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# Different forms of homeostatic plasticity are engaged with distinct temporal profiles

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## Abstract

Global changes in network activity have been reported to induce homeostatic plasticity at multiple synaptic and cellular loci. Though individual types of plasticity are normally examined in isolation, it is their interactions and net effect that will ultimately determine their functional consequences. Here we examine homeostatic plasticity of both inhibition and intrinsic excitability in parallel in rat organotypic hippocampal slices. As previous studies have not examined inhibitory plasticity using a functional measure, inhibition was measured by the ability of evoked inhibitory postsynaptic potentials (IPSPs) to suppress action potentials, as well as IPSP amplitude. We show that manipulations of network activity can both up- and downregulate functional inhibition, as well as intrinsic excitability. However, these forms of plasticity are dissociable. Specifically, robust changes in intrinsic excitability were observed in the absence of inhibitory plasticity, and shifts in inhibition, but not excitability, appear to be sensitive to developmental stage. Our data establish that while the two forms of homeostatic plasticity can be engaged in parallel, there is a specific order in which they are expressed, with changes in excitability preceding those in inhibition. We propose that changes in intrinsic excitability occur first in order to stabilize network activity while optimizing the preservation of information stored in synaptic strengths by restricting changes that will disrupt the balance of synaptic excitation and inhibition.

## Introduction

The output of a neuron is not determined by its synaptic or intrinsic membrane properties in isolation, but rather by the net interaction of all these elements. Both inhibition (Crook & Eysel, 1992; Miles *et al*., 1996; Paulsen & Moser, 1998; Buzsaki, 2001; Wehr & Zador, 2003) and intrinsic excitability (Daoudal & Debanne, 2003; Zhang & Linden, 2003) play an important role in shaping neuronal responses. However, there is significantly less known about plasticity of these properties in comparison with excitatory transmission. In recent years it has been shown that specific patterns of activity lasting seconds to minutes can produce long-term potentiation or depression of both excitability (Armano *et al*., 2000; Ganguly *et al*., 2000; Daoudal & Debanne, 2003; Zhang & Linden, 2003) and inhibitory γ-aminobutyric acid (GABA)ergic synapses (e.g. Stelzer *et al*., 1987; Xie *et al*., 1995; McLean *et al*., 1996; Gaiarsa *et al*., 2002; Chevaleyre & Castillo, 2003; Marder & Buonomano, 2004) in several mammalian brain areas, and particularly the hippocampus. In contrast to these rapidly induced changes, there is now a large body of evidence that neurons also exhibit a form of homeostatic plasticity induced by increases or decreases in overall network activity over the course of hours or days (Turrigiano & Nelson, 2004).

Homeostatic plasticity can regulate evoked excitatory postsynaptic potentials (EPSP) and miniature (m)EPSC amplitudes (Muller *et al*., 1993; O'Brien *et al*., 1998; Turrigiano *et al*., 1998), as well as several other synaptic and intrinsic properties (Desai *et al*., 1999; McKinney *et al*., 1999; Lüthi *et al*., 2001; Ehlers, 2003; Aptowicz *et al*., 2004). Such plasticity of inhibitory processes has also been examined. In dissociated cultures, activity deprivation produces a decrease in expression of GABA (Rutherford *et al*., 1997) as well as a decrease in $GABA_A$ receptors and miniature inhibitory postsynaptic current (mIPSC) amplitudes (Kilman *et al*., 2002). In organotypic hippocampal cultures, changes in the $GABA_A$ receptor subunit populations (Holopainen & Lauren, 2003) and density of GAD65-immunoreactive terminals and inhibitory synapses have been observed (Marty *et al*., 2000). However, studies that examined evoked inhibitory postsynaptic potentials (IPSPs) have not observed changes in amplitude in either organotypic slices or dissociated cultures (Muller *et al*., 1993; Ivanova *et al*., 2003).

Homeostatic plasticity is hypothesized to prevent networks of neurons from entering hyper- or hypoactive states in response to changes in the biochemical environment or levels of synaptic input (Turrigiano & Nelson, 2004). To understand the role of changes in inhibition and intrinsic excitability in this process, it is important to establish whether their plasticity is bi-directional, or whether it is preferentially engaged in response to either decreases or increases in activity. Additionally, it is not known how multiple forms of homeostatic plasticity interact. It is possible that they are modified independently, sequentially, or in parallel. Different mechanisms may be engaged with distinct temporal patterns depending on the stimulus or the condition of the organism.

To address these questions we examined homeostatic changes of both functional inhibition and intrinsic excitability in hippocampal organotypic slices. Our results establish that inhibition and excitability are bi-directionally regulated in these cultures, and that intrinsic plasticity occurs alone under certain conditions. We propose that inhibitory plasticity may be restricted in the hippocampus in order to

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delay changes that can disrupt the excitatory–inhibitory synaptic balance.

## Materials and methods

### *Organotypic slices*

Hippocampal organotypic slices were prepared by the interface technique (Stoppini *et al*., 1991). Sprague–Dawley rats (Charles River) at age P7 were anesthetized with halothane and decapitated. The brain was removed and placed in ice-cold cutting media composed of Mediatech EMEM, 15-010, with the following additions (in mM): HEPES, 25; Tris base, 10; $MgCl_2$, 3; glucose, 10. Both hippocampi were removed and cut into 400-μm transverse slices on a tissue chopper. Slices were placed on a Millipore semipermeable membrane filter (Millicell CM, PICM030), in a dish with 1 mL culture media. Culture media was comprised of Mediatech EMEM 15-010 to which was added (final concentration in mM): $MgSO_4$, 1.85; glucose, 30; HEPES, 30; ascorbic acid, 0.5; $CaCl_2$, 2.5; 20% horse serum (Mediatech). Glutamine (1 mM) and penicillin/streptomycin (Gibco) were also added (10 units or μg/mL). Culture media was changed within 2 h of dissection, then again after 24 h, and on alternate days following that. Slices were incubated at 35 °C in 5% $CO_2$ for 10–12 or 18–20 days prior to recording.

### *Electrophysiology*

Whole-cell recordings were made from CA1 pyramidal neurons using an Axoclamp 2B amplifier (Axon Instruments). Recording electrodes pulled from 1.5-mm outer diameter glass were 4–7 MΩ in resistance, with the following internal solution (in mM): K-gluconate, 100; KCl, 20; ATP-Mg, 4; phosphocreatine, 10; GTP, 0.03; HEPES, 10. Internal solution had a pH of 7.3 and osmolarity of 290–300 mOsm. Recordings were carried out at 32 °C in oxygenated artificial cerebrospinal fluid (ACSF) with (in mM): NaCl, 125; KCl, 2.5; $MgSO_4$, 2.0; $NaHCO_3$, 26.2; $NaH_2PO_4$, 1; glucose, 25; $CaCl_2$, 2.5. The ACSF also contained 10 μM CNQX and 50 μM APV. All chemicals were purchased from Sigma-Aldrich. Recordings were considered acceptable if the cells showed overshooting action potentials and their resting membrane potential did not vary by more than 5 mV. The series resistance of the current clamp recordings (20.8 ± 0.76 MΩ) was compensated by bridge balancing. Cells with an initial series resistance greater than 30 MΩ were discarded. Additional criteria required an input resistance of > 80 MΩ, which did not change by more than 15% during the course of the recording. Input resistance was calculated from the difference between the resting potential and the steady-state potential from a 250-ms hyperpolarizing (−0.1 nA) pulse. All cells were held at a membrane potential of −60 mV.

### *Experimental protocol*

Slices were incubated with media containing either CNQX and APV (20/50 μM), TTX (1 μM) or bicuculline methiodide (10 μM) for 2 or 4 days prior to recording. Recordings were done at either 10–12 days *in vitro* (DIV) or 18–20 DIV. Drugs were refreshed every 24 h, and media for control sister slices from the same animals was changed daily as well.

The effectiveness of functional inhibition was tested in current-clamp by a series of three stimuli with a 10-s interstimulus interval, repeated four–seven times. First, an extracellular electrode placed in the stratum radiatum at the CA3–CA1 border, < 600 μm from the recording electrode, was used to elicit a train of five GABAergic IPSPs with a 25-ms interpulse interval at approximately 60 μA. This was followed by a 250-ms depolarizing current injection in the cell, set at an intensity that produced approximately 6–8 spikes. The number of spikes produced by depolarization alone in the measured window was not significantly different between pharmacological treatments and controls in any set of experiments. The last stimulus consisted of the depolarization paired with the IPSPs. The degree of effectiveness of inhibition was measured as the difference between the number of spikes produced by the depolarization alone, and the number of spikes produced during the depolarization together with the IPSPs (Fig. 1A and B). This value was compared between control and drug-treated populations by unpaired two tailed Student's *t*-tests. The time window used for the difference calculation was defined by the onset of the IPSPs and the offset of the depolarizing current (pictured in the control histogram in Fig. 1B).

To measure evoked IPSC reversal potential, measurements were taken in uncompensated continuous single electrode voltage-clamp. Trains of five IPSCs such as those used for testing functional inhibition were elicited at five–seven holding potentials ranging from −85 to −45 mV. Cells accepted for analysis maintained access resistance of < 20 MΩ during voltage-clamp recording and did not vary by more than 15% in input resistance. Reversal potential for each cell was calculated by first averaging the amplitude of the first PSC across three sweeps at each holding potential. The best linear fit of peak current vs. membrane potential allowed interpolation of the point of reversal. Paired-pulse kinetics were determined by finding the ratio of the slope of the first PSP or PSC in a train to the slope of the second. Slopes were calculated as the absolute value of the maximal onset slope between 5 and 95% of the peak response.

Intrinsic excitability of an individual cell was measured as the number of spikes in response to a series of six fixed 250-ms current injection steps (0.03, 0.05, 0.1, 0.15, 0.2, 0.3 nA). The number of spikes was averaged over four sweeps for each current intensity. Differences between groups were determined by two-way ANOVA across pharmacological treatment and current intensity with intensity as a repeated measure. To measure spike-firing properties, traces with exactly five action potentials during current injection were selected from each experimental group. The first spikes of these sweeps were used to calculate the average spike waveforms. The regularity of spike firing patterns was measured for each cell as the standard deviation of the four interspike intervals (ISIs), and these values were compared across groups with unpaired two-tailed Student's *t*-tests.

## Results

Experiments were conducted in organotypic hippocampal slice cultures, a system that maintains the basic architecture and synaptic organization of tissue *in vivo* (Stoppini *et al*., 1991; De Simoni *et al*., 2003). Though shifts in mIPSC amplitudes have been reported in dissociated culture (Kilman *et al*., 2002), previous work has failed to observe changes in evoked IPSPs in response to changes in network activity (Muller *et al*., 1993; Ivanova *et al*., 2003). Thus, our first goal was to examine whether bi-directional homeostatic plasticity of both inhibition and intrinsic excitability is present in hippocampal organotypic cultures.

### *Homeostatic plasticity of functional inhibition*

Whole-cell current-clamp recordings were carried out in CA1 neurons in slices aged 10–12 DIV. We define functional inhibition here as the efficacy of evoked synaptic inhibition in preventing cells from producing action potentials. To measure this property, for each cell a 250-ms depolarization was set at an intensity that evoked 6–8 spikes

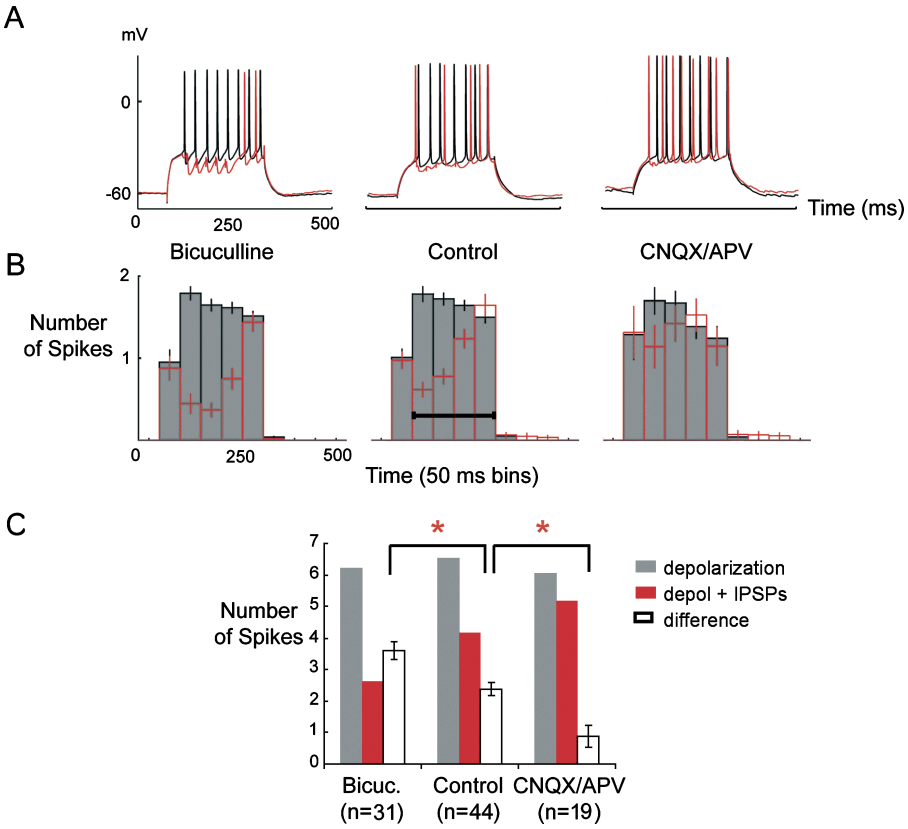


FIG. 1. Functional inhibition shows bi-directional shifts due to pharmacologically induced changes in activity. (A) Individual traces from cells in bicuculline- (left), control (centre) and CNQX/APV-treated slices (right). A 250-ms depolarization produces action potentials (black), which are blocked to varying degrees by a train of five inhibitory postsynaptic potentials (PSPs, red). (B) Average data histograms for bicuculline, control and CNQX/APV experiments. Spikes are grouped in 50-ms bins across the 500-ms window. Grey bars show spikes from depolarization alone, red outlined bars show spikes in response to depolarization with inhibitory postsynaptic potentials (IPSPs). (C) Summary of changes in functional inhibition. Data collapsed across the time window indicated in black on the control histogram in (B) were used for measurement of inhibition. Comparisons of the differences (black outline bars) between depolarization alone and depolarization with IPSPs show that both bicuculline and CNQX/APV groups are significantly different from control (*$P < 0.001$).

on average. The number of spikes produced by depolarization alone (Fig. 1A, black traces) was compared with that produced by simultaneously depolarizing the cell and extracellularly evoking a train of five GABAergic PSPs (Fig. 1A, red traces). Recordings were done in ACSF containing the glutamatergic antagonists CNQX and APV, allowing us to examine IPSPs without contamination from excitatory transmission.

To induce homeostatic plasticity, global network activity was modified by incubating the slice cultures with pharmacological agents for 4 days prior to recording. The $GABA_A$ receptor antagonist bicuculline was added to the culture media to increase activity rates, while the glutamatergic antagonists CNQX and APV were used together to decrease overall slice activity. Figure 1A shows example responses from a control cell, and one from each experimental group. The bi-directional plasticity of inhibition is evident in the histograms showing the average data across all cells for each condition (Fig. 1B). IPSPs appear to block more spikes in the bicuculline-treated slices than in controls, and fewer spikes in the CNQX/APV slices. Figure 1C shows the average difference between the depolarization and depolarization–PSP conditions for the time indicated in black on the control histogram in Fig. 1B. The time window is defined by the onset of the first IPSP, and ends at the offset of the depolarization. This measure of functional inhibition reflects the ability of the train of IPSPs to prevent action potentials. Overall, incubation of the slices with bicuculline increased functional inhibition ($P < 0.001$), while CNQX/APV produced a decrease ($P < 0.001$).

### *Activity-dependent modification of inhibitory reversal potential*

The amplitude of the first IPSP was significantly larger in the CNQX/APV group (3.67 ± 0.91 mV) compared with controls (1.23 ± 0.52 mV; $P < 0.02$). Figure 2A shows the train of IPSPs averaged across cells for each group at −60 mV (left panel), and in conjunction with the depolarizing pulse (with spikes filtered out, right panel). An analysis of short-term plasticity, using the ratio of the slope of the second to the slope of the first PSP in the train, showed no significant difference between the CNQX/APV group (1.07 ± 0.16) and controls (1.04 ± 0.13, $P = 0.95$). There was no significant difference in the bicuculline group's peak first IPSP amplitude (2.51 ± 1.01 mV) and that of the controls ($P = 0.23$). Though bicuculline treatment did cause an increase in paired-pulse depression (0.64 ± 0.05, $P < 0.001$), the overall increase in functional inhibition suggests that the short-term kinetics are not the primary mechanism for this type of homeostatic plasticity. IPSPs appear depolarizing at rest, as the theoretical reversal potential given the experimental solutions is approximately −52 mV.

The increased IPSP amplitude and decreased functional inhibition found in the CNQX/APV group is consistent with a shift in the IPSP equilibrium potential. A more depolarized reversal potential would account for the increased IPSP amplitude, as the driving force would be greater at rest than it would be for controls. It would also explain the decreased efficacy of the IPSPs in preventing spikes given the closer proximity of the shifted equilibrium potential to the firing threshold. To investigate this possibility, we estimated the inhibitory reversal potential in controls and cells treated with CNQX/APV. Under voltage-clamp, trains of IPSCs were evoked using the same extracellular stimulation as described above. Calculations of the reversal potential for each cell based on these data show a significant shift ($P < 0.02$, unpaired Student's $t$-test) for inhibitory currents in CNQX/APV-treated cells, which reversed at −45.5 (± 1.56) mV, in comparison with the −52.9 (± 2.40) mV found for controls (Fig. 2B). In agreement with the current-clamp data, paired-pulse measurements in the two groups do not show any differences in the kinetics of the first two IPSCs of the train ($P = 0.15$).

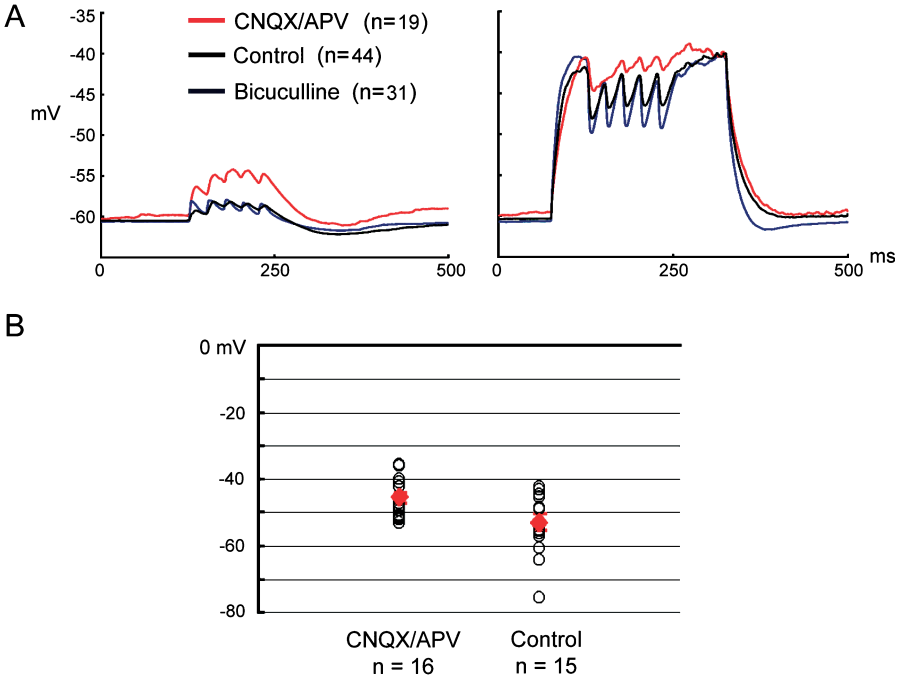


FIG. 2. Changes in IPSP reversal may contribute to shifts in the efficacy of functional inhibition. (A) Average mean filtered traces of the data from the experiments measuring functional inhibition. Left: traces shown represent trains of IPSPs. Right: IPSPs combined with depolarization. Average currents used for depolarization are (in nA): 0.11 ± 0.01 CNQX/APV, 0.15 ± 0.01 control and 0.22 ± 0.01 bicuculline. (B) Plot of the individual reversal potentials (black) calculated for a set of CNQX/APV-treated cells compared with controls. Filled symbols indicate group averages.

### *Bi-directional regulation of intrinsic excitability*

The number of action potentials evoked by a 250-ms depolarization was used as a measure of intrinsic excitability (see Materials and methods), and an input–output profile was developed for each cell by recording these responses across six preset current injection intensities. As in the previous experiments, CNQX and APV were present in the recording ACSF to ensure that measurements of excitability were not affected by responses to glutamatergic synaptic signals. Figure 3A shows responses to the 0.2-nA step from individual cells in the bicuculline-, control and CNQX/APV-treated groups. In accordance with results from previous studies (Desai *et al*., 1999; Aptowicz *et al*., 2004), complete activity block with tetrodotoxin (TTX) for 4 days *in vitro* caused an increase in excitability compared with control slices, as seen in Fig. 3B ($F_{1,65} = 21.77$, $P < 0.001$). Decreases in excitatory network activity produced by CNQX and APV caused a similar increase ($F_{1,76} = 22.06$, $P < 0.001$). In contrast, incubation of the slices with bicuculline caused a decrease in excitability ($F_{1,86} = 27.61$, $P < 0.001$). Therefore, this intrinsic property, like inhibition, exhibits bi-directional plasticity in response to changes in slice activity.

Average spike waveforms, depicted in Fig. 4A and B, revealed no significant differences in spike amplitude between either experimental group and control. However, there was a significant reduction in afterhyperpolarization (AHP) between the CNQX/APV spikes and controls (Fig. 4A, inset ii; $P < 0.001$), and the bicuculline-treated cells showed a slightly depolarized spike threshold (Fig. 4B, inset i;

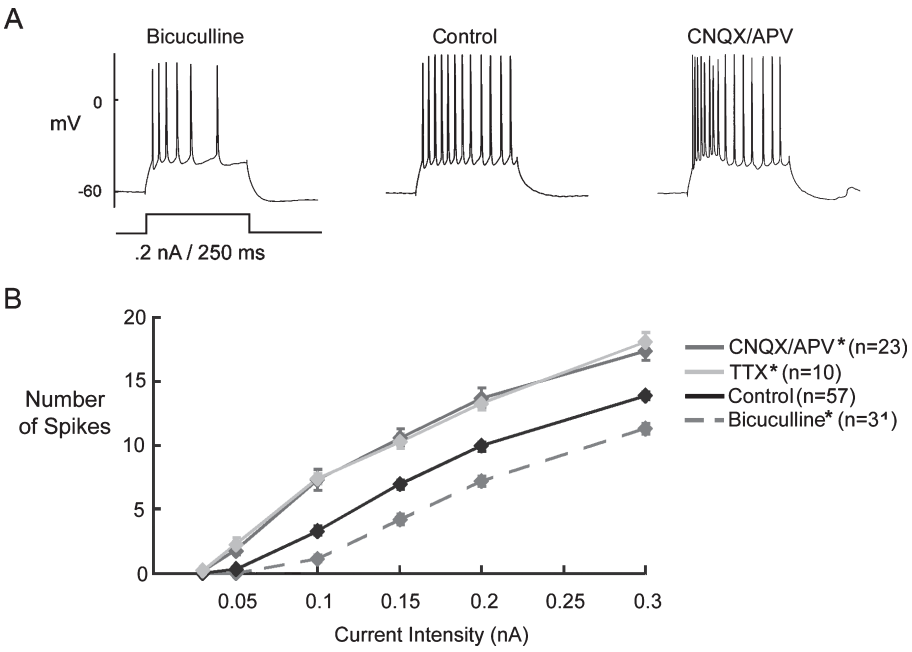


FIG. 3. Homeostatic plasticity of intrinsic excitability. (A) Sample recordings from cells in bicuculline-, control and CNQX/APV-treated slices. All three cells shown here have identical input resistances of 160 MΩ; spikes were produced by a 250-ms 0.2-nA current step. (B) Average data across groups. CNQX/APV- and TTX-treated cells show increases in excitability in comparison with controls. Cells from bicuculline-treated slices show a decrease in excitability. All shifts are significant; *$P < 0.001$.

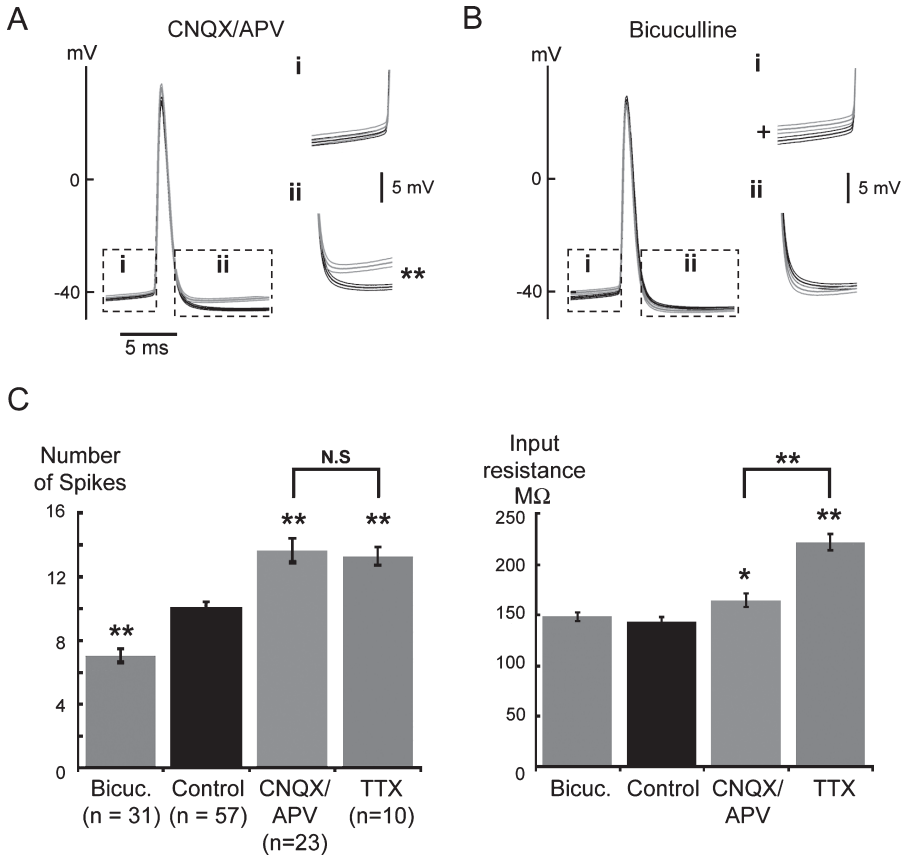


FIG. 4. Activity-dependent shifts in intrinsic properties. (A) Average spike waveforms for CNQX/APV (grey, $n = 14$), control (black, $n = 32$) cells. Fine lines represent standard error. Inset (i) shows a 5-ms magnified window of the spike thresholds. Inset (ii) shows a magnification of 9 ms of the AHP, which is significantly decreased in the CNQX/APV group. (B) As in (A), average spike waveforms for bicuculline (grey, $n = 16$) and control (black, $n = 32$) cells. The bicuculline group shows depolarized thresholds compared with controls. (C) Left panel: average number of spikes produced for each condition in response to a 0.2-nA depolarization. Right panel: input resistances from the same cells. Comparisons of the two indicate that input resistance is not an adequate predictor of excitability. Significant differences from controls are represented by $+P < 0.05$, $*P < 0.01$ and $**P < 0.001$.

$P < 0.05$). Examination of the ISIs also revealed changes in overall spiking patterns. Specifically, the CNQX/APV group showed significantly more variability than controls ($P < 0.001$), while the spike rate in the bicuculline group became less variable ($P < 0.02$) as measured by the standard deviation of the ISIs (see Materials and methods). Therefore, both the net input–output relationships and the actual spike firing patterns are altered by changes in global network activity.

Analysis of input resistance revealed no difference between the bicuculline group and controls ($P = 0.41$, unpaired $t$-test), and an increase in input resistance in CNQX/APV and TTX cells ($P < 0.01$ and $P < 0.001$, respectively; Fig. 4C, right panel). However, in comparison with the excitability results (Fig. 4C, left panel), there is not a direct correspondence between changes in excitability and input resistance. For example, the spike outputs of the CNQX/APV and TTX groups were nearly indistinguishable, but there was a significant difference in input resistance between them ($P < 0.001$). Additionally, in the CNQX/APV group there was no significant correlation between input resistance and excitability ($r_{23} = 0.22$; $P = 0.3$). Coupled with the changes in AHP and threshold across the data, these results suggest that while passive membrane conductances can contribute to intrinsic plasticity, at least in the CNQX/APV group, changes in active conductances as seen by Desai *et al*. (1999) and Aptowicz *et al*. (2004) are involved as well.

### *Parallel changes in excitability and inhibition in the same 10–12 DIV cells*

The data presented suggest that inhibition and excitability are regulated in parallel by changes in slice activity. We next wanted to determine if both mechanisms act cooperatively or in competition within a particular cell. To address this issue we measured the two properties together in the same neurons. As expected from the data described, CA1 cells from slices treated with CNQX/APV showed a decrease in inhibition as compared with controls ($P < 0.001$), which was matched by an increase in excitability ($F_{1,46} = 12.06$; $P < 0.001$). However, the overall correlation between functional inhibition and excitability for the CNQX/APV condition was not significant ($r_{14} = 0.36$, $P = 0.21$). This implies that though both processes are shifted synergistically to compensate for changes in activity, there may be distinct biochemical pathways for the regulation of different forms of homeostatic plasticity.

### *Sequential recruitment of different forms of homeostatic plasticity*

It is not clear from the data presented whether both intrinsic and inhibitory plasticity are engaged at the same time, or if neurons initially use one mechanism to adjust their activity levels and then recruit other mechanisms if necessary. To examine this issue we incubated 10–12 day-old slices with CNQX/APV for 2, as opposed to 4, days. Inhibition and excitability were tested together in the same cells. The 2 days in drugs (DID) treatment did not produce a change in functional inhibition (Fig. 5A, $P = 0.68$), though there were significant changes in intrinsic excitability compared with control slices (Fig. 5B, $F_{1,24} = 7.23$; $P < 0.015$). There was a clear trend for cells in the CNQX/APV group to have an increased input resistance (CNQX/APV: 160.2 ± 10.3; control: 137.4 ± 5.7; $P = 0.054$). Because 4 DID altered both properties together, the dissociation after 2 DID suggests that these mechanisms are

expressed sequentially, with changes in excitability preceding those in inhibition.

### *Homeostatic regulation in slices aged 18–20 DIV*

As the development of organotypic slices seems to mimic that of the hippocampus *in vivo* (e.g. De Simoni *et al*., 2003), it is possible for us to examine the effects of development on inhibitory homeostatic plasticity using slices of age 18–20 DIV. The protocol was identical to that carried out in the initial 10–12 DIV slices; drugs were added to the culture media beginning 4 days prior to the day of recording. As can be seen from the averaged group data in Fig. 6, there is little difference between the evoked IPSP trains at rest and during depolarization. Neither the bicuculline- ($P = 0.77$) nor the CNQX/APV ($P = 0.27$)-treated 18–20 DIV cultures showed a significant change in inhibition compared with controls (Fig. 7A and B). However, intrinsic excitability did exhibit bi-directional plasticity (CNQX/APV: $F_{1,52} = 8.67$, $P < 0.01$; bicuculline: $F_{1,50} = 15.20$, $P < 0.001$, Fig. 7C). Similarly, the other intrinsic properties tested showed changes that mimicked the 10–12 DIV results. Using ISIs from control slices for comparison, spike firing rates became more irregular in the CNQX/APV group ($P < 0.001$), and more regular in the bicuculline group ($P < 0.05$). Additionally, there was a significant difference in input resistance between CNQX/APV-treated slices and controls ($P < 0.01$), but not between bicuculline-treated slices and controls ($P = 0.44$). Analysis of spike waveforms revealed a shift in the spike threshold for the bicuculline group ($P < 0.01$), and a reduced AHP for the CNQX/APV cells ($P < 0.05$).

These results were consistent with a subset of experiments in which both inhibition and excitability were measured in the same cells. The 'matched' data at 18–20 DIV showed no significant change in inhibition after incubation with CNQX/APV ($P = 0.13$) despite an increase in intrinsic excitability ($F_{1,42} = 4.71$; $P < 0.05$; data not shown). These data reveal that homeostatic plasticity may be developmentally regulated.

## Discussion

Using hippocampal organotypic slices, we have shown that functional inhibition can undergo bi-directional homeostatic plasticity and that intrinsic excitability can be similarly modified. Recordings of both

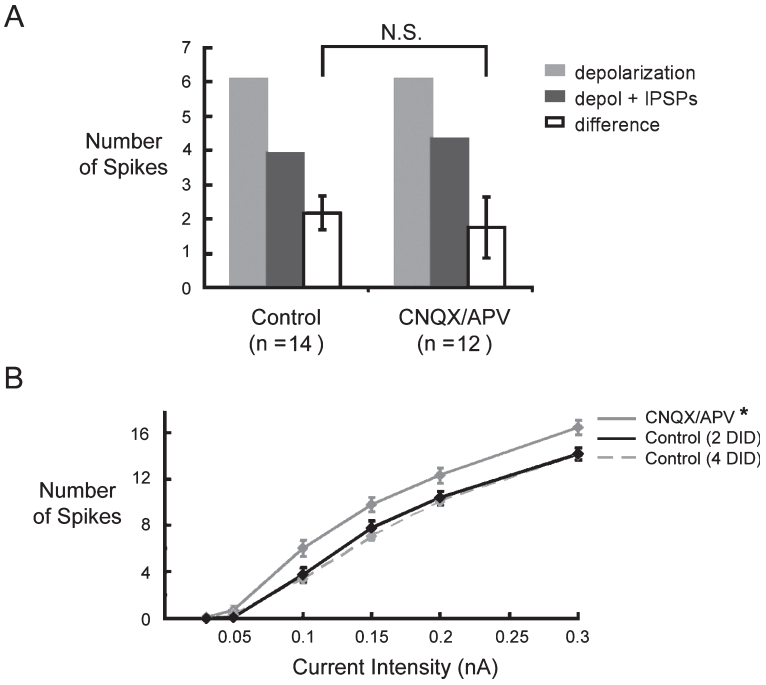


FIG. 5. Dissociation between intrinsic plasticity and inhibition after 2 days in drugs (DID). (A) Summary of average data reveals no differences in functional inhibition after treatment with CNQX/APV in comparison with sister controls. (B) Matched data from the same cells in (A) show an increase in excitability in CNQX/APV-treated neurons (*$P < 0.015$).

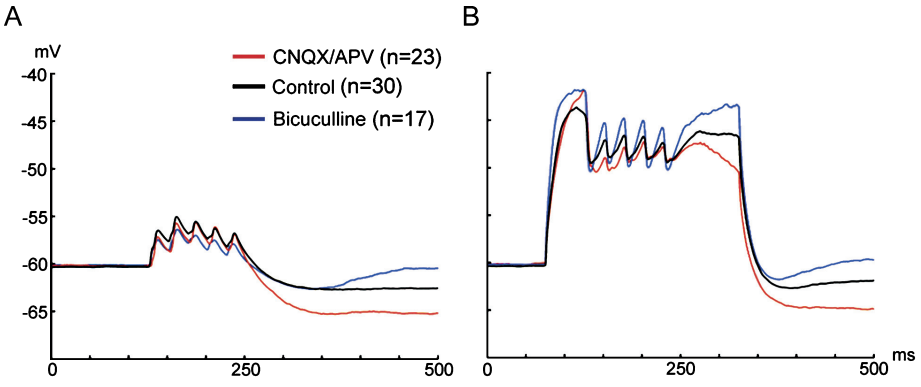


FIG. 6. IPSP responses are not different across groups in 18–20 DIV slices. Average mean filtered traces of the data from the experiments measuring functional inhibition. (A) Traces shown represent trains of IPSPs. There is no significant difference in amplitude between either the CNQX/APV (2.72 ± 1.1 mV) or bicuculline (2.51 ± 1.0 mV) groups and controls (3.74 ± 0.94 mV; $P = 0.48$ and $P = 0.37$, respectively). (B) IPSPs combined with depolarization. Average currents used for depolarization are (in nA): 0.15 ± 0.1 CNQX/APV, 0.16 ± 0.1 control and .25 ± 0.02 bicuculline.

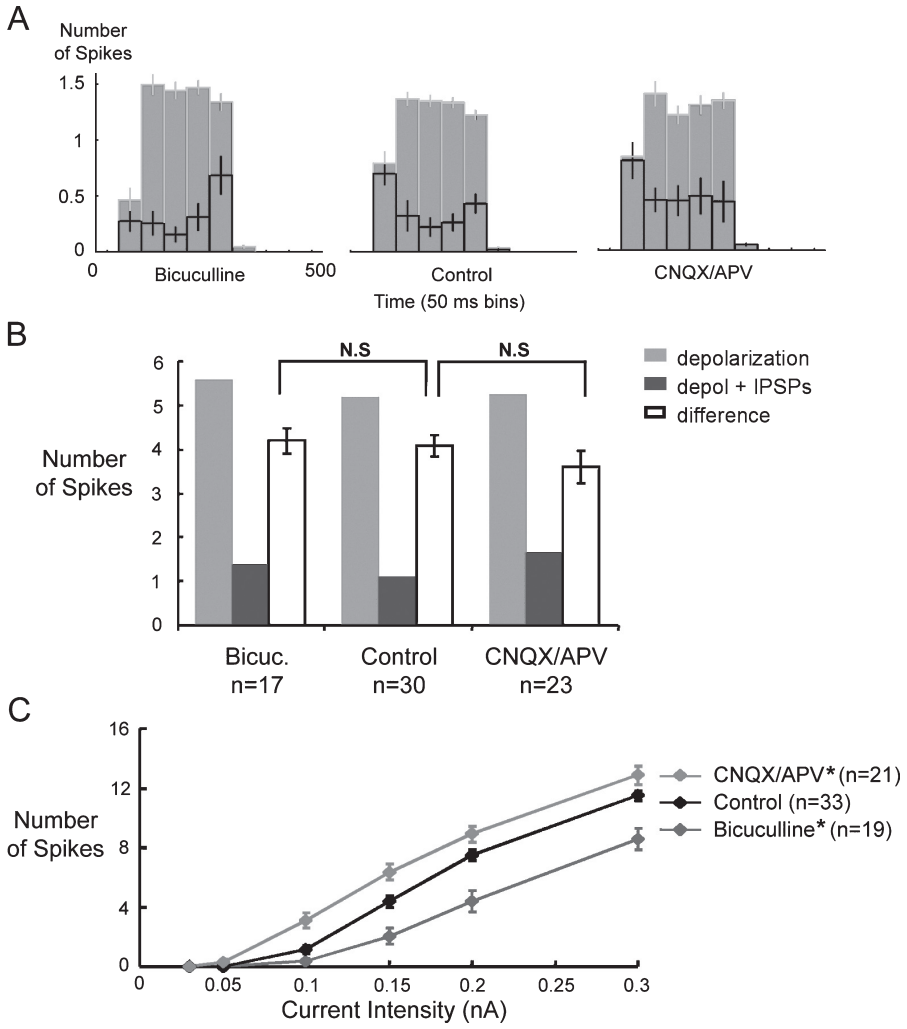


FIG. 7. Presence of intrinsic plasticity in the absence of inhibitory shifts in 18–20 DIV slices. (A) Histograms showing average profile of functional inhibition for bicuculline, control and CNQX/APV groups of 18–20 DIV slices. Grey bars show spikes from depolarization alone, black outlined bars show response to depolarization with IPSPs. (B) Average summary inhibitory data from (A), using the same time window as used in the 10–12 DIV experiments shows no significant changes between either set of drug-treated slices and controls. (C) Average intrinsic excitability data for bicuculline, control and CNQX/APV conditions shows significant shifts in excitability for both experimental groups. Significant differences are shown by $*P < 0.01$.

properties in the same cells show that while both forms of homeostatic plasticity can be expressed together, they are also dissociable. Below we discuss the physiological and computational implications of these findings.

### *Homeostatic plasticity of functional inhibition*

The effects of changes in activity on evoked inhibitory synaptic responses have been examined in two previous studies. In dissociated hippocampal cell cultures, no differences in the amplitude of evoked IPSCs were found after kyunerate treatment for 16–22 days (Ivanova *et al*., 2003). The experimenters did report an increase in paired-pulse depression of IPSCs, which would effectively decrease overall functional inhibition. Muller *et al*. (1993) also reported that no changes in IPSP amplitude occurred in 15–23 DIV hippocampal organotypic slices treated with bicuculline or picrotoxin for 3 days. Similarly, in an *in vivo* protocol for epilepsy, which resembled the induction of homeostatic plasticity via increases in activity, Buhl *et al*. (1996) observed no shift in evoked IPSP amplitude, but reported a decrease in paired-pulse depression. Though we too found no changes when plasticity was measured in slices of a similar age as the above studies (18–20 DIV), we did observe shifts in functional inhibition and IPSP amplitude at 10–12 DIV. Our results may be accounted for by an age-sensitive period for homeostatic plasticity of IPSPs (see 'Developmental changes in homeostatic plasticity' section).

The bi-directional inhibitory plasticity after 4 DID at 10–12 DIV is consistent with evidence of bi-directional changes in the density of synaptic terminals staining positive for GAD65 in CA1 of organotypic slices after 13 days in either bicuculline or the AMPA channel antagonist DNQX (Marty *et al*., 2000). Changes in the overall number of inhibitory terminals could underlie these results, as there is evidence that the homeostatic decreases in mIPSC amplitude observed in dissociated cortical cultures are due to a decrease in $GABA_A$ receptors and synapse density (Kilman *et al*., 2002). It is also possible that there is a presynaptic locus to plasticity of inhibition, such as a shift in the excitability of inhibitory terminals, or in release probability.

Our data, however, support a change in the $Cl^-$-mediated reversal potential as an additional mechanism underlying inhibitory plasticity. This is suggested by the dramatic decrease in functional inhibition in the CNQX/APV-treated cells coupled with an increase in IPSP amplitudes, and confirmed by direct estimation of the IPSCs equilibrium potential (Fig. 2). We should stress that the calculation of reversal potential was not done with perforated patch and was thus subject to washout, which can produce false negatives. Still, as we observed a positive significant difference between the CNQX/APV and control groups, our measures were actually likely to have underestimated the magnitude of the shift.

### Activity-dependent changes in intrinsic excitability

Plasticity of intrinsic excitability has been shown in several systems in response to various patterns of activity (Disterhoft *et al*., 1986; Crow, 1988; Ganguly *et al*., 2000; Daoudal & Debanne, 2003; Zhang & Linden, 2003). In the stomatogastric system, extensive theoretical and experimental studies have shown that neurons can maintain their overall firing patterns and their role in the network through regulation of excitability (e.g. LeMasson *et al*., 1993; Turrigiano *et al*., 1994). Our observations also showed that the timing or overall pattern of action potentials was modified in an activity-dependent manner. The ability of intrinsic excitability to be both up- and downregulated, as shown here, and the flexibility in induction of plasticity suggest a general role in adjusting to changes in network activity.

Though inhibition is regulated in a cooperative manner with intrinsic excitability, the dissociation between these forms of plasticity under specific conditions suggests that shifts in inhibition are moderated by separate mechanisms. Additionally, the increase in input resistance after CNQX/APV treatment does not appear to relate to the observed shifts in inhibition, as higher input resistance would be expected to enhance the effects of inhibitory currents rather than reducing them. However, evidence from granule cells in the cerebellum shows that there can be interaction between GABAergic inhibition and excitability in the form of tonic inhibition (Brickley *et al*., 1996). Extrasynaptic $GABA_A$ receptors have been shown to mediate a tonic current, providing a shunting inhibition that could affect cell firing rates (also see Zhang & Linden, 2003). Homeostatic shifts in tonic inhibition would predict changes in input resistance, which were not observed in the bicuculline condition. Such shifts also do not account for the changes in spike waveforms. Nevertheless, our experiments cannot rule out tonic inhibition as a possible contributor to intrinsic plasticity (Mody, 2005).

Data from activity-suppressed dissociated cortical cultures and organotypic slices suggest that changes in a number of ion channels, most notably $Na^+$- and TEA-sensitive channels, underlie shifts in excitability (Desai *et al*., 1999; Aptowicz *et al*., 2004). In CNQX/APV-treated cells at both developmental stages, we observed decreases in the spike AHP and increases in input resistance. In contrast, input resistance in the bicuculline cells did not shift, but the spike threshold increased. Thus, it is most likely that a number of intrinsic membrane conductances are modulated to induce changes in excitability. It is important to note that though increases and decreases in excitability are presented as two directions along the same functional scale, they are not necessarily achieved by regulation of the same factors.

### Developmental changes in homeostatic plasticity

We have found that homeostatic plasticity of inhibition and excitability are not co-regulated in the 18–20 DIV slices, as there was no significant inhibitory plasticity at this stage. From our data, it appears that age causes an overall increase in inhibition (Figs 1 and 7). This increase and the accompanying decrease in excitability of controls ($F_{1,88} = 24.277$, $P < 0.001$) would actually be predicted by the general increase in spontaneous activity observed as organotypic slices age (Buonomano, 2003). However, more inhibition overall cannot account for the lack of inhibitory plasticity. Treatment with CNQX/APV would be expected to cause decreases in inhibition, which would be easily detectable in comparison with the high levels of inhibition in the controls.

Instead, the dependence on developmental stage is consistent with our observation that the change in inhibition was associated with shifts in the IPSP reversal potential. In the neonatal rat, it has been shown that hippocampal GABAergic PSPs are depolarizing (Ben-Ari, 2002; for review). The reversal potential for GABA-induced currents shifts at approximately P7 to change the GABAergic PSPs from depolarizing to hyperpolarizing as a result of increased expression of the KCC2 transporter (Rivera *et al*., 1999). Thus, during this plastic earlier period, network activity may be able to directly influence KCC2 expression (i.e. Ganguly *et al*., 2001; Wardle & Poo, 2003; but see Ludwig *et al*., 2003). A shift in reversal potential may not be possible at later developmental stages, when the appropriate balance of excitation and inhibition and mature levels of the KCC2 transporter have been established.

Experiments from Ganguly *et al*. (2001) examined dissociated cultures from the hippocampi of E18 rats, in which controls showed the hyperpolarizing shift in the $Cl^-$ reversal potential during the first 2 weeks *in vitro*. Long-term blockade of GABAergic channels with bicuculline actually prevented this change, implying a decrease in inhibition. Additionally, neither TTX nor glutamatergic antagonists had an effect. The reason for the differences from our data may be that their cultures were taken from E18 rats at a point where GABAergic PSPs are depolarizing and the glutamatergic system is still developing. These dissociated cultures would not be expected to be at the same maturational stage as our organotypic slices from P7 rats, in which GABAergic PSPs are already primarily inhibitory. Thus, taken as a whole, these results present a strong case for the influence of developmental stage on the expression of homeostatic plasticity of hippocampal inhibition.

Homeostatic plasticity of other synaptic properties such as miniature excitatory postsynaptic currents (mEPSC) amplitudes also appears to be affected by age, as seen in experiments in the rat cortex *in vivo* (Desai *et al*., 2002). Furthermore, sensitivity to developmental stage has been revealed in experiments that manipulate the activity of a single neuron, as opposed to the entire network (Burrone *et al*., 2002). Suppressing excitability in one cell reduced functional synaptic inputs if done before synapse formation in culture. Instead, if the cell's activity is suppressed after synapse formation, the experimenters observed an increase in mEPSC frequency that agrees with other observations of homeostatic plasticity. In contrast to these synaptic properties, our data show that intrinsic excitability does not have the same developmental restrictions and exhibits bi-directional plasticity regardless of the age of the slice.

### Computational implications

It is hypothesized that information is stored in the strengths of the synapses of a given neuron, and that during learning these strengths are modified (Bliss & Collingridge, 1993; Buonomano & Merzenich, 1998; Martin *et al*., 2000). While most research has focused on plasticity of excitatory neurons, inhibitory neurons have widespread connections that shape individual neurons and network activity, particularly in the hippocampus (Freund & Buzsaki, 1996), and these synapses also undergo plasticity (Gaiarsa *et al*., 2002). Another potential site of plasticity is the intrinsic excitability of neurons, which can be modified by various types of associative protocols as described above (Daoudal & Debanne, 2003; Zhang & Linden, 2003; for review). It is often assumed that changes in intrinsic excitability are less robust than synaptic plasticity in response to associative protocols. In contrast, the data presented here suggest that changes in excitability may be the most robust locus of plasticity due to changes in network activity.

We suggest that homeostatic plasticity relies initially on changes in intrinsic excitability so as not to 'interfere' with previously stored information. The balance of excitatory and inhibitory synaptic inputs has been shown to be crucial for shaping some forms of learning and cortical processing of sensory information (e.g. Zheng & Knudsen, 1999; Wehr & Zador, 2003; Gabernet *et al*., 2005; Wilent & Contreras, 2005). By modifying intrinsic excitability, neurons may maintain the relative balance in their synaptic strengths. In cases where excitability alone is not sufficient to overcome the change in activity, neurons would additionally engage synaptic forms of homeostatic plasticity. It is not yet known whether changes in EPSP strength occur before or after changes in excitability. As the name implies, synaptic scaling, or homeostatic plasticity of mEPSP amplitude, provides a manner to maintain the relative ratios of excitatory synaptic strengths, and potentially preserve stored information (Turrigiano *et al*., 1998). However, given the non-linearities between the interaction between excitation and inhibition (e.g. shunting), excitatory homeostatic synaptic plasticity could still result in the loss of information. We would thus predict that synaptic scaling of EPSPs is also delayed in comparison with changes in excitability.

### *Conclusions*

Using hippocampal organotypic slices, we find that functional inhibition exhibits bi-directional homeostatic plasticity, but that its expression is delayed in comparison with that of intrinsic excitability. This discrepancy in temporal onset and their age-dependent dissociation suggests that there are independent pathways regulating each form of plasticity. However, it is clear that neurons posses multiple loci to up- or downregulate their activity, only two of which have been examined here. Thus, it will be important for future studies to examine the interaction and temporal profiles of other forms of homeostatic plasticity in the same system.

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## Abbreviations

ACSF, artificial cerebrospinal fluid; AHP, afterhyperpolarization; DID, days in drugs; DIV, days *in vitro*; EPSP, excitatory postsynaptic potential; GABA, γ-aminobutyric acid; IPSP, inhibitory postsynaptic potential; ISI, interspike intervals; mEPSC, miniature excitatory postsynaptic current; mEPSP, miniature excitatory postsynaptic potential; mIPSC, miniature postsynaptic current; PSP, postsynaptic potential; TTX, tetrodotoxin.

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