Active cortical networks promote shunting fast synaptic inhibition in vivo

In brief
In this study, Burman et al. demonstrate that the way in which fast synaptic inhibition operates in the intact mammalian cortex is linked to the level of activity within the network. The authors further show that this dynamic inhibition has profound implications for how the cortex responds to incoming stimuli.

Highlights

- In vivo gramicidin recordings reveal the driving force for synaptic GABAARs
- Synaptic $E_{\text{GABAAR}}$ favors shunting inhibition in the awake cortex
- The high levels of cortical activity raise $E_{\text{GABAAR}}$ to promote shunting inhibition
- Shunting inhibition promotes local network desynchronization and response flexibility

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Report

Active cortical networks promote shunting fast synaptic inhibition in vivo

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SUMMARY

Fast synaptic inhibition determines neuronal response properties in the mammalian brain and is mediated by chloride-permeable ionotropic GABA-A receptors (GABAARs). Despite their fundamental role, it is still not known how GABAARs signal in the intact brain. Here, we use in vivo gramicidin recordings to investigate synaptic GABAAR signaling in mouse cortical pyramidal neurons under conditions that preserve native transmembrane chloride gradients. In anesthetized cortex, synaptic GABAARs exert classic hyperpolarizing effects. In contrast, GABAAR-mediated synaptic signaling in awake cortex is found to be predominantly shunting. This is due to more depolarized GABAAR equilibrium potentials (EGABAAR), which are shown to result from the high levels of synaptic activity that characterize awake cortical networks. Synaptic EGABAAR observed in awake cortex facilitates the desynchronizing effects of inhibitory inputs upon local networks, which increases the flexibility of spiking responses to external inputs. Our findings therefore suggest that GABAAR signaling adapts to optimize cortical functions.

INTRODUCTION

Synaptic inhibition is tightly coupled to synaptic excitation and plays a key role in cortical computations,1 including modulating sensory response properties2,3 and oscillatory activities.4 Fast synaptic inhibition in cortex is mediated by ionotropic GABA-A receptors (GABAARs), which are mainly permeable to chloride (Cl−).5–7 The inhibitory effects that GABAARs have upon a neuron therefore depend upon the local transmembrane Cl− gradient, which reflects a dynamic equilibrium between Cl− extrusion and intrusion processes.8–10

When the GABAAR equilibrium potential (EGABAAR) is more negative than the membrane potential (Vm), GABAAR activation will lead to membrane hyperpolarization. If EGABAAR is close to Vm, GABAAR activation would have minimal effects upon Vm, and the inhibitory effects would be primarily mediated by local effects on the membrane resistance (Rm)—a phenomenon known as “shunting inhibition.”11,12 These different forms of signaling determine how inhibition is temporarily integrated with incoming excitatory synaptic inputs.13,14 Thus, appreciating how fast synaptic inhibition operates is fundamental to understanding how neuronal and network activity are regulated in the intact brain.

Previous work has identified the contribution of different transmembrane Cl− fluxes in determining synaptic EGABAAR in cortex. This includes Cl− effluxes mediated by the potassium-chloride co-transporter (KCC2)15,16 as well as Cl− influxes such as those mediated by GABAARs themselves, which can vary depending upon a neuron’s activity.17,18 Previous investigations of synaptic EGABAAR relied heavily upon in vitro investigation and were therefore influenced by the distorted fluxes that operate under these recording conditions.9 Consequently, there is a lack of evidence regarding how fast synaptic inhibition operates in the intact cortex.

To address this gap, we combine optogenetic activation of GABAergic synaptic inputs with in vivo gramicidin perforated patch-clamp recordings. This enables us to measure synaptic EGABAAR and GABAAR driving forces in the intact rodent cortex, under conditions in which transmembrane Cl− gradients are preserved. We demonstrate that, in contrast to the anesthetized cortex, pyramidal neurons of the awake cortex exhibit a relatively high synaptic EGABAAR, which is close to resting Vm and generates a clear preference for shunting fast synaptic inhibition. This depolarized EGABAAR results from the high levels of synaptic activity in the awake cortex, and the resulting shunting synaptic inhibition is well placed to reduce synchrony within local cortical networks and increase the flexibility of responses to external input.
RESULTS

Measuring the equilibrium potential and driving force for synaptic GABA\(_{A}\)Rs in vivo

To study GABA\(_{A}\)-mediated synaptic signaling in vivo, we established gramicidin perforated patch-clamp recordings from layer 2/3 (L2/3) pyramidal neurons in primary visual cortex (V1) of urethane-anesthetized mice aged between six and eight weeks (Figures 1A and S1). Similar to in vitro findings,19,20 the perforation of the neuronal membrane with cation-selective gramicidin pores was marked by a decrease in the series resistance (Rs).

During the 10 min following gigaseal formation we observed a 20-fold decrease in Rs (mean Rs\(_{10}\) min: 1,126.9 ± 35.8 M\(\Omega\) vs. Rs\(_{0}\) min: 50.2 ± 3.8 M\(\Omega\)), which provided stable conditions for studying synaptic responses. By using transgenic mice that express channelrhodopsin-2 (ChR2) in Gad2-positive neurons (Figures 1A and 1C), we were able to initiate presynaptic GABA release by optically activating the axons of nearby GABAergic interneurons.21,22 This resulted in postsynaptic GABA\(_{A}\)R responses in the L2/3 pyramidal neuron, which could be recorded in either current or voltage clamp (Figures 1D and S1C).
To measure synaptic $E_{\text{GABAAR}}$ in vivo, we combined our optogenetic approach with voltage ramp protocols (Figure 1E) to minimize disruption to transmembrane Cl\textsuperscript{−} gradients and generate current-voltage (IV) plots from which the resting $V_m$ (RMP) and $E_{\text{GABAAR}}$ can be determined (Figure 1F). The internal pipette solution contained high Cl\textsuperscript{−} (~150 mM) so that the integrity of the patch could be confirmed at the end of a recording by electing to break through into whole-cell mode (Figures 1E and 1F). To check that our voltage ramp protocols provided an accurate estimate of synaptic $E_{\text{GABAAR}}$, results were compared to voltage step protocols performed in the same neuron (Figure S2). Step protocols take longer to perform but offer the chance to analyze synaptic $E_{\text{GABAAR}}$ at a defined time following presynaptic GABA release, thereby further isolating the GABAAR response (Figure S2).23 The estimates of synaptic $E_{\text{GABAAR}}$ from ramp protocols were equivalent to those made using step protocols (Figure S2E), and measurements from the ramp protocols were not related to the amplitude of the synaptic GABAAR response or the neuron’s $R_m$ (Figure S3). To further validate the setup, we assessed sensitivity to changes in synaptic $E_{\text{GABAAR}}$ caused by altering the balance of transmembrane Cl\textsuperscript{−} fluxes. As expected, application of the selective KCC2 antagonist, VU0463271 (VU), resulted in a depolarizing shift in synaptic $E_{\text{GABAAR}}$ (Figures 1G and 1H), consistent with the blocking of a Cl\textsuperscript{−} efflux. While VU caused a depolarizing shift in $E_{\text{GABAAR}}$, there was no detectable change in RMP (Figure 1I), and consequently, there was a depolarizing shift in the driving force for GABAAR Rs ($D_{\text{GABAAR}} = \text{RMP} - E_{\text{GABAAR}}$, Figure 1J).

Awake cortex exhibits more depolarized synaptic $E_{\text{GABAAR}}$ and shunting fast synaptic inhibition

We next investigated synaptic GABAAR signaling in the awake cortex (Figure 2A). In keeping with previous whole-cell studies,24,25 our gramicidin recordings in head-fixed mice revealed that L2/3 pyramidal neurons in the awake cortex exhibit high levels of synaptic activity. Compared with anesthetized cortex, awake neurons exhibited more depolarized $V_m$ (Figures 2B and 2C) and larger fluctuations in their subthreshold $V_m$ (Figure 2D). This is consistent with the elevated levels of excitatory and inhibitory synaptic activity reported in the awake cortex.24,25 The $R_m$ was also lower in the awake recordings (An.: 87.2 ± 6.1 MΩ, n = 14 neurons, 8 mice vs. Aw.: 50.6 ± 6.4 MΩ, n = 13 neurons, 10 mice, p < 0.0001, unpaired t test), consistent with the increased membrane conductance associated with high levels of synaptic activity.26 The membrane properties we observed in the anesthetized state were consistent with what has been reported with other anesthetics,3,25,26 suggesting that a more hyperpolarized mean $V_m$ and lower levels of synaptic activity are a common feature of anesthetized cortex.

The high level of synaptic activity in awake cortex includes strong activation of GABAAR-containing synapses.3 This generates conditions that are predicted to increase the Cl\textsuperscript{−} influxes (i.e., a “Cl\textsuperscript{−} load”) experienced by a cortical neuron,9,27 particularly as more depolarized $V_m$ values will increase the driving force for Cl\textsuperscript{−} to enter via the GABAAR (Figure 2E). Indeed, strong GABAAR activation can alter intracellular Cl\textsuperscript{−} levels and affect $E_{\text{GABAAR}}$ via changes in the equilibrium potential of Cl\textsuperscript{−} ($E_{\text{Cl}}$),9,27 while the equilibrium potential for bicarbonate is more stable owing to buffering mechanisms.28 We hypothesized that the conditions of the awake cortex would impact transmembrane Cl\textsuperscript{−} gradients and thereby affect synaptic GABAAR signaling. To test this prediction, we used our optogenetic approach to compare synaptic GABAAR responses between the anesthetized cortex and awake cortex. First, current clamp recordings revealed that synaptic GABAAR responses in the awake cortex produced $V_m$ deflections that remained close to the RMP and could be depolarizing or hyperpolarizing (Figure 2F). This indicated a net shunting effect for synaptic GABAAR in the awake cortex, consistent with a different $D_{\text{GABAAR}}$ that could be caused by a more depolarized synaptic $E_{\text{GABAAR}}$. We confirmed this by performing ramp protocols in voltage clamp, demonstrating that synaptic $E_{\text{GABAAR}}$ was more depolarized in the awake cortex (Figures 2G and 2H). While the RMP was also more depolarized in the awake cortex (Figure 2I), the net effect was to move the synaptic $D_{\text{GABAAR}}$ in a depolarized direction when compared with the anesthetized cortex (Figure 2J). Importantly, the synaptic $D_{\text{GABAAR}}$ in the awake cortex was not different from zero, consistent with the conclusion that GABAAR-mediated synaptic signaling favors shunting inhibitory effects in the awake state. These differences were not related to how the synaptic GABAAR Rs were activated because the amplitude of the GABAAR conductances were comparable in the anesthetized cortex and awake cortex (Figure 2K). Meanwhile, modeling of synaptic GABAAR responses in biologically realistic neurons confirmed that differences in synaptic $E_{\text{GABAAR}}$ could not be explained by the intrinsic membrane properties in anesthetized and awake cortices (Figure S4). Finally, more detailed analysis revealed modest synaptic $E_{\text{GABAAR}}$ changes within anesthetized recordings, which were consistent with the general principle9,27 that increased synaptic activity and depolarized $V_m$ result in more depolarized synaptic $E_{\text{GABAAR}}$ because of an increased Cl\textsuperscript{−} load (Figure S5).

Network activity in awake cortex raises synaptic $E_{\text{GABAAR}}$ and promotes shunting fast synaptic inhibition

Having established that awake cortex favors shunting synaptic GABAAR responses, we explored whether the more depolarized synaptic $E_{\text{GABAAR}}$ is caused by the high levels of synaptic activity that exist in the awake state. To test this, we examined the effects of reducing local network activity in awake cortex by blocking glutamatergic signaling with a local injection of the AMPA receptor antagonist, NBQX (Figure 3A). Following NBQX injection, the distribution and mean $V_m$ of cortical neurons were more hyperpolarized (Figures 3B and 3C), and fluctuations in subthreshold $V_m$ were greatly decreased (Figure 3D), consistent with the effective suppression of synaptic activity to the recorded neuron. The reduction in local network activity was also reflected in the neuron’s $R_m$, which was higher in NBQX, again consistent with decreased synaptic activity (Aw.: 50.6 ± 6.4 MΩ, n = 13 neurons, 10 mice vs. +NBQX: 136.2 ± 11.9 MΩ, n = 10 neurons, 7 mice, p < 0.0001, unpaired t test).

To investigate whether the levels of local network activity determine the nature of GABAergic signaling in the awake cortex, the polarity of synaptic GABAAR responses was compared across recordings performed with and without...
Depolarized membrane potential (RMP) and synaptic GABA conductance differences in anesthetized (An.) and awake (Aw.) cortex. (A) Current clamp recording of spontaneous activity from a L2/3 pyramidal neuron in an anesthetized (An., black, top) and awake mouse (Aw., blue, bottom).

(B) Probability density function for Vm in anesthetized (n = 13 cells, 7 mice) and awake (n = 12 cells, 10 mice) cortex. Awake data are also used in Figure 3.

(C) Mean Vm was more depolarized in awake cortex (An.: 69.1 ± 1.4 mV vs. Aw.: 60.3 ± 1.1 mV; p < 0.001, one-way ANOVA with Bonferroni correction).

(D) Mean change in subthreshold Vm was greater in awake cortex (An.: 4.9 ± 0.2 mV/ms vs. Aw.: 6.6 ± 0.3 mV/ms; p < 0.001, one-way ANOVA with Bonferroni correction).

(E) Illustration of how a more depolarized Vm and higher synaptic activity are conducive to greater Cl− correction.

(F) Averaged light-evoked postsynaptic IC responses in anesthetized (n = 18 cells, 10 mice) and awake (n = 14 cells, 11 mice) cortex. Responses in awake cortex produced Vm deflections that remained close to the RMP and could be depolarizing or hyperpolarizing (An.: 0/18 vs. Aw.: 6/14; p = 0.003, Fisher-Exact test).

(G) Summary IV plot of all anesthetized (n = 10 cells, 6 mice) and awake (n = 10 cells, 9 mice) light-evoked GABA currents reveal a more depolarized synaptic E_{GABAAR} in awake cortex.

(H) Synaptic E_{GABAAR} is more depolarized in awake cortex (An.: −81.1 ± 1.7 mV vs. Aw.: −63.3 ± 1.4 mV; p < 0.001, one-way ANOVA with Bonferroni correction).

(I) Voltage-clamp recordings confirmed a more depolarized RMP in awake cortex (An.: −70.7 ± 1.9 mV vs. Aw.: −64.2 ± 1.1 mV; p = 0.02, one-way ANOVA with Bonferroni correction).

(J) GABAAR driving force (DF_{GABAAR} = RMP − E_{GABAAR}) was more depolarized in awake cortex (An.: 10.4 ± 2.0 mV vs. Aw.: −0.9 ± 1.7 mV; p < 0.001, one-way ANOVA with Bonferroni correction). Awake DF_{GABAAR} was not different to zero, consistent with synaptic GABAARs exerting a predominantly shunting effect (An.: p = 0.0006, one-sample t test; Aw.: p = 0.61, one-sample t test).

(K) Light-evoked synaptic GABA conductances did not differ between the anesthetized and awake cortex (An.: 11.04 ± 1.8 nS vs. Aw.: 11.4 ± 1.5 nS, p = 0.92, one-way ANOVA with Bonferroni correction). ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.

NBQX (Figure 3E). In the quietened network state, light-evoked responses recorded in current clamp were found to be exclusively hyperpolarizing, which differed from the active awake state (Figure 3E). Consistent with this, reducing local network activity caused a hyperpolarizing shift in synaptic E_{GABAAR} (Figures 3F and 3G). When combined with the negative shift in RMP (Figure 3H), the net effect of reducing local network activity was to cause a hyperpolarizing shift in DF_{GABAAR}, such that the synaptic DF_{GABAAR} was now strongly hyperpolarizing (Figure 3I). Importantly, the effects of reducing local network activity with NBQX were not related to effects on the optogenetic paradigm itself, as the amplitude of the light-evoked synaptic GABAAR conductances were unaffected by the AMPA receptor antagonist (Figure 3J). More generally, this confirmed that the measurements of synaptic E_{GABAAR} were not contaminated by glutamatergic conductances. Also, the differences in synaptic E_{GABAAR} could not be explained by the neurons’ intrinsic membrane properties (Figure S4).
Shunting inhibition promotes local network desynchronization and response flexibility

The nature of synaptic inhibition affects how neurons integrate incoming excitatory input.\textsuperscript{12,13} To explore the implications of the shunting synaptic EGABAAR observed in the awake state, we first analyzed a publicly available data set of high-density Neuro-pixels (NPXs) recordings from regular spiking neurons in mouse cortex.\textsuperscript{29,30} These data allowed us to compare how the same population of neurons respond to a stimulus under awake and isoflurane-anesthetized conditions (Figure 4A). In the awake state, electrical stimuli delivered to cortex evoked spiking activity, with neurons exhibiting variability in terms of when they spiked relative to one another. In contrast, in the anesthetized state, the same stimuli evoked less overall activity, and spiking

Figure 3. High network activity raises synaptic EGABAAR and promotes shunting fast synaptic inhibition in awake cortex

(A) Setup (top) and current-clamp (IC) recording (bottom) showing the effects of reducing local network activity by acute, local delivery of NBQX (100 μM) in awake cortex.

(B) Probability density function for Vm in control awake cortex and following NBQX (Aw. blue: n = 12 neurons, 10 mice vs. +NBQX, turquoise: n = 10 neurons, 7 mice). Control data from awake group presented in Figure 2.

(C) Reducing local network activity caused a hyperpolarizing shift in mean Vm (Aw.: \(-60.3 \pm 1.1\) mV vs. +NBQX: \(-72.6 \pm 1.0\) mV; p < 0.001, one-way ANOVA with Bonferroni correction).

(D) Reducing local network activity caused a decrease in the mean change in subthreshold Vm (Aw.: 6.6 ± 0.3 mV/ms vs. +NBQX: 0.7 ± 0.1 mV/ms; p < 0.001, one-way ANOVA with Bonferroni correction).

(E) Averaged light-evoked postsynaptic IC responses (top; Aw., blue: n = 14 neurons, 11 mice vs. +NBQX, turquoise: n = 10 neurons, 7 mice). Reducing local network activity caused a hyperpolarizing shift in the polarity of light-evoked GABA currents (bottom; Aw.: Depol. 6/14 vs. +NBQX: Depol. 0/10; p = 0.03, Fisher-Exact test).

(F) Summary IV plots of light-evoked GABA currents reveal more hyperpolarized synaptic EGABAAR values following local NBQX (Aw.: n = 10 neurons, 9 mice vs. +NBQX: n = 11 neurons, 7 mice).

(G) Reducing local network activity led to more hyperpolarized synaptic EGABAAR (Aw.: \(-63.3 \pm 1.4\) mV vs. +NBQX: \(-78.1 \pm 1.3\) mV; p < 0.001, one-way ANOVA with Bonferroni correction).

(H) Reducing local network activity caused a hyperpolarizing shift in RMP (Aw.: \(-64.2 \pm 1.1\) mV vs. +NBQX: \(-71.4 \pm 1.4\) mV; p = 0.009, one-way ANOVA with Bonferroni correction).

(I) Reducing local network activity caused a hyperpolarizing shift in DFGABAAR (Aw.: \(-0.9 \pm 1.7\) mV vs. +NBQX: \(6.7 \pm 1.5\) mV; p = 0.03, one-way ANOVA with Bonferroni correction); DFGABAAR became different from zero (Aw.: p = 0.61, one-sample t test; +NBQX: p = 0.001, one-sample t test).

(J) Reducing local network activity did not affect light-evoked synaptic GABA conductances (Aw.: 11.4 ± 1.5 nS vs. +NBQX: 10.4 ± 0.7 nS; p = 0.78, one-way ANOVA with Bonferroni correction). ns, not significant; *p < 0.05; **p < 0.01; ***p < 0.001.
responses were more closely aligned to the spiking of other neurons (Figure 4B). To quantify this, we (1) determined the degree of neuronal synchrony, which relates the firing of a single neuron to the activity of the other neurons on a trial-by-trial basis,

and (2) computed the peri-stimulus histogram entropy, as a measure of the flexibility (or variability) of each neuron’s responses. This revealed that in the awake state, stimulus-evoked synchrony was lower and entropy was higher (Figures 4C and 4D). As the mean firing rate was greater in the awake state, we also established that the differences in synchrony and entropy were still evident after regressing out the effect of firing rate (Figure S6).

To compliment these in vivo recordings, we constructed simple computational network models of recurrently connected excitatory pyramidal neurons and inhibitory interneurons. This enabled us to selectively vary synaptic $E_{\text{GABA}}$ and thereby compare the effects of shunting and hyperpolarizing $E_{\text{GABA}}$-mediated synaptic inhibition upon responses to excitatory input (Figure 4E). We observed that networks utilizing shunting $E_{\text{GABA}}$ exhibited more variable input-evoked spike times, whereas networks with hyperpolarizing $E_{\text{GABA}}$ exhibited less activity and the input-evoked spiking was more closely aligned to the spiking of other neurons in the network.

Figure 4. Shunting inhibition promotes local network desynchronization and response flexibility
(A) High-density Neuropixels (NPXs) recordings were used to compare spiking activity in the same cortical neurons under awake and anesthetized conditions. (B) Example raster plots show the spiking activity of a population of neurons in somatosensory cortex (SS). Spiking activity is shown across three stimulation trials for each condition. (C) Neuronal synchrony on a trial-to-trial basis (top) and the entropy of the peri-stimulus histogram (bottom) were calculated for each of the 662 recorded neurons under awake and anesthetized conditions (17 probe recordings in 16 mice). Each dot corresponds to a single neuron and the dashed line indicates the line of equality. (D) Schematic of network model consisting of interconnected excitatory pyramidal neurons (Pyr.) and inhibitory interneurons (IN). $E_{\text{GABA}}$ was adjusted relative to the RMP to create two different conditions: a shunting (Shunt.) and a hyperpolarizing (Hyperpol.) $E_{\text{GABA}}$ condition. Spiking activity was evoked by delivering brief depolarizing currents (input) of varying amplitudes to each neuron in the network. (E) Schematic of network model consisting of interconnected excitatory pyramidal neurons (Pyr.) and inhibitory interneurons (IN). $E_{\text{GABA}}$ was adjusted relative to the RMP to create two different conditions: a shunting (Shunt.) and a hyperpolarizing (Hyperpol.) $E_{\text{GABA}}$ condition. Spiking activity was evoked by delivering brief depolarizing currents (input) of varying amplitudes to each neuron in the network. (F) Raster plots for the same population of pyramidal neurons (n = 50) in the shunting (left) and hyperpolarizing (right) $E_{\text{GABA}}$ conditions. (G) Synchrony (top) and entropy (bottom) for pyramidal neurons in the shunting and hyperpolarizing $E_{\text{GABA}}$ conditions (n = 1,000 randomly selected). Dashed line indicates the line of equality. (H) Neurons in the shunting $E_{\text{GABA}}$ condition exhibited lower synchrony (Shunt: 0.784 ± 0.001 vs. Hyperpol: 0.881 ± 0.001, p < 0.001, paired t test) and higher entropy (Shunt: 0.451 ± 0.002 nats vs. Hyperpol: 0.197 ± 0.002 nats, p < 0.001, paired t test) (n = 16,000 pyramidal neurons from the model). **p < 0.001.
optogenetic activation of synaptic GABA ARs, we have (Figures 4G and 4H). The higher entropy associated with shunt-entropy, similar to what was observed in the awake state (Figures 4G and 4H). The higher entropy associated with shunting E_{GABAAR} would also be predicted to improve a network’s ability to encode different stimuli. Indeed, when we assessed a network’s ability to encode different patterned inputs, we observed that networks with shunting E_{GABAAR} could be more accurately decoded by a downstream classifier (Figure S7). Taken together, these observations support the idea that shunting inhibition promotes the desynchronization of local cortical networks and enables greater flexibility in terms of neuronal responses.

**DISCUSSION**

To determine how fast synaptic inhibition operates in vivo, we require measurements that preserve the ionic driving forces acting upon GABAARs, while providing information that can relate a neuron’s synaptic GABAAR conductance, synaptic E_{GABAAR}, and V_{m}. Whole-cell patch-clamp recordings have revealed prominent GABAAR synaptic conductances in awake cortex, but they compromise the ionic gradients underlying synaptic GABAAR transmission. Imaging approaches have provided estimates of intracellular Cl\(^-\) levels in vivo, but they do not offer a readout of E_{GABAAR} as they do not account for extracellular ion concentrations or the permeability of the GABAAR receptor; furthermore, they typically provide information on Cl\(^-\) throughout the soma, not at GABAergic synapses. Efforts have also been made to infer synaptic GABAAR function from whole-cell recordings or extracellular recordings, although these approaches do not provide direct measurements of E_{GABAAR} or D_{GABAAR}.

By combining in vivo gramicidin perforated recordings with optogenetic activation of synaptic GABAARs, we have measured synaptic D_{GABAAR} in the intact brain. While in vivo gramicidin recordings have been performed in early developing systems, this is the first demonstration in the intact mammalian brain. Our recordings demonstrate that the nature of GABAAR signaling is linked to the network’s activity. Synaptic GABAARs in awake cortex exhibit relatively depolarized E_{GABAAR} and low D_{GABAAR}, such that their inhibitory effects are more likely to result from local changes in R_{m}, (i.e., shunting effects). This provides experimental support for theoretical predictions regarding how E_{GABAAR} reflects a dynamic equilibrium between Cl\(^-\) extrusion and intrusion processes, and how Cl\(^-\) influxes associated with high GABAAR activity can increase the Cl\(^-\) load experienced by a neuron.

Our observations have implications for how fast synaptic inhibition might vary under other in vivo network conditions that are associated with changes in ongoing synaptic activity, and how such short-term, activity-dependent changes may interact with longer-term fluctuations in Cl\(^-\) homeostasis. In addition, while acknowledging the space-clamp limitations associated with in vivo patch-clamp recordings, future work could explore GABAAR synapses in different neuronal compartments.

A synaptic E_{GABAAR} that favors shunting has important implications for neural computation. This includes effects upon synaptic integration, gain modulation, and network synchronization. In the case of synaptic inhibition that is purely shunting, the duration of inhibition is restricted to the time course of the GABAAR conductance. By contrast, hyperpolarizing GABAAR-mediated inhibition is more long-lasting, meaning it can strongly synchronize the recovery of neurons within the network. In addition to these temporal differences, the spatial effects of shunting inhibition depend upon where the GABAAR conductance occurs within the neuron and with respect to other synaptic inputs, which introduces further diversity for neural computations. Our analysis of in vivo spiking activity and network modeling experiments suggest that the shunting synaptic E_{GABAAR} observed in active awake cortical networks can promote a desynchronization of local networks and increase their ability to encode external inputs. These findings support the general idea that dynamic changes in GABAAR-mediated synaptic inhibition can be used to optimize cortical function.

**STAR METHODS**

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**SUPPLEMENTAL INFORMATION**

Supplemental information can be found online at https://doi.org/10.1016/j.neuron.2023.08.005.

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AUTHOR CONTRIBUTIONS

DECLARATION OF INTERESTS

The authors declare no competing interests.

INCLUSION AND DIVERSITY
We support inclusive, diverse, and equitable conduct of research.

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REFERENCES


STAR METHODS

KEY RESOURCES TABLE

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RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Colin Akerman (colin.akerman@pharm.ox.ac.uk).

Materials availability
No new materials were generated in this study.

Data and code availability
Custom code used in analysis of data is publicly available as of the date of publication (links provided in the key resources table). Any additional materials required to reanalyze data are available on request to the lead contact.
EXPERIMENTAL MODEL AND SUBJECT DETAILS

All mice were bred, housed and used in accordance with the United Kingdom Animals (Scientific Procedures) Act (1986). Homozygous Gad2-ires-Cre mice were crossed with homozygous Ai32(Rcl-ChR2(H134R)/EYFP) mice. This produced a heterozygous colony expressing channelrhodopsin-2 (Chr2(H134R)-YFP) in Gad2-positive neurons, which includes the main subclasses of GABAergic interneurons. Mice were purchased from Jackson Laboratory (Maine, USA). Both male and female mice were used in the experiments. Mice were maintained under a 12-hour:12-hour light-dark cycle and fed ad libitum. For all experiments both males and females were used, and mice were between six to eight weeks postnatal age at the time of recording.

METHOD DETAILS

Surgical procedures

The preparation for anesthetized recordings was adapted from previously published protocols. Mice were anesthetized with an intraperitoneal (IP) injection of 25 % urethane (1 g/kg, diluted in sterile PBS). To counteract adverse events caused by urethane, a bolus of the anticholinergic agent, Glycopyrronium Bromide (0.01 mg/kg) was administered subcutaneously (SC). Local anesthetic (Marcain 2 mg/kg) was applied intradermally to the scalp and topically in the ears prior to mounting the mouse into the head holding apparatus (Narisighe) under a surgical stereoscope (Olympus). The mouse’s body temperature was maintained at 37 degrees Celsius using a heating mat and rectal probe. The animal’s head was shaved and eye-protecting ointment (Viscotears) was applied to both eyes. An incision in the scalp was made using surgical scissors and the area expanded with blunt dissection to expose the skull. The site of the craniotomy was marked over the primary visual cortex (V1). Tissue adhesive was applied to fix the surrounding scalp to the skull and to secure cranial sutures. Multiple layers of dental cement (Simplex Rapid) were applied to create a recording chamber on top of the skull. A 0.5 mm craniotomy was drilled over the marked region using a dental drill (Foredom). The craniotomy was submerged in cortex buffer (containing, in mM: 125 NaCl, 5 KCl, 10 HEPES, 2 MgSO4 7H2O, 2 CaCl2 2H2O, 10 Glucose). The bone flap and dura were removed. The animal was then transferred to the in vivo patch setup and the recording session typically lasted 3 hours between zeitgeber time 3 (ZT3) and ZT6, at which point the animal was culled. Throughout the recording session, the depth of anesthesia was monitored by testing for pedal reflexes.

The preparation for awake recordings consisted of three phases based on published protocols. The first phase involved the fixation of the head plate. Mice were anesthetized with isoflurane (Zoetis) and mounted into a stereotaxic frame (Kopf). Subcutaneous analgesia (meloxicam 5mg/kg and buprenorphine 0.1mg/kg) was administered along with intradermal local analgesic (Marcain 2 mg/kg) into the scalp. The scalp was washed (Wahl) and cleaned (Hibiscrub). Eye-protecting ointment (Victear) was applied. The scalp was then removed and the site was washed with sterile cortex buffer (containing, in mM: 125 NaCl, 5 KCl, 10 HEPES, 2 MgSO4 7H2O, 2 CaCl2 2H2O, 10 Glucose). After drying the skull with absorbent swabs (Haag-Streit), the periosteum was removed using a micro curette (Fine Science Tools). Tissue adhesive was applied to secure cranial sutures and to fix the surrounding scalp to the underlying bone. A custom-designed aluminum headplate with a 7 mm well was bonded to the skull, first with adhesive glue (Locite), and then followed by serial layers of dental cement (Super-Bond). The well was then covered with silicone sealant (Kwik-Cast). The animal was singly housed and allowed to recover. From day three following head plate fixation, the animal was habituated to head-fixation for increasing time intervals up to 60 minutes. On the day of the recording, the animal was briefly anesthetized with isoflurane and mounted onto a stereotaxic frame. The silicone sealant was removed and the area washed with sterile cortex buffer. A 0.5 mm craniotomy was created using a dental drill (Foredom) and the bone flap removed. A durectomy was performed and the site was covered with a soft dressing soaked in cortex buffer. This step in the procedure was limited to 20 minutes. The animal was then removed to the head-fixation setup and transferred to the in vivo patch setup. The mouse was allowed to fully recover for at least 30 minutes before recording was commenced. Recording sessions typically lasted 2-3 hours between ZT3 and ZT6, at which point the animal was culled. During the recording session, the experimenter visually monitored the mouse to confirm that the animal was awake.

Electrophysiological recordings

All electrophysiological recordings were performed using a Multiclamp 700B amplifier (Molecular Devices) and digitized at 20 kHz (Digitimes 15500, Molecular Devices). A HumBug noise eliminator (Digitimer) was used to remove 50 Hz noise. To perform perforated patch clamp recordings, patch pipettes were back-filled with K+ methanesulfonate internal solution (mM: 110 KMeSO3, 6 NaOH, 3 MgCl2 6H2O, 0.02 CaCl2, 40 HEPES, 0.05 EGTA, 2 Na2ATP, 0.5 NaGTP, 2 MgATP, 10 Biocytin). To perform perforated patch clamp recordings, the internal pipette solution was prepared immediately prior to recording by combining a high chloride (150 mM) solution (mM: 141 KCl, 9 NaCl, 10 HEPES) heated to 37 degrees Celsius, with a stock solution of gramicidin A (4 mg/ml - dissolved in dimethyl sulfoxide, DMSO, Merck) to achieve a final concentration of 80 µg/mL gramicidin. The solution was then vortexed (40 seconds) and sonicated (20 seconds). The patch pipette was back-filled with the gramicidin solution and mounted on a Otopatcher pipette holder (A-M System) mounted to a 473 nm laser (MBL-FN-473-150mW, CNI Laser). Pipettes were lowered onto the brain surface and blind patching commenced. Once the gigaseal had formed, perforation was then monitored by observing changes in series resistance. Recording protocols were started once the series resistance had stabilized at <100 MΩ. In our experience, recordings that failed to reach these series resistance values within
the first 10 minutes never achieved a perforation quality that was suitable for recordings. Rupture or breakthrough of the perforation in to whole-cell configuration was detected by a sudden and persistent depolarization of the equilibrium potential of the GABA\text{\textsubscript{A}}R (E\text{\textsubscript{GABA\text{\textsubscript{A}}R}}), consistent with dialysis of the neuron with the high chloride pipette solution. In a subset of experiments, KCC2 was blocked by injecting the selective antagonist, VU0463271,\textsuperscript{52} directly into the cortex. The injection pipette contained 100 \mu M VU0463271 (Tocris) in ACSF, which was delivered at a rate of 33 nL/min to a total volume of 200 nL. In a subset of experiments where local network activity was reduced, the \alpha\text{-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) receptor blocker 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo (F) quinoxaline (NBQX) was injected directly into the cortex.\textsuperscript{53} The injection pipette contained 100 \mu M NBQX (Tocris) in ACSF, which was delivered at a rate of 33 nL/min to a total volume of 250 nL.

Data was acquired using recording protocols configured in Clampex (Molecular Devices) and analyzed using custom code written in Python. Online series resistance compensation was not used, as the high amounts of activity in the \textit{in vivo} brain would cause large fluctuations in input current, which increases the rate of perforation rupture.\textsuperscript{28,54} Therefore, to correct for series resistance effects, offline correction was performed.\textsuperscript{55} The voltage drop caused by the series resistance (R\textsubscript{s}) was calculated by multiplying the measured current response with 90 \% of the R\textsubscript{s}. The voltage drop was then subtracted from the command voltage to estimate the neuron’s membrane potential. Membrane and recording properties were calculated by measuring the change in current in response to a -10 mV step during voltage clamp recordings. R\textsubscript{s} was calculated from both the peak current elicited by the -10 mV voltage step, and by estimating the peak after fitting an exponential to the decay of the current transient response to the -10 mV step. These methods gave similar values and so the numerical average was used as a final estimate of R\textsubscript{s}. To calculate the membrane resistance (R\textsubscript{m}), R\textsubscript{s} was subtracted from the measured input resistance.

**Biocytin labelling**

For reconstruction of biocytin-filled neurons, mouse brains were fixed via transcardial perfusion of phosphate buffered solution (PBS, 0.1 M) and 4 \% paraformaldehyde solution (PFA). Brains were stored for 24 hours at 4 °C in 4 \% PFA and then washed and stored in PBS containing 0.05 \% sodium-azide. Within a week of perfusion, brains were washed in PBS and mounted onto a microtome (HM650V, ThermoScientific) before being sectioned into 100 \mu m thick coronal slices whilst bathed in PBS. For biocytin labelling, sections were incubated in Streptavidin-Cy3 (1:1000, Thermo Fisher) for 2 hours at room temperature before being mounted with Vectashield (Vectorlabs) onto glass slides (Avantor). Images were acquired using a LSM 880 microscope (Zeiss). All images were captured using a 20x water-immersion objective (W Plan-Apochromat NA 1.0) through the ZEN software (Zeiss). Image processing was performed in ImageJ software (NIH).

**Neuronal network simulations**

Computational models were used to explore the effect of synaptic E\text{\textsubscript{GABA\text{\textsubscript{A}}R} upon neuronal synchrony, entropy, and stimulus discrimina-

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Set value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Equilibrium potential of the leak current (E\text{\textsubscript{Leak}}, equivalent to RMP)</td>
<td>-60 mV</td>
</tr>
<tr>
<td>Equilibrium potential of the glutamatergic current</td>
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</tr>
<tr>
<td>Excitatory postsynaptic conductance decay time constant</td>
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<tr>
<td>Inhibitory postsynaptic conductance time constant</td>
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<tr>
<td>Membrane capacitance</td>
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</tr>
<tr>
<td>Membrane time constant</td>
<td>5 ms</td>
</tr>
<tr>
<td>Refractory period</td>
<td>5 ms</td>
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Synaptic E\text{\textsubscript{GABA\text{\textsubscript{A}}R} in glutamatergic neurons was set to -60 mV for the first half of each simulation and to -80 mV for the remainder, to represent the synaptic E\text{\textsubscript{GABA\text{\textsubscript{A}}R recorded experimentally in the awake and anesthetized states, respectively. E\text{\textsubscript{GABA\text{\textsubscript{A}}R was set to -60 mV in the GABAergic neurons. All external inputs were modelled as random, independent currents with values drawn from log-normal distributions with the scale parameter \mu set to zero, and the shape parameter sigma set to 1.}

\[ X = M \cdot \exp(\mu + \sigma \cdot Z) \]
Z represents a standard normal variable. For the stimulus inputs, we generated 100 distinct input patterns. Each pattern consisted of a set of 1 ms long depolarizing current pulses delivered simultaneously, but with variable amplitudes (M = 500 pA), to each neuron in the network. The delivery of each input pattern was followed by a 25 ms long pause and each pattern was presented 100 times in each condition (shunting or hyperpolarizing E_{GABAAR}). To prevent full synchronization of all neurons, each neuron independently also received a noise input with variable amplitude (M = 25 pA), varied every 10 ms. Each simulation was repeated 20 times.

Simulating measurements of synaptic E_{GABAAR}
To investigate the effect of intrinsic membrane properties on our estimates of synaptic E_{GABAAR} under different network conditions, a multi-compartment model of an adult pyramidal neuron from L2/3 of mouse primary visual cortex was constructed using the NEURON simulation environment. The neuron’s morphology was sourced from NeuroMorpho and based on a reconstruction (NMO_62358) that was shared by Madisen and colleagues. Model parameters are shown in Table 2.

Table 2. Parameters used for modeling effects of membrane resistance

<table>
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<th>Parameter</th>
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<tr>
<td>Membrane capacitance C_m</td>
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<tr>
<td>Axial resistance (R_{axial})</td>
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<td>Passive membrane reversal (equivalent to RMP)</td>
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<tr>
<td>An. e_{pas} = -70.7 mV</td>
<td>A_w. e_{pas} = -64.2 mV</td>
</tr>
<tr>
<td>+NBQX e_{pas} = -71.4 mV</td>
<td></td>
</tr>
<tr>
<td>Voltage clamp electrode series resistance (R_{L})</td>
<td>47.5 MΩ</td>
</tr>
<tr>
<td>Passive membrane resistance</td>
<td></td>
</tr>
<tr>
<td>An. R_m = 10^3.784 Ωcm²</td>
<td>A_w. R_m = 10^3.540 Ωcm²</td>
</tr>
<tr>
<td>+NBQX R_m = 10^3.988 Ωcm²</td>
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</table>

With these parameters, the membrane resistance (R_m) measured by simulated voltage clamp at the soma was 87.2 MΩ in the anesthetized condition, 50.6 MΩ in the awake condition, and 136.2 MΩ in the Awake + NBQX condition. These values matched the average experimentally measured membrane resistance from data (Figure S3). Activation of GABA_ARs was simulated by placing twenty GABA_A-containing synapses randomly within a 75 μm radius of the center of the soma. E_{GABAAR} was set to between -85 mV and -35 mV (iterated by 5 mV for each simulation). Activation of GABA_AR synapses was simulated by using an alpha function with a tau of 150 ms. The peak local conductance of each GABA_AR synapse was set to 2 nS. To simulate the experimental estimation of synaptic E_{GABAAR}, a simulated voltage clamp was placed at the soma. Two consecutive voltage ramps were then applied, one before and one during simulated activation of GABA_ARs (to reproduce the experimental protocol). Synaptic E_{GABAAR} was estimated using IV plots, either with 0% R_s correction, or 90 % R_s correction, to replicate the experimental data acquisition and analysis process.

QUANTIFICATION AND STATISTICAL ANALYSIS
Membrane potential measurements from current-clamp recordings
To determine the resting membrane potential (RMP) during current clamp recordings, spontaneous activity was recorded for a continuous period of five minutes. During analysis, membrane potential values greater than ~40 mV were removed to avoid distortions caused by action potentials. Average distribution plots were created by concatenating all membrane potential values across all neurons in each group, to which a Gaussian kernel-density estimate was then fitted.

Background synaptic activity
The level of subthreshold synaptic activity was determined by measuring the rate of change in the membrane potential over time, after excluding action potentials. The rate of change in membrane potential was calculated by taking the first derivative (\(V_m \frac{dV}{dt}\) in mV/ms). The first derivative was winsorized and the mean of the modulus of the \(V_m \frac{dV}{dt}\) calculated for each neuron.

Polarity of GABAergic responses
Synaptic GABA_A-mediated responses in current clamp were evoked with a 10 ms light pulse, presented during a one second sweep. The average of 15 sweeps was then calculated and normalized to the mean membrane potential during the 100 ms preceding the light pulse. Current clamp recordings from a subset of the neurons in the NBQX condition also contributed to another study. The polarity of the synaptic GABA_A response was defined as the mean normalized membrane potential during the 100 ms following the light pulse. If the mean value was greater than zero, the response was classified as depolarizing, whereas a mean value below zero was classified as hyperpolarizing.
Two types of voltage clamp protocol were used to measure synaptic $E_{GABA_A}$: a ramp protocol and a step protocol. The ramp protocol involved clamping the neuron at -70 mV, and then imposing two consecutive voltage ramps, each lasting 150 ms, which extended from 60 mV below the holding voltage to 40 mV above the holding voltage (i.e. from -130 mV to -30 mV, at a rate of 0.7 mV/ms). The first ramp (i.e. the ‘baseline’ ramp) sampled the neuron’s intrinsic membrane currents and the second ramp (i.e. the ‘light’ ramp) included a light-evoked synaptic GABA$_A$R conductance. The light-evoked synaptic GABA$_A$R conductance was elicited with a 10 ms light pulse that coincided with the start of the ramp, to ensure the evoked GABA$_A$R current was at its peak during the ramp. During analysis, the first and last 15 ms of each ramp were excluded to avoid transient currents caused by the discharge of the pipette capacitance. The currents from both ramps were then superimposed on a current-voltage (IV) plot using the series corrected membrane potential and, to avoid action potentials and capacitance transients, the current responses were cropped to only include regions that were clear of these sources of contamination. A straight line was fitted to both currents. The current from the baseline ramp was used to infer the equilibrium potential of the holding current (from which the resting membrane potential, RMP, could be inferred), defined as the voltage at which the fitted line was equal to zero. The point at which the fitted lines for the two ramps intersected is $E_{GABA_A}$. This can also be calculated by subtracting the current response during the baseline ramp, from the current response during the light ramp. The voltage at which a fitted line to this subtracted current is equal to zero, is equivalent to $E_{GABA_A}$. The driving force can then be calculated by subtracting the measured $E_{GABA_A}$ from the RMP. The slope of the subtracted current is equal to the GABA$_A$R conductance.

The step protocol meanwhile, involved estimating synaptic $E_{GABA_A}$ from a voltage clamp protocol in which the neuron was exposed to a series of voltage steps, whilst eliciting a light-evoked GABA response during each step. The voltage was stepped in 10 mV increments from -130 mV to -30 mV. Each step lasted 500 ms, with a 10 s interval between steps. The light-evoked synaptic GABA conductance was evoked 100 ms after the start of each voltage step, by delivering a 100 ms light pulse. For analysis purposes, the membrane current was measured immediately before the light pulse (i.e. ‘baseline’ current) and then 20 ms after the onset of the light pulse, which corresponds to the peak of the GABA$_A$R conductance and current (i.e. ‘light’ current, Step20ms). A further current measurement was made at the time when $E_{GABA_A}$ was estimated from the ramp protocol in the same cell (StepRamp). The currents were then used to estimate $E_{GABA_A}$, in a similar way to that described for the ramp protocol. The difference between the two $E_{GABA_A}$ values ($E_{GABA_A}$ at Step20ms and $E_{GABA_A}$ at StepRamp) allowed us to assess the stability of $E_{GABA_A}$ and the contribution of GABA$_A$Rs to our estimates of $E_{GABA_A}$, as previously described.

Pre-processing of Neuropixels dataset

We analyzed a recent dataset, made publicly available by the Allen Institute. Surgical procedures, habituation, Neuropixels recordings, electrical stimulation, and data processing including spike sorting are described in the manuscript accompanying the dataset. Briefly, up to three Neuropixels probes were inserted per animal in order to target cortical regions and thalamic nuclei of interest. Electrical stimuli were delivered via a bipolar platinum-iridium stereotrode, which was located within 0.5 mm of the Neuropixels probe and targeted either layer 5/6 or layer 2/3 of the respective area of cortex. From the resulting dataset, we sub-selected all neurons that were in somatosensory cortex (annotations “SSp-ll” & “SSp-tr”) or secondary motor cortex (annotations “MOs”), depending on which two areas had been electrically stimulated. As in Claar et al., regular spiking neurons were identified as having a waveform duration $\geq 0.4$ ms. The evoked firing rate, synchrony, and peri-stimulus histogram entropy were computed using spikes that occurred within an interval of 2–12 ms following an electrical stimulus. The start and end of this interval were chosen to remove artifacts arising from the electrical stimulation and to exclude spikes elicited by recurrent activity from the thalamus, respectively. Neurons with an average evoked firing rate below 0.1 Hz or above 200 Hz in either condition (awake/anesthesia) were excluded from further analysis (203 neurons). The 662 remaining neurons derived from 17 recordings in 16 mice, each contributing a median of 34 neurons (minimum: 1, maximum: 93).

Characterization of stimulus-evoked spiking responses

We applied measures of synchrony and entropy to describe the stimulus-evoked spiking activity from the Neuropixels in vivo recordings. To quantify the degree to which each neuron was in synchrony with the neuronal population as a whole, we adapted a measure used in Bharioke et al. For each stimulus, we computed the population response by constructing the peri-stimulus spike histogram across all recorded neurons during the 12 ms following stimulus onset, using a bin width of 1 ms. For each neuron, we then computed the average magnitude of the population response each time the neuron spiked. Our measure differed from its previous definition in as much as we normalized each stimulus-evoked population response by the total spike count for the corresponding trial rather than the average firing rate. To quantify the variance in each neuron’s stimulus-evoked responses, we computed the peri-stimulus spike histogram for the 12 ms following stimulus onset, using a bin width of 1 ms. We then calculated the normalized histogram’s entropy using the standard formula for discrete variables, as implemented in the scipy, stats python module. The same measures of synchrony and entropy were used to describe the input-evoked spiking activity in the neuronal network simulations. To determine the separability of population responses to different external inputs in the neuronal network simulations, we stimulated the network using 100 different input patterns, each repeated 100 times. For each trial we computed the spike count in 50 randomly chosen neurons. Using the resulting 10000 population vectors we then trained and tested a k-nearest neighbor classifier using 5-fold stratified cross-validation.
Statistical analyses

All data is reported as mean ± standard error of the mean (SEM). For comparative statistics, the distribution of the continuous data was first established using the Shapiro-Wilk test for normality, which guided the subsequent use of appropriate parametric and non-parametric tests. For data used in both Figures 2 and 3, a one-way ANOVA with post-hoc correction with the Bonferroni method was used. All statistical analyses was performed using the Python SciPy library (provided in key resources table). Details of the data values, sample sizes and statistical measurements are provided in the figure legends.