inferred from our experiments. Likely candidates for this part of the burst process are cation-sensitive and/or voltage-sensitive hyperpolarizing currents. At
burst termination, all cells are more or less synchronized by activity offset. Then the slow
depolarization/recharging of the neurons restarts, and the whole three step process repeats itself.
The ongoing spontaneous network activity that is characterized by alternating periods of
long interburst intervals and shorter network-wide bursting activity, shows highly correlated
firing across all electrodes in the MEA during the network bursts. During such synchronous
network activation, different synaptic receptors are associated with different transmission delays.
These delays were estimated from the cross-correlation functions of firing activity at all
electrodes within the MEA. Since the MEA electrodes capture a very local sample of neuronal
activity, correlations between electrodes must come from direct or indirect biological
connections i.e., from the network and not volume conduction (see Methods section 2.7). Our
principal focus is on the examination of both strength and delays governing synaptic
transmission effects during burst activity. Since we have conditions with slow and fast excitatory
receptors, as well as conditions with enabled and disabled inhibition, all of which may affect
burst propagation, we investigated correlation delays in our data set. Figure 9A depicts the two
simplest scenarios: networks purely driven by AMPA or NMDA receptors. Here, we tested a
simple hypothesis that a significant part of the correlations represented ionotropic monosynaptic
connections. Given that synaptic transmission delays via AMPA and NMDA receptors occur in
the ranges of 2-10 ms and 20-250 ms respectively (Destexhe et al. 1995; Myme et al. 2003), we
investigated these specific time delays under different synaptic connectivity conditions. We
found indeed that short-delay correlation (2–10 ms) dominate in purely AMPA driven networks
while long-delay correlations (20-150 ms) were more prominent in networks with purely
functional NMDA receptors (Fig. 9A). Although this finding supports our hypothesis, the
examples in Figure 9A show that there were clearly exceptions: i.e. we also found long-delay
correlations in the AMPA case and short-delay ones in the NMDA driven network. Multiple explanations for these exceptions can be proposed. In any condition, a fraction of the inter-channel correlations can be thought of as random occurrences. Long-delay correlations in a condition with only AMPA receptors, can be explained by polysynaptic connections or metabotropic glutamate connections. Several network motifs (e.g. divergence) can explain short-delay correlations between channels connected to a common source, whether synaptic transmission is slow or fast. Last but not least, gap junctions, known to exist in mature cultures (Hales et al. 2012), may play a role in creating short-delay correlations across the network. In our case, however, a principal role for the gap junctions is somewhat unlikely since bursting and inter-channel correlation vanished if glutamate transmission was disabled. The fast-slow dominance of connectivity was quantified across all conditions (Fig. 9C), and it is remarkable that the only scenarios dominated by fast correlations are the above mentioned AMPA driven networks but also the disinhibited case NMDA + AMPA.

We found that blocking the GABA_A receptors modified spontaneous bursts in terms of increased spike rates and prolonged burst duration (Fig. 6B, D). It has been reported previously that in much older cultures (10 weeks in-vitro) than we employed, blocking these receptors resulted in a transition of synchronized bursting into a random spiking pattern (Li et al. 2007). In contrast, Chen and Dzakpasu (2010) found that an increased proportion of inhibitory neurons in hippocampal cell cultures caused spikes to cluster into burst events. Although the findings described above appear contradictory, part of this contradiction may be due to the different age and type of cultured network and perhaps to different equilibrium potentials of Cl⁻ across the different experiments (Fritschy 2008). Furthermore, our finding that blocking inhibitory activity using GABA antagonists induces epileptiform activity is in agreement with previous reports on
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both in-vivo and in-vitro hippocampal networks (e.g. Wong et al. 1985; Traub and Miles 1991; Wells et al. 2000; Colombi et al. 2013). We observed intense and often prolonged burst activity with an initial high intensity activity followed by a long tail of seizure-like regular bursts (Figs. 5-B2 and 6D). There is a high level of excitation, which was evident from increased spike activity (Fig. 6B) and a strong presence of low frequencies (<4Hz) in the LFP (Fig. 6-A2). These low frequencies were fairly synchronous as indicated by the relatively high amplitude of the MEA-wide averaged L-LFP signal (Fig. 5-C2). Furthermore, in this disinhibited condition, the spike and burst activities were strongly correlated with the higher LFP frequencies rather than the delta component in the LFP (Fig. 8). This is an interesting finding, especially since high-gamma has been implicated as a signature feature of seizure activity (Ochi et al. 2007; Bragin et al. 2010; Weiss et al. 2013). Our intracellular recordings showed that the disinhibited condition NMDA+AMPA, was associated with neural saturation, i.e. the paroxysmal depolarization (Fig. 5-A2). In this context, it is very interesting to note that in the mixed-effects model, while the coefficients for the linear terms of NMDA and AMPA are positive, the coefficient for the interaction term NMDA+AMPA is negative (Fig. 7, row 2). These intracellular and modeling results are in agreement: each individual receptor has a positive contribution towards network activation, but the two together create a saturation effect causing their interaction term to become negative.

It has been reported that NMDA receptor antagonists suppress pathologic activity during epileptic seizures, both in in-vivo and in-vitro epilepsy models (Mody et al. 1987; Robinson et al. 1993; Jimbo et al. 2000; Bonzano et al. 2006). In recent work by Verstraelen et al. (2014), Ca^{2+} imaging was employed in combination with NMDA antagonists as well as agonists, and it was reported that NMDA receptors make significant contributions towards synchronized network
activity in mouse hippocampal cultures. Our data, albeit a different modality, confirms this finding. Activity levels in terms of spike rates and bursting were significantly reduced in the NMDA-disabled condition (GABA\textsubscript{A}+AMPA), relative to the control condition (Figs. 6B, C, D). Of course our sub-millisecond time resolution is much higher compared to the imaging studies reported by Verstraelen et al. (2014), but considering that we determined these metrics across individual bursts, the similarity of our findings is encouraging.

Niedringhous et al. (2013) found that increase in AMPA receptors was associated with increased overall spike and burst rates and decreased burst durations. Although we don’t have a condition with increased AMPA receptor density, our data suggests that this receptor is indeed a principal contributor to propagate network activity and plays a very important role in mediating fast excitatory neurotransmission. This is very clearly demonstrated in the condition GABA\textsubscript{A}+NMDA, where AMPA receptors are disabled, resulting in total absence of any spike (Fig. 6B) or burst activity (Fig. 6D). Other groups have also reported that blocking AMPA receptors is enough to abolish this activity (Bonzano et al. 2006; Reinartz et al. 2014).

Furthermore, in a network that is driven uniquely by AMPA receptors, we did find maximal spike rates (Fig. 6B), burst indices (Fig. 6C), and fast correlations (Fig. 9C).

It is interesting to note that in the presence of inhibition from GABA\textsubscript{A} receptors, blocking just the NMDA receptors (i.e., condition GABA\textsubscript{A}+AMPA) does not completely suppress bursting activity as indicated by non-zero values of spike rates, burst indices and burst duration (Fig. 6B, C, D). However, combined functional GABA\textsubscript{A} and NMDA receptors with blocked AMPA receptors (i.e., condition GABA\textsubscript{A}+NMDA) completely shut down any activity within the network. Perhaps the fast dynamics of the AMPA receptors can overcome the slower inhibitory GABA\textsubscript{A} effects, whereas the slower NMDA receptors cannot (Fig. 9). It seems to be necessary to
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block both AMPA and NMDA receptors, or even just AMPA receptors to eliminate any kind of
activity within the network. Several studies using slice models have reported similar results (Lee
development of repetitive bursting in 2-3 week old cultures shows parallels to the process of
epileptogenesis. This observation has also been described in organotypic cultures of
hippocampus (Staley et al. 2011). In our cultures, we found that AMPA receptor antagonist
CNQX is more effective than NMDA receptor antagonist CPP in blocking burst activity. This
finding is in agreement with reports involving comparison of AMPA and NMDA receptor
antagonists in anticonvulsant therapy (Rogawski 2011). Such agreement between our
experimental findings and the clinically reported effects indicate that developing network models
may be useful for the study of mechanisms that govern pathological network activity in diseases
such as epilepsy, and in testing new drug targets to treat them.

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References


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Figure Legends

Figure 1. Experimental recording setup and network burst analysis.

(A1) The multi-electrode array (MEA) and a light microscope-view of the MEA showing four (out of sixty) electrodes. (A2) We recorded four 15-min epochs from each culture: 1. control condition (no drugs), 2. after adding first drug, 3. after adding second drug, 4. after adding third drug, with a 5-min pause in between the four recordings. In this particular depiction, the four 15-min epochs correspond to conditions with: 1. No drugs, 2. CPP (first drug), 3. CPP+PTX (second drug), 4. CPP+PTX+CNQX (third drug). All recordings from the MEA were done in neurobasal medium. (B1) To ensure stable bursting, a 5-min period (highlighted by rectangular box) is selected, >5 min after onset of each 15 min recording session. Then 10-sec epochs, surrounding each burst present in the selected 5-min period is the basis of burst analysis. (B2) From the raw signal (corresponding to the 10-sec burst epoch), we generated filtered signals: one for spike detection (300-1500 Hz, first-row trace) and one for the low frequency component of the LFP (<4 Hz, fourth-row trace). We generated spike rasters based on spike detection (second-row trace). A burst index was obtained by rectifying and integrating the spike signal (using a leaky integrator with a time constant of 50ms, third-row trace). (C) Power spectral analysis was performed on the same 10-sec epoch, in the following frequency bands - delta (δ):<4Hz, theta (θ): 4-8Hz, alpha (α): 8-12Hz, beta (β): 12-20Hz, gamma (γ): 20-80Hz, high-gamma (high-γ): 80-150Hz. (D1) Raster plots from all electrodes within the array were used to obtain an estimate of the network correlation and connectivity. (D2, D3) Two typical examples of cross-correlation that were computed between pairs of electrodes. Grey trace indicates cross-correlation computed between the spike rasters (discrete signal), black trace represents cross-correlations between the
integrated spike activity (continuous signal) of the same pairs of channels. (D4) This procedure was performed for all channel pairs and for each correlogram, the maximum correlation was plotted against its delay.

Figure 2. Effect of synaptic transmission on network-wide bursting activity.
Representative MEA activity from four out of sixty channels (bandwidth 1–3000 Hz) depicted across eight different synaptic transmission (pharmacological) conditions 1) GABA<sub>A</sub>+NMDA+AMPA receptors functional (No drugs added), 2) NMDA+AMPA receptors functional (PTX added), 3) GABA<sub>A</sub>+AMPA receptors functional (CPP added ), 4) GABA<sub>A</sub>+NMDA receptors functional (CNQX added), 5) only AMPA receptors functional (PTX+CPP added ), 6) only NMDA receptors functional (PTX+CNQX), 7) only GABA<sub>A</sub> receptors functional (CNQX+CPP added) and 8) none of the three receptors functional. Recording medium was neurobasal.

Figure 3. Effect of riluzole on bursting activity.
(A1) Network activity (raw signal) recorded from a representative single electrode within the multi-electrode array, before and after the addition of riluzole (20 µM) (indicated by arrow). (All MEA recordings were done in neurobasal medium). (A2) Integrated spike activity computed from the raw signal was used to identify bursts. Burst detection threshold was set at four standard deviations. (B) Burst frequency, calculated based on threshold crossing of the integrated spike activity, before and after riluzole application, shows that addition of riluzole gradually abolishes bursting activity. Note that A1, A2 and B, all correspond to the same 10 min window. (C) Intracellular recording of a pyramidal cell's response to current injection before and after bath
application of riluzole (20 µM) demonstrated that fast sodium currents are not abolished by addition of this drug.

**Figure 4. Variability in spatial burst onset.**

(A) Example of four spontaneous network-wide bursts (labelled as Burst1-4), shown on four out of sixty MEA channels; no drugs added. (B) Details of these burst sequences during the first few seconds of burst onset show that spike sequences vary across the channels from one burst to the next suggesting that sequence of channel activation varies considerably across each burst. (Recording medium in all MEA recordings was neurobasal).

**Figure 5. Effect of synaptic transmission on intracellular activity and extracellular network burst patterns, L-LFP and power spectrum.**

This figure depicts intracellular and multi-electrode array (MEA) activity observed in the five out of eight synaptic connectivity conditions that exhibited network-wide bursting:

- GABA<sub>A</sub>+NMDA+AMPA receptors functional (No drugs added),
- NMDA+AMPA receptors functional (PTX added),
- GABA<sub>A</sub>+AMPA receptors functional (CPP added),
- only AMPA receptors functional (PTX+CPP added), and only NMDA receptors functional (PTX+CNQX).

Remaining conditions i.e.,

- GABA<sub>A</sub>+NMDA receptors functional (CNQX added),
- only GABA<sub>A</sub> receptors functional (CNQX+CPP added), and none of the three receptors functional (CNQX+CPP+PTX added) are omitted because no bursting activity occurred. Note that intracellular and MEA recordings were made from two independent experimental groups of cultures. A1-A5) Sample intracellular recordings within a 10-sec epoch surrounding a burst. The inset for panel A2 depicts a detail with paroxysmal depolarization. All intracellular recordings
were done in ACSF. B1-B5) Representative spike-filtered recordings, containing a 10-sec epoch surrounding a sample burst from one of the channels in the MEA. Recording medium was neurobasal. C1-C5) Low frequency component of the local field potential (L-LFP, also referred to as delta frequency band, <4Hz), averaged across all MEA electrodes within the 10-sec burst epoch. D1-D5) Power spectra averaged across all MEA electrodes within the same 10-sec burst epoch. The grey rectangular strips in the spectral plots highlight the differences in power in the delta frequency band (<4Hz). All MEA recordings were done in neurobasal.

Figure 6. Quantification of the effect of synaptic transmission on spectro-temporal burst properties.
Overview of mean and SEM for the frequency and time domain burst metrics, calculated from MEA recordings across the eight experimental conditions. (A1) Spectral parameters quantified as the average power (in arbitrary units) in different frequency bands (0-150 Hz). (A2) Average power in the delta (<4Hz) band. (A3) Average power in the theta (4-8Hz) band. (B) Average intra-burst spike rate. (C) Average burst index. (D) Average burst duration. (* p-value < 0.01, adjusted by the Bonferroni correction).

Figure 7. Mixed-effects modeling of network burst metrics in terms of synaptic functionality.
Results from the mixed-effects model was used to investigate both linear and interaction effects of AMPA, NMDA, and GABA\textsubscript{A} receptors on different time (burst parameters, network connectivity) and frequency domain (LFP frequency) network metrics obtained from the MEA recordings. The complete model included an intercept, linear terms for each factor and the 2\textsuperscript{nd}
and 3rd order cross terms of the factors. The X-axis represents the coefficients for each of these terms - (A) Spike rate, (B) Burst index, (C) Burst duration, (D) Normalized sum of maximal correlations (see Methods, Section 2.7), (E) Delta frequency (<4 Hz), (F) Non-Delta frequency (4-150 Hz). (*) p-value < 0.0006, adjusted by the Bonferroni correction.) See Table 3 for p-values and coefficients.

**Figure 8. Effect of synaptic transmission on correlations between LFP power and spike/burst activity.**

Correlations between (A) spike rate, (B) burst index and delta, non-delta powers in the LFP measured across eight experimental conditions. (**) denotes p<0.01

**Figure 9. Network connectivity analysis - Effect of synaptic transmission on correlations and delays.**

(A) Representative examples of connectivity graphs characterizing networks with AMPA (top row) and NMDA (bottom row) only connections. Each of the 60 MEA channels is depicted as a vertex in the graph and the delay associated with the maximum cross-correlation was employed to determine each of the edges. It can be seen that networks governed by AMPA are predominantly determined by fast (lags between 2-10ms) correlations, while the networks with NMDA receptors show a dominance of slow (lags between 20-150ms) correlations. (B) Normalized sum of maximal correlations (NSMC) between all pairs of the n active channels; note that summed values were normalized using the factor 100/(n^2-n)/2 (for details see Methods 2.7). The histogram shows the mean NSMC across the eight conditions. (C) Mean ratio
of maximum correlations with fast/slow delays (indicated as black bars); Mean ratio of  
maximum correlations with slow/fast delays (indicated as grey bars). Error bars indicate SEM.
Multi-electrode array (MEA) recordings - Experimental setup

A1. MEA
MEA under light microscope

Network activity recorded from an electrode

100 μm

A2. No Drugs
1. Start rec.
15 mins
2. Stop rec.
Add Drug 1
3. Start rec.
15 mins
No rec. 5 min

Drug 1
4. Stop rec.
Add Drug 2
5. Start rec.
15 mins
No rec. 5 min

Drug 1+Drug 2
6. Stop rec.
Add Drug 3
7. Start rec.
15 mins
No rec. 5 min

100μV
5 min

Burst Analysis

B1. Within every 15-min recording a stable 5-min period is selected to detect bursts
5 min

10-sec epoch around each detected burst is used for burst analysis

Power Spectral Analysis - LFP

C. EEG frequency bands (0-150 Hz)

D. Cross Correlation

Network connectivity Analysis

D1. Raster Plot

D2. Maximum Correlation vs Delay

D3.
1. GABA\textsubscript{A}, NMDA, AMPA (No drugs)

2. NMDA, AMPA (PTX added)

3. GABA\textsubscript{A}, AMPA (CPP added)

4. GABA\textsubscript{A}, NMDA (CNQX added)

5. AMPA (PTX + CPP added)

6. NMDA (PTX + CNQX added)

7. GABA\textsubscript{A} (CPP + CNQX added)

8. No receptor functional (PTX + CNQX + CPP added)
Spectral burst metrics

A1. Power in all EEG bands (0-150 Hz)

A2. Power in Delta band (< 4 Hz)

A3. Power in Theta band (4-8Hz)

Temporal burst metrics

B. Spike Rate

C. Burst Index

D. Burst Duration

Drug Conditions

Mean Power (AU)

Mean Delta Power (AU)

Mean Theta Power (AU)

Mean Spike Rate (spikes/s)

Mean Burst Index (AU)

Mean Burst Duration (s)

GABA, NMDA, AMPA

High-gamma  ■ Gamma  □ Beta  ■ Alpha  ■ Theta  ■ Delta (<4Hz)

Non-delta (4-150 Hz)

Error Bars: +/- 1 SE
A. Connectivity Graphs

Fast Correlations
(r > 0.5, short delay 2-10 ms) (r > 0.5, long delay 20-150 ms)

Slow Correlations

Condition 5: AMPA

Condition 6: NMDA

B. Normalized sum of maximal correlations

C. Mean Fast/Slow, Slow/Fast correlations

Error Bars: +/- 1 SE
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Experimental sample sizes per culture per drug condition

Our experiment comprised of a total of 17 cultures, each of which is indicated under the column marked “culture ID”. Within each culture, drugs were added in different sequences, as shown under the column marked as “condition”. The total number of bursts that were recorded within
Network Burst Activity in Hippocampal Neuronal Cultures

Each culture, for a given drug condition, are shown in the last column. Each of these bursts was analyzed and used to report the different network parameters. Note that you only see 7 different pharmacological conditions here. The eighth condition corresponding to CPP+CNQX is not included here because, there is only inhibition in this case, which fails to elicit any kind of spiking or bursting activity.
## Network Burst Activity in Hippocampal Neuronal Cultures

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<th>Drug(s)</th>
<th>(n)</th>
<th>Functional Receptors</th>
<th>Excitation Level</th>
<th>Outcome</th>
<th>Inter Burst Interval (s)</th>
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<td>Bursting</td>
<td>25.6±2.7</td>
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<td>PTX</td>
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<td>NMDA, AMPA</td>
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<td>(6)</td>
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<td>(6)</td>
<td>GABA&lt;sub&gt;A&lt;/sub&gt;, NMDA</td>
<td>-(0)</td>
<td>Almost No Activity</td>
<td>36.1±7.7</td>
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<td>5</td>
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<td>(8)</td>
<td>AMPA</td>
<td>+(1)</td>
<td>Bursting</td>
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<td>83.0±20.1</td>
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<td>GABA&lt;sub&gt;A&lt;/sub&gt;</td>
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<td>36.1±7.7</td>
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<td>None</td>
<td>0(0)</td>
<td>Almost No Activity</td>
<td>36.1±7.7</td>
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### Table 2

**Overview of pharmacological experimental conditions**

The condition numbers (1-8) denote the drugs that were applied and (consequently) the receptors that remained functional. The number of experiments (n) carried out for each condition is indicated in between brackets. For each condition, the excitation level indicates the overall state of excitation (+) and inhibition (-) in the network. The indicator number within parenthesis indicates the state by adding one for + and subtracting one for -. Except for conditions 4, 7, and 8 all resulted in bursting activity that formed the basis for the analysis in this study. The inter burst intervals are indicated as mean ± SEM.
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<th>BurstDur</th>
<th>SumCorRel</th>
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**Table 3**

Mixed-effects model analysis.

Contribution of the potential predictors (indicated under model terms, in the left column), towards the observed output parameters (indicated by variables in the top row). Coefficients as well as p-values of the linear (GABA, NMDA, AMPA) and higher order interaction terms (GABA:NMDA, GABA:AMPA, NMDA:AMPA, GABA:NMDA:AMPA) are indicated for each output parameters i.e., spike rate, burst index, burst duration, normalized sum of maximal correlations, delta and non-delta.