Elevated serotonergic signaling amplifies synaptic noise and facilitates the emergence of epileptiform network oscillations

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Abstract

Serotonin fibers densely innervate the cortical sheath to regulate neuronal excitability, but its role in shaping network dynamics remains undetermined. We show that serotonin provides an excitatory tone to cortical neurons in the form of spontaneous synaptic noise through 5-HT3 receptors, which is persistent and can be augmented using fluoxetine, a selective serotonin reuptake inhibitor. Augmented serotonin signaling also increases cortical network activity by enhancing synaptic excitation through activation of 5-HT2 receptors. This in turn facilitates the emergence of epileptiform network oscillations (10-16 Hz) known as fast runs. A computational model of cortical dynamics demonstrates that these two combined mechanisms, increased background synaptic noise and enhanced synaptic excitation, are sufficient to replicate the emergence fast runs and their statistics. Consistent with these findings, we show that blocking 5-HT2 receptors in vivo significantly raises the threshold for convulsant-induced seizures.

Keywords: 5-HT2 receptors, 5-HT3 receptors, synaptic noise, cortical network dynamics, epileptic seizures.
Introduction

Various neurotransmitter systems converge onto the neocortex to regulate the activity of cortical neurons in a process known as neuromodulation (Kaczmarek and Levitan 1987). Through the regulation of neuronal excitability, neuromodulation shapes the patterning of neural activity (Gil et al. 1997; Puig et al. 2010). Of the gamut of neuromodulators, serotonin (5-hydroxytryptamine; 5-HT) exerts a powerful influence on cortical neurons owing to an extensive distribution of 5-HT receptors (Dougherty and Aloyo 2011; Nichols and Nichols 2008; Santana et al. 2004) juxtaposed with dense innervation by serotonergic axons from the midbrain (Blue et al. 1988). In cortical neurons, metabotropic 5-HT2 and ionotropic 5-HT3 receptors (5-HT2Rs & 5-HT3Rs) are the primary mediators of excitatory responses to 5-HT (Nichols and Nichols 2008; Roerig et al. 1997; Tanaka and North 1993). The effects of these receptors are well characterized at the level of intrinsic neuronal and synaptic excitability (Araneda and Andrade 1991; Fink and Gothert 2007; Zhou and Hablitz 1999). However, the consequences of these effects on cortical network dynamics remain largely unexplored (Puig and Gulledge 2011).

Cortical network activity emanates from complex interactions between synaptic inputs (Economo and White 2012; Fernandez et al. 2013) and cell-intrinsic excitability (Grashow et al. 2010; Kolind et al. 2012) – both are subject to neuromodulation (Araneda and Andrade 1991; Gil et al. 1997). The temporal dynamics of such activity vary between irregular (Shadlen and Newsome 1998) and oscillatory (Buzsaki and Draguhn 2004) patterns of activation, which may coexist simultaneously (Burns et al. 2011; Frohlich et al. 2010; Hansel 1996) or under different operational modes within a network (Poulet and Petersen 2008). Investigation of the invertebrate nervous system has revealed that network dynamics are reconfigured with neuromodulation to switch between distinct dynamical regimes, resulting in changes to behavior (Marder 2012). Complementary findings in vertebrates remain scarce. Despite recent advances in understanding the role of 5-HT in regulation of cortical network activity (Andrade 2011; Celada et al. 2013; Nakamura and Wong-Lin 2014; Puig and Gulledge 2011), comprehensive evidence is still lacking owing to serotonin’s complex and combinatorial effects on neuronal excitability.

Theoretical studies have suggested that network dynamics are sensitive to random fluctuations of synaptic conductances or in other words, synaptic “noise” (Parga and Abbott 2007; Stacey et al. 2009). Introduction of randomly fluctuating synaptic inputs to simulated neuronal networks facilitates transitions between distinct network states (Destexhe et al. 2001; Frohlich et al. 2010), but to date there is little empirical evidence for this. Within the neocortex, synaptic noise emerges from spontaneous release of synaptic vesicles (Otsu and Murphy 2003; Wasser and Kavalali 2009). Based on the theoretical
considerations, we sought to understand how 5-HT contributes to synaptic noise in the cortex and, more specifically, how this serotonergic tone influences network excitability and network oscillations.

Here we show that synaptic noise can be augmented with the antidepressant fluoxetine (FLX), resulting in enhanced cortical network activity. Importantly, the increase in noise is accompanied by the emergence of epileptiform cortical dynamics that arise from a 5-HT2R-dependent enhancement of synaptic excitatory coupling. Using computational simulations, we demonstrate that enhanced synaptic excitation and noise are sufficient to account for the observed dynamics. In accord with these findings from in vitro experiments, we show that blocking 5-HT2Rs in mice significantly delays the onset of convulsant-induced epileptic seizures in vivo, and may even completely prevent them.

Materials and Methods

All experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at Case Western Reserve University.

**Thalamocortical slice preparation.** Thalamocortical slices (350 µm) of somatosensory cortex were prepared as previously described from juvenile (P13-21) C57BL/6 wild-type mice (Agmon and Connors 1991; Pucilowska et al. 2012). Animals were anesthetized with vapor isoflurane and decapitated with a guillotine. The brain was then submerged in ice-cold artificial cerebrospinal fluid (ACSF) saturated with 95% O₂ 5% CO₂ containing the following: 125 mM NaCl, 2.5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 25 mM NaHCO₃, 1.25 mM NaH₂PO₄ and 25 mM glucose. Brain slices were cut on a vibratome (Leica VT1200). All chemical salts and reagents were purchased from Fisher Scientific (Pittsburgh, PA) and Sigma-Aldrich (St. Louis, MO). After the vibratome, slices were transferred to a bath containing room temperature ACSF for 20 minutes to incubate. Subsequently, slices were moved to the recording chamber and perfused with standard ACSF warmed to 31°C with a TC-324B Automatic Temperature Controller (Warner Instrument Corporation; Hamden, CT) at a rate of 2 mL/minute. Slices were then incubated for one hour before beginning electrophysiological recordings.

**In vitro electrophysiology.** Pyramidal cells within L2/3 were visually identified at 63× magnification using Kohler illumination with an upright microscope (Zeiss Axioskop 2 FS+; Germany). Whole-cell patch clamp recordings under the current-clamp configuration were established in single neurons using borosilicate glass electrodes (6-10 MΩ) filled with standard internal solution containing the following: 120 mM potassium gluconate, 2 mM KCl, 10 mM HEPES, 10 mM sodium phosphocreatine, 4 mM MgATP, 0.3 mM Na₃GTP, 25 mM QX314, and adjusted to pH 7.4 with KOH. For voltage-clamp recordings, a cesium-based internal solution was used to improve space clamp and contained the
following: 120 mM cesium gluconate, 2 mM CsCl, 10 mM HEPES, 10 mM sodium phosphocreatine, 4 mM MgATP, 0.3 mM Na3GTP, 20 mM BAPTA, and 25 mM QX314 to block voltage-gated sodium channels and adjusted to pH 7.4 with CsOH. Voltage and current clamp experiments were performed using Multiclamp 700B amplifier (Molecular Devices, Foster City, CA) digitized at 10 KHz with Digidata 1400 data acquisition interface. The data was low-pass filtered online at 1 KHz.

Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in the voltage-clamp configuration for 60 seconds. To isolate the excitatory synaptic currents, the membrane potential of the neuronal membrane was biased to the reversal potential for inhibitory postsynaptic currents (E_{IPSC}), which was experimentally determined to be -80 mV, consistent with previous reports (Chagnac-Amitai and Connors 1989; Hasenstaub et al. 2005). Recordings were excluded if access resistance exceeded 30 MΩ throughout the duration of the recordings and/or if the resting membrane potential was more positive than -60 mV. All pharmacological agents were washed into the bath. The slices were then given one hour to incubate before beginning recordings.

Current-clamp recordings were performed to assess cortical network activity in a disinhibited slice preparation. To partially disinhibit the cortical network, the ACSF was modified to contain 1 µM gabazine (GZN), a selective GABA_A receptor antagonist. Under control conditions, spontaneous network activity appeared as paroxysmal depolarizing shifts (PDS), which had a stereotypical voltage profile containing an initial plateau depolarization (~60-80 mV relative to baseline) lasting 400-500 ms succeeded by a decaying tail lasting ~500 ms. Recordings of cortical network activity lasted 10 minutes and were obtained without current injection (I_{hold} = 0 pA). The concentration of GZN was chosen so to elicit on average 1-2 PDS per minute, thus making the recordings amenable to statistical analysis. At this concentration, the network was not sufficiently disinhibited to spontaneously exhibit fast run epileptiform oscillations.

Sampling from individual animals for slice experiments involved taking one slice per animal and recording from 5-7 cells from each slice. No more than one pharmacological challenge was presented to a given slice. Statistical tests were carried out on groups of 30-40 cells (corresponding to 5-6 animals/group).

**Data analysis and statistics.** Spontaneous excitatory postsynaptic currents (sEPSCs) were detected using a custom algorithm written and implemented in Matlab (Mathworks). Detection was based on a threshold for the sEPSC derivative with threshold values for events obtained empirically. sEPSC detection also included criteria for event kinetics. Events with rise time longer than 5 ms and decay time constant longer than 30 ms were excluded; also the rise time was not allowed exceed the decay time constant. Events
were visually inspected after being subjected to the exclusion criteria to ensure they exhibited the typical profile of an alpha function. Network events in current-clamp were detected manually using a custom interactive program written in Matlab. Voltage deflections during PDS network activity typically were between 50-80 mV, depending on the resting potential of the neuron (typically ~ -70 mV), thus could be detected easily by a simple amplitude threshold-based detection. Reverberant afterdischarges during fast runs were detected by setting an absolute amplitude threshold of 30 mV relative to the resting potential and a 10 mV threshold relative to the local minimum (i.e. if the voltage deflection occurred on top of a depolarization plateau). Network-mediated postsynaptic currents (nEPSCs) were detected using an automated custom script in Matlab that detected currents larger than 100 pA. In the instance of reverberant network currents, the first current in a single network event always exceeded 1 nA and was substantially larger than the succeeding currents. Thus, to separate the first event from the smaller reverberant currents, we used an amplitude threshold of 1 nA. This procedure allowed us to separate the initial network current (i.e. the trigger event) and the sequential afterdischarges (i.e. reverberant currents). We determined statistically significant differences between distributions using the nonparametric Wilcoxon rank-sum test, which tests the null hypothesis that two independent groups of samples come from distributions with equal medians.

**Spectral analysis.** To compute the power spectrum of PDS, we first convert the membrane potential traces into series of discrete events

\[ s(t) = \sum_{p=1}^{N} \delta(t - t_p), \quad (1) \]

where \( \delta(x) \) is Dirac’s delta function; \( t_p \) denotes the time of the \( p \)-th PDS onset; and \( N \) denotes the number of PDS in 10 min. The Fourier transform of (1) yields

\[ \tilde{s}(\omega) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{\infty} s(t) \exp(i\omega t) dt = \frac{1}{\sqrt{2\pi}} \sum_{p=1}^{N} \exp(i\omega t_p), \]

where \( i \) is the imaginary unit and \( \omega \) is the angular frequency. The power spectrum is then given by

\[ P(\omega) = |\tilde{s}(\omega)|^2 = \frac{1}{2\pi} \sum_{p=1}^{N} \sum_{q=1}^{N} \exp\left(i\omega(t_p - t_q)\right) = \frac{N}{2\pi} + \frac{1}{\pi} \sum_{p=1}^{N} \sum_{q=p+1}^{N} \cos\left(\omega(t_p - t_q)\right). \quad (2) \]
Expression (2) is computed for each cell and then averaged for all cells recorded under the same conditions (more than 30 in each group). Note that at $\omega=0$ the power is $N^2/(2\pi)$. Moreover, for stochastic binary processes, as $\omega \rightarrow \infty$ the power converges to the baseline $N/(2\pi)$. The x-axis in Fig. 2f shows the linear frequency, $f = \omega/(2\pi)$.

**Computational model.** The model consists of an excitatory neuron coupled to an inhibitory neuron as depicted in Fig. 4a. The model also includes recurrent excitation and inhibition. The rate of change of the membrane potential for each neuron obeys the equation $C \cdot dV/dt = -I_{\text{total}}$, where $C$ is the membrane capacitance, $V$ is the membrane potential, and $I_{\text{total}}$ is the total current, i.e. the sum of all membrane and synaptic currents. We first note that during a PDS the current is completely dominated by synaptic currents, in other words, the neurons are in a high-conductance state. Similarly, in the absence of PDS the neurons are close to their resting potential whose fluctuations are dominated by synaptic noise. Consistent with these observations, all voltage-gated membrane currents can be neglected for the purpose of this study. We thus have that the total current consists of an excitatory and inhibitory component. The excitatory component is mediated by AMPA and NMDA currents, whereas the inhibitory component is mediated by $\text{GABA}_A$ current and by a slow outward $K^+$ current similar to the slow afterhyperpolarizing current ($I_{sAHP}$) (Andrade et al. 2012; Villalobos et al. 2005). In addition, there is a leak current and synaptic noise in the form of mixed excitatory and inhibitory barrages. The total current takes then the form: $I_{\text{total}} = I_{\text{AMPA}} + I_{\text{NMDA}} + I_{\text{GABA}_A} + I_{sAHP} + I_{\text{leak}} + I_{\text{noise}}$. The parameters for all these currents are defined below and their values are listed in Table 1. The functional form of the AMPA, $\text{GABA}_A$ and sAHP currents is the same, $I_k = G_k(t)(V - E_k)$, where the subindex $k$ refers to the current type, $G_k(t)$ is the time dependent synaptic conductance, as explained below, $V$ is the postsynaptic membrane potential and $E_k$ is the reversal potential for that synapse type. The time dependence of the synaptic conductances is modeled as a double low-pass filter so that the impulse-response is the well-known “alpha function”. Specifically, the integration of the synaptic dynamics is given by

\[
\begin{align*}
\tau_k \frac{dF_k}{dt} &= -F_k + g_k H(V_{\text{pre}} - \theta) \\
\tau_k \frac{dG_k}{dt} &= -G_k + F_k
\end{align*}
\]
Where $V_{\text{pre}}$ is the membrane potential of the presynaptic neuron, $\theta$ is the voltage threshold for the presynaptic neuron to release neurotransmitter, $g_k$ is the maximal synaptic conductance, and $\tau_s$ is the synaptic time constant (see Table 1). The function $H(x-a)$ is the Heaviside function; its value is 1 when $x>a$ and 0 otherwise. The functional form of the NMDA current contains an additional factor, $B(V) = 1/(1+0.7\exp(-V/16.13))$, that accounts for the Mg$^{2+}$-blockade of NMDA receptors dependent on the postsynaptic membrane potential: $I_{\text{NMDA}} = G_{\text{NMDA}}(t) \cdot B(V) \cdot (V-E_k)$. The leak current has the form $I_{\text{leak}} = g_L(V-E_L)$, where the leak conductance, $g_L$, is constant. The synaptic noise has two independent contributions, both excitatory: 1) small-amplitude noise; and 2) large-amplitude noise. The former is responsible for fluctuations of the membrane potential that at rest are not sufficient to cause large depolarizations. The latter is responsible for large but infrequent membrane potential depolarizations. Both noises are modeled as Poisson processes that are statistically independent from each other. The small noise impinges also independently on the excitatory and inhibitory neurons, whereas the large noise impinges on the excitatory neuron only. The events of the Poisson process are processed through a double low-pass filter to model the kinetics of synaptic noise. The rates of the Poisson processes, $\lambda$; the peak amplitudes of the events, $g$; and their time constants, $\tau$, are listed on Table 1.

**Seizure induction.** Juvenile C57BL/6 mice (P18-24) were administered the convulsant, pentetrazol (PTZ; i.p. 80 mg/kg) dissolved in 0.1 M phosphate-buffered saline (PBS). Some animals were treated with ketanserin (KSN; i.p. 5 mg/kg) dissolved in 5% DMSO 0.1 M PBS solution one hour prior to PTZ injection and a control group was pre-treated with DMSO solution alone to exclude the potential effect of the drug vehicle in modulating seizure parameters. Control animals were not subsequently used in testing the effects of KSN. The starting point of behavioral seizures was considered as the appearance of generalized clonic convulsions with loss of righting reflex (GCC-LOR). The endpoint for each GCC-LOR seizure was considered as either complete release from tonus (in animals presenting with tonus) or as cessation of generalized clonic convulsions and recovery of righting (Loscher et al. 1990). The time elapsed between PTZ injection and the first GCC-LOR seizure was taken as the seizure latency. Time elapsed between each subsequent seizure was considered to be the inter-seizure interval (ISI). In the majority of acute seizure experiments the animal died after experiencing several seizures. If the seizures persisted for over one hour after PTZ injection, the animals were euthanized by isoflurane anesthesia and decapitation. Animals that did not experience seizures within an hour after PTZ injection were not included in the data analysis. For the electroencephalography recordings (see next section), the time limit for the experiment was extended to two hours.
In vivo electrophysiology. We obtained electroencephalographic (EEG) recordings of PTZ-induced seizures from 13 juvenile (P20-24) C57BL/6 mice. Using techniques described elsewhere (Berkeley et al. 2002; Papale et al. 2013), we implanted a fine wire EEG recording electrode (Vintage Machine Supplies, Inc., Medina OH) subdurally over somatosensory cortex and a reference electrode was placed over the ipsilateral sensorimotor cortex. The tip of the electrodes (~ 1 mm) was fed through two small holes in the parietal bone of the calvarium and positioned parallel to the surface of the cortex. Postsurgical pain management was maintained with bupivacaine (2 mg/kg every 12 hours, s.c.) and carprofen (5 mg/kg every 24 hours, s.c.). Animals were allowed to recover 1-2 days before the seizure experiments were performed. EEG recordings were collected using a CP511 AC amplifier (Grass Technologies, Middleton, WI) using 100× amplification. The raw signal was band-pass filtered on-line between 0.1-100 Hz and digitized at 1 KHz using a Power 1401 mkII data acquisition system (Cambridge Electronic Design Limited; Cambridge, United Kingdom).

EEG recordings were then processed offline using Matlab. Raw EEG waveforms were mean-subtracted and passed through a Butterworth filter in the passband of 8-16 Hz. This passband was selected based on the relevant physiological frequencies of fast run oscillations in vivo (Steriade et al. 1998). The onset of electrographic seizures was detected by rectifying and integrating the band-pass filtered EEG waveform with a time constant of $\tau = 5$ s. An amplitude threshold was then set for the filtered and integrated EEG traces from individual animals due to the variable signal-to-noise ratios between different experiments. The latency to the first electrographic seizure was taken as the time from injection of convulsant to the first threshold crossing in the processed EEG signal.

Biocytin staining, histology, and imaging. Layer 2/3 PCs were filled with 20 mM biocytin (Sigma-Aldrich) during patch-clamp recordings. At the end of the experiments, slices were preserved in 0.1 M PBS solution containing 4% paraformaldehyde for over 24 hours. Slices were then subjected to 3 washes in 0.1 M PBS solution containing 0.5% triton-X 100 for 15 minutes and left to incubate for 24 hours in the same solution containing avidin-biotin complex (ABC Staining Kit – Elite Vector Labs). Slices were then washed 3 times in the PBS-triton solution for 15 minutes and stained with a diaminobenzidine (DAB Peroxidase Substrate Kit – Elite Vector Labs) solution until desired staining intensity was achieved. Slices were rinsed 3 times for 10 minutes in 0.1 M PBS solution followed by series of alcohol and xylene washes to dehydrate the tissue. Slices were stored in methyl salicylate. Stained neurons were visualized under brightfield illumination with 20× and 40× magnification using a Leica DM5000 B upright microscope and imaged with a Leica DFC420 C digital camera and Leica Application Suite imaging software. Images were further processed and refined using ImageJ (NIH) graphics editing software.
Results

Spontaneous synaptic activity in mouse neocortex is partially mediated by 5-HT in vitro

As a first step to investigate the role of serotonin in cortical network excitation, we measured the contribution of serotonergic signaling to spontaneous synaptic activity in layer 2/3 pyramidal cells (PCs) in thalamocortical slices of mouse somatosensory cortex (Agmon and Connors 1991). We chose somatosensory cortex as a target region owing to its distinct organization of neuronal populations into barrel columns, which specialized for local (< 300 µm) neuronal interactions and have a high degree of recurrent connectivity (Lefort et al. 2009; Petersen et al. 2003; Thomson and Lamy 2007). Pyramidal cell morphology was determined visually and verified post-hoc with biocytin labeling (Fig. 1a). Spontaneous excitatory postsynaptic currents (sEPSCs) were recorded in voltage-clamp using a cesium-based internal solution while blocking glutamatergic synaptic transmission through AMPA and kainate receptors with 2,3-dyhydroxy-6-nitro-7-sulfamoyl-benzof[ quinoxaline-2,3-dione (NBQX, 10 µM). NMDA receptor contribution was eliminated by holding the cell at a holding potential (-80 mV) at which NMDA receptors are blocked by Mg$^{2+}$ ions. We show that bath application of NBQX significantly reduced sEPSC amplitudes (p < 0.01, Wilcoxon rank-sum test; Fig. 1b,c) and increased the inter-event intervals (IEI) (p < 0.01; Wilcoxon rank-sum test; Fig. 1b,d), but failed to eliminate all sEPSCs, consistent with previous reports implicating other neurotransmitters involved in spontaneous synaptic release (Pankratov et al. 2007; Roerig et al. 1997). We investigated 5-HT as a potential source of the NBQX-resistant sEPSCs based on a previous report showing 5-HT as a mediator of fast synaptic transmission in cortical PCs through 5-HT3 ionotropic receptors (5-HT3Rs)(Roerig et al. 1997). In agreement with that report, bath application of the 5-HT3R antagonist, granisetron (GSN; 1 µM), with NBQX resulted in a dramatic reduction of sEPSC amplitudes (p < 0.01, Wilcoxon rank-sum test; Fig. 1b,c) and a substantial increase in the IEIs (p < 0.01, Wilcoxon rank-sum test; Fig. 1b,d). To test whether the serotonergic component depends on neuronal firing, we co-administered tetrodotoxin (TTX; 1 µM), a voltage-gated sodium channel blocker, with NBQX and observed no significant difference in event amplitudes between events recorded in the presence of NBQX alone or with NBQX and TTX in tandem (p > 0.05; Wilcoxon rank-sum test; Fig. 1b, c). The event amplitudes in the presence of NBQX and GSN together were significantly smaller than with NBQX and TTX (p < 0.01, Wilcoxon rank-sum test; Fig. 1b, c). While no significant change in IEIs was observed between spontaneous activity recorded with NBQX and NBQX with TTX (p > 0.05, Wilcoxon rank-sum test; Fig. 1b, d), there was a significant increase in IEIs recorded with NBQX and GSN as compared to the group recorded with NBQX and TTX (p < 0.01, Wilcoxon rank-sum test; Fig. 1b, d). This suggests that the fast ionotropic 5-HT3R signaling observed in cortical PCs arises independently of neuronal firing. Combined, these results indicate that 5-HT signaling through 5-HT3Rs
significantly contributes to the NBQX-resistant component of the ongoing spontaneous synaptic activity in the mouse neocortex.

In line with this hypothesis, raising endogenous 5-HT levels should increase spontaneous synaptic activity. Indeed, bath application of the selective serotonin reuptake inhibitor, fluoxetine (FLX, 4 µM), dramatically augmented sEPSC amplitudes (p < 0.01, Wilcoxon rank-sum test; Fig. 1e,f) and reduced the IEIs (p < 0.01; Wilcoxon rank-sum test; Fig. 1e,g) relative to control. Co-application of FLX and GSN substantially reduced the FLX-mediated increase in sEPSC amplitudes (p < 0.01, Wilcoxon rank-sum test; Fig. 1e,f), but had no effect on the FLX-mediated decrease in IEIs (p > 0.05; Wilcoxon rank-sum test; Fig. 1e,g). Note that the FLX and FLX+GSN IEI distributions are overlapping. To further verify that activating 5-HT3 receptors increases the amplitude and rate of spontaneous synaptic activity in cortical PCs, we bath applied a 5-HT3 receptor-specific agonist, m-chlorophenylbiguanide (mCPBG) and recorded sEPSCs. Indeed, administration of mCPBG resulted in a dramatic increase in the amplitude and frequency of sEPSCs in cortical neurons relative to control conditions (p < 0.01, Wilcoxon rank-sum test; Fig. 1e, f, g). The effect of mCPBG on sEPSCs amplitude exceeded that of FLX (p < 0.01, Wilcoxon rank-sum test), but the effect on frequency was comparable (p > 0.05, Wilcoxon rank-sum test). One potential confound of these results is the possibility that incomplete voltage-clamp across the somato-dendritic axis (i.e. incomplete space-clamp) would result in contamination of sEPSCs with inhibitory GABA_A receptor-mediated postsynaptic currents. This scenario is plausible considering that a large proportion of cortical inhibitory interneurons express 5-HT3Rs and could be activated by mCPBG to trigger sIPSCs in PCs (Lee et al. 2010; Morales and Wang 2002; Vucurovic et al. 2010). To control for this possible confound, in addition to using a Cs-based internal solution that minimizes space-clamp effects, we recorded the sEPSCs elicited by mCPBG in the presence of gabazine (GZN; 5 µM), a GABA_A receptor-specific antagonist. If the sEPSCs recorded at the reversal potential for inhibition are contaminated by GABAergic currents due to incomplete space clamp, then GZN would lead to the abolition of these events. mCPBG co-administered with GZN did not lead to a reduction in sEPSC amplitude compared with mCPBG treatment alone but modestly increased the IEI (data not shown), suggesting that though a small fraction of the recorded events may arise from GABAergic currents escaping voltage-clamp, the majority of these events result from 5-HT3 receptor signaling at the level of cortical PCs. This finding is consistent with the idea that spontaneous synaptic release, or synaptic noise, in the cortex is mediated in part by 5-HT signaling through 5-HT3Rs and offers a valuable tool to explore how altered serotonergic signaling affects network activity in cortical slices.

*Augmenting endogenous 5-HT signaling transforms cortical network dynamics via 5-HT2 and 5-HT3 receptors*
What are the consequences of increasing endogenous 5-HT levels on cortical network activity? To address this question, we employed a disinhibited slice preparation (Gutnick et al. 1982; Hablitz 1984) using bath application of the GABA_A receptor antagonist, gabazine (GZN; 1 µM). In this paradigm, the normally quiescent cortical network initiates bursts of activity in the form of paroxysmal depolarizing shifts (PDS; Fig. 2a). The PDS reflects synchronous activation of local cortical networks, thus enabling recordings from single neurons to probe network activity. The voltage profile of a PDS under a current-clamp recording configuration is characterized by a large and rapid depolarization (ca. 60-80 mV from baseline) lasting ca. 0.5 s followed by a tail that decays to baseline within 0.5-2 s (Fig. 2a). The power spectrum density of the voltage traces (see Methods and Materials) revealed that PDS events occur randomly in time, represented by a flat spectrum lacking frequency peaks as shown in Fig. 2g (Control case). Occurrence of these events can thus be considered a stochastic process, reflecting a lack of temporal organization in cortical dynamics. To determine the effect of enhanced endogenous 5-HT signaling on network dynamics, we bath-applied FLX (4 µM) for the duration of the recordings and found that FLX dramatically increased the number of network bursts recorded in a 10 min interval relative to control conditions (p < 0.01, Wilcoxon rank-sum test; Fig. 2b,f). Moreover, we show that enhanced serotonergic signaling transforms the patterning of cortical activity from temporally sparse and random to clustered and periodic network dynamics as described below (Fig. 2b). These clusters of network activity have a voltage profile characterized by an initial long burst (~500 ms) comparable to the PDS seen in control conditions followed by a sequence of shorter afterdischarges (duration ~40 ms) superposed on a long depolarizing plateau. The intra-cluster bursts occurred with a preferred frequency in the low beta range (10-16 Hz; Fig. 2b,g), while inter-cluster intervals showed no temporal patterning. The shift in the baseline of the power spectrum in Figure 2g corresponds to an increase in the total number of events, consistent with findings shown in Figure 2f. Note that the afterdischarges are of synaptic origin and persist in the absence of spiking when the recorded neuron is filled with QX-314 to block voltage-gated sodium channels. This pattern of activity has been observed previously in disinhibited rat cortex in vivo and slices in vitro (Castro-Alamancos 2000; Castro-Alamancos and Rigas 2002), animal models of spontaneous epileptic seizures (Timofeev et al. 1998), as well as in certain forms of childhood onset epilepsies (Camfield 2011) and has been termed “fast runs”, a designation we will employ here.

FLX increases serotonergic signaling by blocking presynaptic reuptake of 5-HT, thereby increasing transmitter availability for postsynaptic receptors. Thus, if the alteration of cortical network activity observed here is a bona fide serotonergic phenomenon, then some 5-HT receptor(s) must be responsible for the effect. Our observation that 5-HT3Rs generate a significant portion of spontaneous synaptic noise prompted us to ask whether this enhanced noise in the presence of FLX can account for the observed
changes in cortical network activity. Co-application of GSN with FLX in disinhibited slices led to a
significant reduction of the number of network events (p < 0.01, Wilcoxon rank-sum test; Fig. 2f), but did
not eliminate the fast runs, the persistence of which is represented by the beta peak in the power spectrum
(Fig. 2g). This finding suggested the involvement of another 5-HT receptor in the emergence of the
oscillatory fast runs. 5-HT2Rs are densely expressed in the cortex (Santana et al. 2004) and mediate
excitatory neuronal responses (Araneda and Andrade 1991; Tanaka and North 1993), making them a
suitable candidate. We blocked 5-HT2Rs with ketanserin (KSN, 10 μM) in the presence of FLX and
reduced the total number of network events back to control levels (control vs. KSN; p = 0.13; Wilcoxon
rank-sum test; Fig. 2f). Importantly, this led to complete attenuation of fast runs represented by the
elimination of the 10-16 Hz peak in the power spectrum (Fig. 2g). This effect was tested with a gamut of
5-HT2 receptor antagonists, which were all able to eliminate the fast runs albeit with different effects on
network excitability. These included ritanserin and glemanserin to selectively antagonize 5-HT2a
receptors, and RS-102,221 to antagonize 5-HT2c receptors. Receptor antagonists targeting 5-HT2a
receptor subtypes were more effective at reducing network excitability than those targeting the 5-HT2c
subtype, with ketanserin being the most effective (data not shown). We also tested the effect of 5-HT1
receptor (5-HT1R) activation on fast run activity owing to their dense expression in the cortex and their
inhibitory effects on neuronal excitability via activation of K+ currents (Parks et al. 1998; Tanaka and
North 1993). 5-HT1Rs were activated using the specific agonist 8-hydroxy-2-(dihydroxyamino)tetralin
(8-OH-DPAT; 4 μm) in the presence of FLX. 8-OH-DPAT treatment resulted in a pronounced reduction
in the number of recorded network events relative to FLX treatment alone (p < 0.01, Wilcoxon rank-sum
test; Fig. 2e, f), an effect statistically indistinguishable from that seen in the presence of FLX and GSN. It
also led to an almost complete abolition of the 10-15 Hz oscillation (Fig. 2e, g), consistent with a slowing
of network bursting seen in the voltage profile in Figure 1e. In the presence of 8-OH-DPAT, the network
bursts occurred either as single PDS events or as pairs of bursts like those shown in Figure 2e. These
results suggest that 1) 5-HT1R activity exerts an inhibitory effect on cortical network activity by
precluding the occurrence of fast run depolarizations and reducing network bursting; and that 2) in the
presence of FLX alone, activation of 5-HT1Rs by endogenous 5-HT release is insufficient to prevent the
occurrence of fast runs. Thus, our findings show that elevating endogenous 5-HT leads to an increase in
cortical network excitability and the emergence of epileptiform network oscillations. The former effect is
mediated in part by 5-HT3Rs and 5-HT2Rs while the latter effect is exclusively attributable to activation
of 5-HT2Rs. These observations provide a novel insight into how altered serotonergic tone in the cortex
leads to the transformation cortical network dynamics.

Fluoxetine enhances excitatory synaptic inputs mediating cortical network activity
The current-clamp experiments used in the previous section provide a proxy for the network behavior at the level of neuronal output. Voltage-clamping, on the other hand, offers insight into the magnitude, structure, and pharmacology of the inputs arriving at cortical neurons during network activity. We sought to determine the network mechanisms underlying the emergence of fast runs by measuring the network-mediated synaptic inputs in L2/3 PCs during disinhibition-induced activity in voltage-clamp before and after FLX treatment. With GZN alone, excitatory network inputs manifested as massive currents (nEPSCs; Fig. 3a) that were presumably dominated by AMPA receptor-mediated currents (Lee and Hablitz 1991), since NMDA receptor contribution was eliminated by holding the cell at a command potential (-80 mV) at which NMDA receptors are blocked by Mg\(^{2+}\) ions. Addition of FLX (4 \(\mu\)M) altered the network inputs in the following way. As expected, each network event had two phases corresponding to the two phases of the voltage profile in Figure 2b. The first phase consisted of a large nEPSC lasting ca. 500 ms followed by the second phase comprised of substantially smaller nEPSCs which occurred at the same frequency as the fast runs seen in Fig. 2 (Fig. 3b). This latter phase has been previously demonstrated to arise from recurrent glutamatergic synaptic transmission, which is expressed strongly in the superficial cortical layers and can be unmasked with sufficient disinhibition (Castro-Alamancos and Rigas 2002). We found that the charge transferred to each cell per event, taken as the integral of the total synaptic current, was substantially increased (\(p < 0.05\), Wilcoxon rank-sum test; Fig. 3c) after FLX treatment. This suggests that each cell received on average more synaptic excitation during a network event when FLX was present. Importantly, we compared the amplitude of the first nEPSC within each event between the two groups by setting a threshold that clearly separated the first and second phases. Our results show that the first nEPSC within each network event is significantly larger in the presence of FLX (\(p < 0.01\), Wilcoxon rank-sum test; Fig. 3d). Since this massive event acts as a trigger for the subsequent 10-16 Hz afterdischarges, these findings suggest that a shift in favor of more synaptic excitation during the initial burst leads to increased network activity and the onset of reverberant oscillations within the cortical circuit. We tested this hypothesis in the following section.

*Enhanced excitatory coupling and synaptic noise are sufficient to simulate fluoxetine-modulated network activity in a model of a cortical microcircuit*

Are the increased synaptic noise and enhanced excitatory coupling observed in vitro sufficient to account for the emergence of fast runs, or is it secondary to some unknown biophysical process? This question poses a technical problem since precise tuning of synaptic excitation and noise in the slice is impractical. Therefore, we tested this question using a computational model of a cortical microcircuit.
To this end we adopt a minimal modeling approach and focus on capturing the essential biophysical properties of neurons leading to realistic cortical dynamics. The inspiration for our model is taken from a previous computational study (Parga and Abbott 2007) showing that the key feature of cortical network dynamics is the bistability of the neurons’ membrane potential, as explained below. Bistability can be attained by multiple combinations of the excitatory and inhibitory synaptic conductances (Grashow et al. 2010; Prinz et al. 2003) and hence, does not critically depend on their specific values.

For our purposes, those network models can (Parga and Abbott 2007) be simplified by noting that cortical microcircuits can to a good approximation be considered translation invariant, i.e. the connectivity pattern repeats itself across cortical columns. Fig. 4a depicts the connectivity for layer 2/3. Because PDS occur synchronously across PCs within at least 200 μm (data not shown) the study of the dynamics of the microcircuit can be reduced to its simplest structural motif, consisting of a PC and an interneuron (Fig. 4a, right). The inputs of the missing neighbors are compensated by proportionately scaling the recurrent excitation and inhibition so that the net synaptic currents are the same as they would be when embedded in the full circuit. When the membrane potential of the neurons fluctuates around the resting (low conductance) state, the dominant current is the leak, as shown in Fig. 4b (straight dashed line). During the early phase of the PDS, the current driving the membrane potential is dominated by the AMPA, NMDA and to a lesser degree by GABA_A components (high-conductance state; early phase). The dependence of the current on the membrane potential is now given by the solid curved line which crosses I/C=0 at three points. The two crossings with a positive slope denote transiently stable points of the membrane potential, endowing the network with bistable dynamics (i.e. low/high conductance states). In the late phase of the PDS inhibition becomes more dominant and the membrane potential decays slowly (high-conductance state; late phase). The dependence of the current on the membrane potential is now given by the dashed curved line with only one stable point close to the resting potential, driving the neurons to repolarize quickly. It is important to note that in the absence of synaptic noise the neurons would never develop PDS. Noise is necessary to make the transition to a depolarized state. Once in the depolarized state, the recruitment of inhibition and decay of excitation bring the membrane potential back to the resting state. Figure 4c shows the connections of the motif in the control case as well as its dynamics. Figure 4d depicts the motif with increased synaptic excitation, mimicking the effect of FLX shown in Figure 2b. The activity pattern now switches into a fast run mode in which network activation occurs repetitively at ∼15 Hz. How does the increase in synaptic excitation make a qualitative change in the dynamics? Figure 4b shows that while the membrane potential decays, the dependence of the current on the membrane potential smoothly morphs from the solid curved line to the dashed curved line, mainly by moving upwards. In this process, the membrane potential switches from a bistable regime to a monostable
regime. If the synaptic noise is strong enough on the verge of this transition, it will shift the curve back
down, bringing the membrane potential again to a depolarized state. The slower the decay of the
membrane potential, the more likely it is that synaptic noise will depolarize it again. By this mechanism,
increased synaptic noise creates reverberant fast run oscillations within circuit.

Figure 4g shows that the model replicates the experimental results shown in Fig. 2f. The first group
corresponds to control conditions in which synaptic noise and excitatory coupling are kept at basal
conditions. Network events within this group manifest as single PDS-like bursts (Fig. 4c). The second
group mimics the effects of FLX: an increase in synaptic noise mediated by 5-HT3Rs and an increase in
synaptic excitation by 5-HT2Rs. As in vitro, the total number of network events increases significantly
relative to control (p < 0.01; Wilcoxon rank-sum test) and so does the variability in network excitability
as seen by a substantially larger spread in the distribution. Importantly, under these conditions the
network events appear as fast run-like oscillatory bursts with a frequency of ~15 Hz. A third group
models the effect of FLX in the presence of GSN, or equivalently the effect of increasing synaptic
excitation with only a marginal increase in synaptic noise. Clearly, the reduction in synaptic noise alone
reduces the excitability of the network but does not bring it back to the control level nor does it abolish
fast run-like oscillations.

To further validate the model’s ability in predicting the effects of pharmacological manipulations
presented in Figure 2, we modeled the effects of 5-HT1R activation with 8-OH-DPAT, which in the slice
had a combined effect of reducing network excitability and abolishing fast runs. This effect is consistent
with its effect on neuronal excitability (Araneda and Andrade 1991), which results to a large extent from
an increase in potassium conductance through G-protein-coupled inwardly rectifying potassium (GIRK)
channels that contribute to the resting leak conductance (Luscher et al. 1997). We thus simulated the
postsynaptic effect of 5-HT1R activation in the presence of FLX by increasing the leak conductance in
the excitatory neuron within the model while maintaining high levels of synaptic excitation and noise.
This manipulation was able to mimic the effect of 8-OH-DPAT in the slice in two ways: Firstly, the
number of network events dropped substantially relative to the group with increased synaptic excitation
and noise alone (p < 0.01, Wilcoxon rank-sum test; Fig. 4g). This reduction in number of events was
statistically indistinguishable from the one observed in the group simulating effects of FLX and GSN (p =
0.14, Wilcoxon rank-sum test), reproducing the results observed in vitro. Secondly, the fast run oscillation
was replaced by single bursts or burst pairs, similar to those recorded in the slice (Fig. 4e), suggesting that
a 5-HT1R-mediated enhancement of leak current is capable of suppressing network activity and
epileptiform network dynamics.
5-HT2Rs are also known to inhibit outward potassium current mediating the slow afterhyperpolarization (I_{AHP}) in cortical neurons (Satake et al. 2008; Villalobos et al. 2005; Villalobos et al. 2011). To test whether inhibition of I_{AHP} in the model alone can simulate the effects of FLX observed in the slice, we reduced this current while keeping control levels of synaptic excitation. Surprisingly, reduction of the I_{AHP} generated the fast runs oscillation (Fig. 4f) and increased the number of network bursts to levels observed with increased synaptic excitation and noise (i.e. FLX group)(p = 0.87; Wilcoxon rank-sum test). These results suggest that a parallel mechanism mediated by 5-HT2R-dependent inhibition of I_{AHP} could potentially generate epileptiform fast runs similar to those seen under conditions of increased excitatory coupling. Whether FLX actually decreases I_{AHP} in the in vitro preparation remains an open question.

Combined together these results support our hypothesis that increased synaptic noise and excitatory coupling resulting from altered 5-HT signaling are sufficient to account for the increased network excitability and emergence of epileptiform fast run oscillations. The synaptic noise increases the likelihood of transitioning into a depolarized high-conductance state, while the enhanced synaptic excitation facilitates the emergence of oscillatory dynamics. Furthermore, the model predicts the effects of 5-HT1R agonist 8-OH-DPAT by manipulating one of its known targets, namely the potassium current controlling the resting membrane potential, and provides a parallel mechanism by which fast run oscillations can be generated in the presence of elevated 5-HT tone, namely 5-HT2R-mediated inhibition of the slow afterhyperpolarization current. These results underscore the utility of reduced models of cortical circuits, which in our case is capable of reproducing the in vitro results and making accurate predictions about network behavior under various pharmacological manipulations without fine-tuning the model’s parameters.

**In vivo blockade of 5-HT2R activity delays behavioral and electrographic seizure onset and reduces seizure incidence**

Fast runs are not an epiphenomenon of the cortical slice preparation, but have been observed during spontaneous epileptic seizures in cats (Steriade et al. 1998; Timofeev et al. 1998) and are a hallmark of EEG recordings from children with Lennox-Gastaut syndrome, a childhood-onset encephalopathy (Camfield 2011). Based on the in vitro efficacy of KSN in abolishing fast runs, we hypothesized that blocking 5-HT2Rs before convulsant-induced seizures in mice may increase the threshold to epileptic seizures or modulate other seizure parameters. To test this hypothesis, we induced seizures in mice with the convulsant, pentetrazol (PTZ; 80 mg/kg, i.p.), and measured the latency to the first seizure characterized by loss of righting and generalized clonic convulsions (LOR-GCC; Fig. 5a) as well as the
duration of each seizure. Consistent with our hypothesis, mice that received an injection of KSN (10 mg/kg; i.p.) one hour prior to PTZ injection showed a more than five-fold increase in latency to the first LOR-GCC seizure (p < 0.01, Wilcoxon rank-sum test; Fig. 5b) compared to mice injected with the vehicle control. The duration of seizures was not significantly different (p = 0.59; Wilcoxon rank-sum test; Fig. 5c) suggesting that blockade of 5-HT2Rs in vivo raises the threshold to seizure onset but does not affect its termination. Furthermore, KSN treatment reduced seizure incidence in mice (control: 100% seized, n = 17; KSN: 78% seized, n = 18; p = 0.058, Fisher Exact Test).

The acute behavioral seizures accompanied by loss of righting and generalized clonic convulsions are an indirect proxy for the oscillatory brain dynamics that beget them. To directly test whether fast run oscillations occur during acute seizures in mice treated with PTZ and whether the behaviorally measured seizure latencies have a correspondence to the onset of such oscillatory regimes in the cortex we implanted 12 mice with subdural electrodes over the surface of somatosensory cortex and recorded the cortical electroencephalogram (EEG) during seizures with and without pre-treatment with KSN. Figure 5d shows raw EEG recordings from individual mice pre-treated with the drug vehicle alone (left) or with KSN (right). As visible from both traces, the onset of cortical seizures corresponds to a massive increase in activity in the EEG signal relative to the preceding baseline. The two traces also show that the onset of electrographic seizures in the mouse pre-treated with KSN is substantially delayed (latency = 1765 s) as compared with the control mouse (latency = 71 s). We filtered the EEG traces in the passband of 8-16 Hz to capture the fast run oscillations in the beta band as done previously by others (Steriade et al. 1998) and then determined the latency to the emergence of fast runs using a simple threshold (see Materials and Methods). To ensure that the latencies to behavioral seizures are an accurate proxy for the emergence of electrographic fast runs, we compared the latencies to the behavioral seizures with the latency to the onset of 8-16 Hz oscillations in the EEG. Figure 5e shows a very close and highly significant pairwise correlation between behaviorally and electrographically measured seizure latencies (R = 0.9967, p < 0.01, Student’s t-test). We thus proceeded to compare the latency to the onset of cortical fast runs between control and KSN-treated animals and observed a significant delay in the onset of fast runs after pre-treatment with KSN (vehicle: n = 5, KSN: n = 7; p < 0.05, Wilcoxon rank-sum test; Fig. 5f), a finding consistent with the those shown in in Fig. 5b.

Thus, just as 5-HT2R blockade prevents the emergence of epileptiform fast runs in vitro, it is also able to prevent or delay the onset of epileptic fast runs in vivo, lending support to the idea that serotonergic signaling in a disinhibited cortex facilitates the emergence of epileptiform activity and providing evidence across synaptic, network, and behavior levels of observation.
Discussion

Despite decades of investigation on the role of 5-HT in neuronal excitability, its effects on dynamics of cortical networks remain unclear (Celada et al. 2013). We sought to further this understanding by determining how changes in endogenous 5-HT affect cortical network dynamics. Our findings demonstrate that cortical neurons are persistently bombarded with spontaneous excitatory synaptic inputs mediated in part by 5-HT3 receptors. This synaptic noise can be augmented with the SSRI, fluoxetine, at concentrations close to those measured in medicated human patients (Bolo et al. 2000). This results in enhanced network excitability and the transformation from temporally random to transiently oscillatory network dynamics, i.e. from PDS to fast runs, a transition which is facilitated by 5-HT2Rs. We show that in cortical slices such a shift is accounted for by increased excitatory coupling between pyramidal cells. A computational model corroborates that increased synaptic noise and synaptic excitation are sufficient to explain the increased network excitability and transitions between both network regimes. Consistent with these results, blocking 5-HT2 receptors in vivo prior to PTZ-induced convulsions significantly delays the onset of epileptic seizures and reduces seizure incidence. Together, our findings provide a potential mechanism for how altered 5-HT tone can bias cortical network activity to transition into an epileptiform state.

The prevailing view of spontaneous excitatory neurotransmitter release onto cortical neurons held glutamate as its chief mediator (Espinosa and Kavalali 2009; Maffei and Turrigiano 2008; Sutton et al. 2004; Vyleta and Smith 2011), overlooking the contribution of other neurotransmitters (Pankratov et al. 2007; Roerig et al. 1997). Our findings show that 5-HT3Rs contribute substantially to spontaneous activity in the form of background synaptic noise, providing cortical PCs with a considerable excitatory tone. What is the source of 5-HT in the slice preparation? While the cortical slice lacks the dorsal raphe nucleus containing the forebrain-projecting serotonergic neurons, it preserves their dense projections to cortical neurons. These remaining axons are the putative source of endogenous 5-HT in our preparation and, based on results presented in Figure 1, support spontaneous vesicle release.

It is important to mention that our results from Figure 1 are in conflict with the presently held assumption that 5-HT3 receptors are expressed exclusively on cortical inhibitory interneurons. The evidence for this assumption comes from several studies that definitively show robust 5-HT3 receptor expression on cortical interneurons (Jakab and Goldman-Rakic 2000; Lee et al. 2010; Morales and Wang 2002; Puig et al. 2004; Vucurovic et al. 2010) but fail to detect expression of these receptors on the principal pyramidal neurons. While it is undeniable that a subclass of cortical interneurons express 5-HT3 receptors, we posit that the evidence is inconclusive pertaining to their expression on pyramidal cells. We first draw attention
to the study by Puig et al. (Puig et al. 2004) which used in situ hybridization and immunohistochemistry to assess 5-HT3 receptor expression in GABAergic interneurons of the rat prefrontal cortex. They report in their results that a minority of 5-HT3 receptor-expressing cells did not express GAD mRNA, a marker of inhibitory neurons and reported unpublished results in which some of these cells expressed vGluT1 mRNA, a marker of glutamatergic neurons. The authors state that they “[…] cannot exclude that a minority of 5-HT3 receptor-positive cells are pyramidal neurons.” These results are consistent with those of Roerig & Katz who were the first to show spontaneous and evoked 5-HT3 receptor-dependent currents on pyramidal cells of the ferret cortex and carried out post-hoc analysis of neuronal morphology to verify that both pyramidal and non-pyramidal neurons functionally expressed these currents (Roerig et al. 1997).

A study by Sung et al. used a transgenic mouse line in which 5-HT3 receptor expression was driven in forebrain pyramidal neurons using the CamKII promoter to investigate the modulation of 5-HT3 receptors by alcohol (Sung et al. 2000). Using patch clamp recordings, they first report no 5-HT3 receptor-dependent currents in most principal cells from wild-type animals; however, they mention in the results that 7 out of 48 recorded cells in wild-type mice showed evoked 5-HT3 receptor-mediated currents. That is, 15% of the cells which presumably should not express 5-HT3 receptors do, thus, providing supporting evidence for expression of functional 5-HT3 receptors on cortical pyramidal neurons.

Some of the studies addressing the molecular expression of 5-HT3 receptors in the cortex, such is that of Lee et al., have employed transgenic mouse lines that express enhanced green fluorescent protein (eGFP) in 5-HT3a receptor-expressing neurons and were able to demonstrate eGFP expression that was confined to cortical neurons expressing GAD, but not Satb2, a marker of cortical pyramidal cells (Lee et al. 2010). A similar finding was reported by Vucurovic and colleagues (Vucurovic et al. 2010). A critical caveat in these studies pertains to the subunit composition of 5-HT3 receptors of which there are two types, 5HT3a and 5-HT3b. 5HT3 receptors can exist as homomers composed of only 5-HT3a subunits (Brown et al. 1998) or as heteromers containing both subunit types (Davies et al. 1999; Kelley et al. 2003). It has been postulated based on previous work (Morales and Wang 2002; Sudweeks et al. 2002) that the heteromeric form of the receptor was not expressed in the CNS. However, a follow-up study by Doucet et al. showed cortical expression of the 5-HT3b subunit using a polyclonal antibody targeted to the intracytoplasmic loop of the 5-HT3 receptor and demonstrated that this expression differed considerably from localization of 5-HT3a receptors in previous studies (Doucet et al. 2007). The findings of Doucet et al. underscore two important points, namely, that methodological scope of previous studies investigating 5-HT3 receptor expression was too limited to detect 5-HT3b-containing serotonin receptors. Secondly, the lack of the expected anatomical overlap between 5-HT3b and 5-HT3a receptor subunits in their findings suggests that the previous studies may have inadvertently overlooked a population of heteromeric 5-HT3 receptor-
containing cortical neurons. The findings of Doucet et al. are consistent with another study that supports
the existence of heteromeric 5-HT3 receptors in rodent neurons (Hanna et al. 2000). Lastly, we note that
early studies that confirmed 5-HT3 receptor expression on cortical interneurons (Jakab and Goldman-
Rakic 2000; Morales and Wang 2002) used in situ hybridization and immunohistochemistry to find
overlap between markers of inhibitory interneurons and 5-HT3 receptors, but did not rigorously
investigate overlap with any markers of pyramidal neurons, thus, limiting the reach of their conclusions
that 5-HT3 receptors are expressed exclusively on interneurons. Given these caveats, we hold that
previous studies leave the possibility open that there are a subset of 5-HT3 receptors, likely to be
heteromeric, expressed on cortical pyramidal cells. Our findings in Figure 1 provide another compelling
piece of evidence in support of this idea.

An important finding reported herein is that the synaptic noise generated by 5-HT enhances cortical
network activity. That noise can enhance network activity may seem counterintuitive, yet it has been
explored thoroughly in numerous nonlinear dynamical systems, including neurons (McDonnell and Ward
2011; Stacey and Durand 2001). Importantly, synaptic noise can not only enhance network activity but
also network performance, specifically in the context of encoding information (Ermentrout et al. 2008;
Puzerey and Galán 2014). In this context, stochastic resonance is probably the clearest example, in which
the detection of subthreshold periodic inputs to a nonlinear system can be enhanced with the introduction
of noise, but it has been expanded to encompass a variety of dynamic phenomena in which noise
facilitates transitions between distinct system states under a broader concept of stochastic facilitation
(McDonnell and Ward 2011). A novel finding of our study is that synaptic noise provided by 5-HT3Rs
promotes transitions in a bistable network moving between quiescent and active states. This finding is
consistent with theoretical work implicating synaptic noise in the generation of epileptiform oscillations
(Stacey et al. 2009). Whether other sources of noise modulate network dynamics remains an open
question. We note, for instance, that previous work has shown that 5-HT enhances local glutamatergic
transmission in recurrent cortical circuits (Aghajanian and Marek 1999; Beique et al. 2004; Beique et al.
2007). Thus, determining the contribution of other neurotransmitters to synaptic noise and network
activity will be useful in determining if stochastic facilitation occurs as a general phenomenon of fast
neurotransmission.

The notion that neuromodulation plays an active role in transforming network behavior has been explored
thoroughly in the invertebrate central nervous system (CNS), particularly the crustacean stomatogastric
system (Marder and Thirumalai 2002). Consequently, several fundamental principles governing network
function were brought to light. An indispensable principle to our understanding was that networks, while
constrained by the anatomical architecture, exhibit highly dynamic functional connectivity (Getting 1989;
Marder and Thirumalai 2002). In short, the concerted behavior of neuronal networks operating within a defined anatomical circuit can be reconfigured with neuromodulation through changes in cellular, synaptic, or network properties. Our work shows that elevating endogenous cortical 5-HT leads to enhanced signaling through 5-HT2Rs, resulting in increased excitatory synaptic transmission during network activity and the emergence of fast run oscillations. Though previous reports have demonstrated that endogenous 5-HT may modulate network oscillations (Celada et al. 2008; Puig et al. 2010), our work reveals the role of 5-HT as facilitating the emergence of distinct cortical dynamics. While we provide a network mechanism for the emergence of epileptiform fast runs under elevated 5-HT tone, the cellular cascades downstream of 5-HT2R activation responsible for the enhancement of synaptic excitation require elucidation. 5-HT2Rs have distinct downstream targets in cortical neurons including NMDA receptors (Rahman and Neuman 1993), GABA_A receptors (Huidobro-Toro et al. 1996), Ca^{2+}-activated K^+ channels (Villalobos et al. 2005), and M-channels (Tanaka and North 1993). Determining the targets responsible for enhanced network responses will be important in understanding how changes in cellular properties can lead to transformation of network activity. One could then use computational models to test the robustness of the network dynamics to changes in these parameters and determine at which parameter boundaries transitions occur between states. It may also be possible that correlated variation in synaptic or membrane conductances may yield similar network behavior (Grashow et al. 2010; Marder 2011; Prinz et al. 2003). Given the prediction made by our computational model, the potassium currents underlying the slow afterhyperpolarization may be a likely effector downstream of 5-HT2 receptors underlying generation of fast runs, although the enhancement of excitatory synaptic coupling in vitro suggests that NMDA receptors may play a role as well.

Previous reports have suggested that FLX suppresses intrinsic cortical microcircuits in slices of non-epileptic human prefrontal cortex (PFC) (Komlosi et al. 2012). While this finding is certainly relevant for understanding how FLX affects sparsely active cortical microcircuits, it stands in contrast to our findings that FLX enhances the activity of a partially disinhibited cortical network. This discrepancy may be attributable to several factors including: 1) the use of a disinhibited slice in which the release of endogenous 5-HT is likely to be much higher during active states; 2) using somatosensory instead of PFC slices, which have different 5-HT receptor densities (Blue et al. 1988; Weber and Andrade 2010); and 3) comparing effects of FLX on sparse versus global and synchronized cortical activation, the relationship between which is ambiguous. Another important consideration is that elevating endogenous 5-HT is one of several ways in which the balance between excitation and inhibition can be shifted to elicit fast run oscillations. For instance, Castro-Alamancos and Rigas have shown that partial blockade of GABA_A receptors in cortical slices is sufficient to elicit PDS but not fast run activity, while co-administration with
GABA_B antagonists generates fast runs (Castro-Alamancos and Rigas 2002), which, as the authors argued, resulted from the unmasking of recurrent excitatory synaptic activity in the superficial cortical layers. In support of this idea, we posit that elevated cortical 5-HT enhances synaptic excitation during network activity, thereby promoting the expression of fast run oscillations.

Our study focused exclusively on changes in excitatory drive to the pyramidal cells of the cortex under conditions where intrinsic cortical inhibition is altered. Therefore, our interpretation of the results above must be confined to the sphere of cortical function under conditions in which inhibition is diminished, such as those in which epileptiform activity emanates from such an imbalance. Nevertheless, it is important to place our results in the context of the present literature of 5-HT function on cortical circuits in which inhibition is intact. Though there are several comprehensive reviews on this topic (Andrade 2011; Celada et al. 2013; Nakamura and Wong-Lin 2014), we will briefly discuss the main principles gathered from past investigations. It has become increasingly clear that making general claims about 5-HT’s role in cortical circuits is far from trivial. This difficulty arises from the heterogeneity in regional, laminar, cellular, and subcellular serotonin receptor expression (Amargos-Bosch et al. 2004; Blue et al. 1988; Jakab and Goldman-Rakic 2000; Santana et al. 2004; Weber and Andrade 2010). It has been understood, however, that the most frequent responses of cortical neurons to 5-HT are 5-HT1 receptor-mediated hyperpolarizations and 5-HT2 receptor-mediated slow depolarizations (Araneda and Andrade 1991; Tanaka and North 1993) The former effect is mediated by activation of outward potassium currents mediated by G-protein coupled inwardly-rectifying potassium (GIRK) channels (Luscher et al. 1997) while the latter is attributed to inhibition of a slow outward M-type potassium current (Tanaka and North 1993). 5-HT1 receptors are typically expressed at the axon hillock where they are poised to regulate neuronal output, while 5-HT2 receptors are expressed along the apical dendrite where they can modulate neuronal gain in response to synaptic inputs (Amargos-Bosch et al. 2004; Zhang and Arsenault 2005). Often neurons expressing both receptors can have either facilitation or suppression of firing depending on the site of stimulation in the DRN, the afferent source of serotonergic fibers innervating the forebrain, suggesting that precise circuits underlie the direction of the cortical response to 5-HT (Amargos-Bosch et al. 2004). The actions of serotonin receptors extend beyond modulation of cell-intrinsic properties to the level of synaptic interactions. It is by now well established that 5-HT2 receptors enhance spontaneous postsynaptic glutamatergic currents in cortical neurons, a process attributed to the enhancement of activity in intrinsic cortical circuits by 5-HT (Beique et al. 2007; Lambe et al. 2000). 5-HT also modulates the activity of inhibitory interneurons via excitation by 5-HT3 receptors and 5-HT2 receptors (Puig et al. 2004; Zhou and Hablitz 1999), though interneurons expressing these two receptors may have distinct molecular, anatomical, and functional profiles. Activation of 5-HT2 receptors on interneurons leads to
sustained enhancement of spontaneous inhibitory postsynaptic currents onto pyramidal neurons, while
activation of cortical interneurons through 5-HT3 receptors leads to transient increases of inhibitory
currents on pyramidal cells (Zhou and Hablitz 1999). The action of 5-HT goes as far as modulating the
concerted activities of ensembles of cortical neurons. Puig et al. showed that by stimulating the DRN and
recording local field potentials (LFP) in the PFC of anesthetized rodents leads to acceleration of the
cortical slow oscillation (< 2 Hz) in a 5-HT2 receptor-dependent manner (Puig et al. 2010). They also
demonstrated with the same paradigm that inhibition of 5-HT1 receptors increased cortical gamma (30-80
Hz) oscillations while 5-HT2 receptor inhibition decreased their power. Our results find agreement with
this work in that our experiments show a facilitating effect of 5-HT2 receptors and a suppressive effect of
5-HT1 receptors on cortical network activity. Nevertheless, it is important to draw the distinction between
the role of 5-HT as a modulator of cortical dynamics as shown by Puig et al. and the role of 5-HT as a
facilitator to the emergence of said dynamics. Furthermore, given the strict focus on the effects of 5-HT
on pyramidal neurons in our study, it will be important to determine the role of inhibitory cortical
interneurons in the emergence of oscillatory cortical dynamics and how their activity is shaped by altered
5-HT tone.

The transition between PDS and fast run activity is not an artifact of the disinhibited slice. The PDS is a
direct intracellular correlate of the inter-ictal spike that often precedes epileptic seizures and is the most
basic unit of epileptiform activity (McCormick and Contreras 2001). Fast runs have been demonstrated in
disinhibited rat cortex (Castro-Alamancos 2000), during epileptic seizures in cats (Timofeev et al. 1998),
and are an electrographic signature of epileptic seizures in children with Lennox-Gastaut syndrome, a
childhood-onset encephalopathy which comprises ~10% of all childhood epilepsies (Camfield 2011).
Therefore, taking into consideration that 5-HT2R blockade abolishes fast runs in vitro, increases seizure
threshold and delays the onset of electrographic fast runs in vivo lends support to the idea that excessive
5-HT in the CNS facilitates the onset of epileptiform network dynamics. These findings are in line with
reports supporting 5-HT’s pro-convulsive effects (Bercovici et al. 2006; Freitas et al. 2006; O’Dell et al.
2000; Wada et al. 1992), but stands in opposition to others who claim that central 5-HT is anti-convulsive
(Bagdy et al. 2007; Yan et al. 1995). This controversy can be attributed to the lack of consistent seizure
paradigms, animal models, or pharmacological agents employed by different investigators (Loscher et al.
1990). Importantly, the studies mentioned above lack a detailed mechanism for how 5-HT may modulate
epileptic seizures. Our results clearly support a pro-convulsive role for enhanced 5-HT signaling by
providing a mechanistic description for how changes in synaptic noise and excitatory coupling can lead to
epileptiform network dynamics. An important subtlety in the interpretation of our in vitro findings is that
5-HT2R activation alone is insufficient to generate fast runs – the cortex must be partially disinhibited. In
other terms, the cortical network must be close to the border of a transition between two states (i.e. PDS and fast runs) near which a shift in favor of more excitation is sufficient to push the network into an epileptiform regime. This subtlety highlights the role of 5-HT neither as an initiator nor as a modulator of epileptiform oscillations, but as a facilitator for their emergence. In the context of human epileptic seizures, our findings imply that 5-HT2R blockade may prove to be therapeutic for epileptic patients in general and, in particular, for those treated with SSRIs, which are often prescribed to patients comorbid for epilepsy and depression (Kanner 2003). Though the association between SSRIs and epileptic seizures in humans remains anecdotal, largely correlative, and confounded by factors such as co-administration of SSRIs with anti-epileptic drugs (Braitberg and Curry 1995; Gross et al. 1998; Pisani et al. 1999; Prasher 1993; Thome-Souza et al. 2007), there is substantial evidence for a pro-convulsive role of fluoxetine in animal models of epileptic seizures and epilepsy (Ferrero et al. 2005; Macedo et al. 2004; Morita et al. 2005; Raju et al. 1999; Zienowicz et al. 2005). Furthermore, our findings may be relevant for other neurological disorders in which 5-HT signaling and network dynamics are altered such as certain cases of autism (Veenstra-VanderWeele and Blakely 2012) and schizophrenia (Aghajanian and Marek 1999).

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Author Contributions

All experiments were conceived and designed by PAP and RFG. PAP carried out the in vitro electrophysiological experiments and animal behavior. PAP and MJD performed the surgeries and recordings for the in vivo electrophysiology. PAP and RFG analyzed the experimental data. RFG
conceived and implemented the computational model. The manuscript was written by PAP and RFG.

Figures were designed by PAP and RFG.
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**Figure Captions**

**Fig. 1.** Spontaneous excitatory transmitter release is mediated in part by 5-HT3 receptors in mouse neocortex. (a) Images of biocytin-labeled pyramidal neurons in L2/3 of mouse somatosensory cortex. Right panel shows neurons under 20× magnification with layer boundaries delineated by opaque white lines. Cortical laminae are labeled accordingly. Left panel shows neurons under 40× magnification with a clear view of the apical dendrites projecting to L1, basal dendrites projecting laterally, and, in some neurons, the axon is visibly projecting towards the deeper layers. (b) Raw traces of sEPSCs recorded under three conditions: 1.) control (no drugs); 2.) 10 µM NBQX (AMPA receptor blockade); and 3.) 10 µM NBQX with 1 uM GSN (AMPA and 5-HT3R blockade). (c) Cumulative distribution function of sEPSC amplitudes under three experimental conditions (control: n = 28; NBQX: n = 27; NBQX+GSN: n = 25). (d) Cumulative distribution function of sEPSC inter-event intervals (IEIs) for the data presented in (b). (e) Raw traces of sEPSCs recorded under: 1.) control conditions; 2.) 4 µM FLX; 3.) 4 µM FLX and GSN; and 5 µM mCPBG. (f) Cumulative distribution function of sEPSC amplitudes under the described experimental conditions (control: n = 28; FLX: n = 24; FLX+GSN: n = 29; mCPBG: n = 22). (g) Cumulative distribution function of sEPSC inter-event intervals for the data presented in (e). Note that the FLX and FLX+GSN distributions are overlapping.

**Fig. 2.** Elevated endogenous 5-HT in cortical slices enhances network excitability and transforms network dynamics. (a-e) Raw traces of network activity recorded over 10 minutes from L2/3 PC in a disinhibited cortical slice under (a) control conditions (GZN only), (b) with FLX, (c) with FLX and GSN, (d) with FLX and KSN, and (e) with FLX and 8-OH-DPAT. Top trace
represents entire recording and bottom trace zooms in on a single network event. Note that in a
and d network activity manifests as a single PDS, while in b and c it appears as oscillatory fast
runs. (f) Box plots of the number of network bursts observed per recording for the four groups
(control: n = 30; FLX: n = 31; FLX+GSN: n = 31; FLX+KSN: n = 29; FLX+8-OH-DPAT: n =
27). Asterisks above distributions denote level of significance (* = p < 0.05; ** = p < 0.01, n.s. =
not significant). (g) Power spectrum obtained from recordings of network activity exemplified in
a-e. Baseline of power spectrum corresponds to the baseline level of network activity in each
group.

Fig. 3. Enhanced synaptic excitation onto cortical neurons underlies transformation of network
dynamics in the presence of fluoxetine. (a-b) Raw traces of nEPSCs recorded over 10 min
interval under (a) control conditions and (b) with FLX. Note, traces in (a) and (b) were obtained
from different slices. Top trace represents entire recording and bottom trace zooms in on a single
network event. (c) Box plot of excitatory charge transferred to cortical PCs during each network
event under control and FLX conditions (control: n = 28; FLX: n = 26). (d) Cumulative
distribution functions of the amplitude of the initial nEPSC during each network event under
control and FLX conditions.

Fig. 4. Computational model accounts for emergence of fast runs with increased synaptic noise
and excitatory coupling. (a) Cortical microcircuit of layer 2/3 and reduction to its core motif.
Gray triangles represent cortical pyramidal neurons while gray circles represent cortical
interneurons. Excitatory connections are denoted by arrow heads and inhibitory connections by
small circles. (b) Dependence of the total current on the membrane potential at the three phases
of the PDS. The straight dashed line corresponds to the low conductance state preceding a PDS.
During this state, the membrane potential dynamics are dictated by the leak conductance. The
curved solid line corresponds to the early phase of the PDS and is equivalent to the high-
conductance state during which synaptic conductances dominate the membrane potential
dynamics. The curved dashed line corresponds to the late phase of the PDS during which slow
inhibitory conductance competes with fast excitation to repolarize the membrane potential (c)
PDS generated by the model. (d) PDS turn into fast runs when synaptic excitation is increased.
(e) Increasing the leak current in the presence of enhanced synaptic excitation prevents
generation of fast runs, recapitulating in vitro results with FLX + 8-OH-DPAT. (f) Decreasing
the slow afterhyperpolarizing current generates fast run oscillations (g) Box plot of the number
of network events under control conditions (control; n = 30 trials), under increased synaptic noise and synaptic excitation (FLX; n = 30 trials), under increased synaptic excitation and moderate synaptic noise (FLX+GSN; n = 30 trials), under increased synaptic excitation and enhanced leak current (FLX+DPAT; n = 30 trials), and under decreased slow afterhyperpolarizing current (n = 30 trials). The model replicates the statistics of network bursts from the experiments in Fig. 2f.

**Fig. 5.** 5-HT2 receptor blockade increases seizure threshold *in vivo*. (a) Schematic of experimental design for convulsant-induced seizure protocol. Control mice are given PTZ injection alone, while the test group is given the KSN 1 hour prior to PTZ injection. Lightning bolts represent appearance of spontaneous LOR-GCC seizures some time after PTZ injection. (b) Box plot of latency to first seizure in control and KSN conditions (control: n = 17; KSN: n = 14) (c) Box plot of mean seizure duration for each animal in control and KSN groups. (d) Raw traces of electroencephalographic (EEG) recordings of epileptic seizures obtained from mouse treated with PTZ alone (left) and those pre-treated with KSN one hour before PTZ injection (right). t = 0 corresponds to the time of PTZ injection. (e) Comparison of latency to the onset of behavioral and electrographic siezures. Dashed gray line corresponds to the identity line (x = y) and solid black line corresponds to the linear fit of the two variables (n = 12 mice). (f) Box plot of latency to the onset of 8-16 Hz (beta band) activity in the EEG during electrographic seizures in control and KSN-treated mice (control: n = 5 mice; KSN = 7 mice)
- a) Control (1 μM Gabazine)
  - 1 nA
  - 2 min
  - 0.5 nA
  - 1 s

- b) 4 μM Fluoxetine
  - 1 nA
  - 2 min
  - 0.5 nA
  - 1 s

- c) Excitatory charge per network event (nA·ms)
  - Log scale
  - Control vs. FLX

- d) Cumulative distribution function (F(x))
  - nEPSC amplitude (nA)
  - Control vs. FLX
### Tables

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**Table 1:** Parameters of the computational model. Arrows indicate the change of parameters used for Fig. 4g. Values in parentheses correspond to the inhibitory neuron, if different from the excitatory neuron.