Coincident Pre- and Postsynaptic Activity Modifies GABAergic Synapses by Postsynaptic Changes in Cl⁻ Transporter Activity

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Summary

Coincident pre- and postsynaptic activation is known to induce long-term modification of glutamatergic synapses. We report here that, in both hippocampal cultures and acute hippocampal slices, repetitive postsynaptic spiking within 20 ms before and after the activation of GABAergic synapses also led to a persistent change in synaptic strength. This synaptic modification required Ca²⁺ influx through postsynaptic L-type Ca²⁺ channels and was due to a local increase in K⁺-Cl⁻ cotransport activity, effectively reducing the strength of inhibition. Thus, GABAergic synapses can detect and be modified by coincident pre- and postsynaptic spiking, allowing the level of inhibition to be modulated in accordance to the temporal pattern of postsynaptic excitation.

Introduction

Activity-induced modification of synaptic strength is believed to be a cellular mechanism underlying the refinement of developing connections (Katz and Shatz, 1996) as well as the learning and memory functions of the nervous system (Bliss and Collingridge, 1993; Martin et al., 2000). Persistent activity-dependent changes in synaptic strength are often referred to as long-term potentiation (LTP) or long-term depression (LTD), which are best characterized at hippocampal excitatory synapses (Bliss and Lomo, 1973; Nicoll and Malenka, 1995). In different brain regions and at different developmental stages, GABAergic synapses may also undergo bidirectional short-term changes in synaptic strength following repetitive presynaptic stimulation (Fitzsimonds et al., 1997; Komatsu and Iwakiri, 1993; Komatsu, 1996; Marty and Llano, 1995; McLean et al., 1996). In the cerebellum, brief high-frequency stimulation of presynaptic fibers results in a rebound depolarization-induced spiking and LTP of inhibitory postsynaptic potentials (Kano et al., 1992), whereas the same presynaptic activation with reduced postsynaptic spiking results in LTD (Alzenman et al., 1998). While these studies suggest that postsynaptic activity can regulate the strength of inhibitory synapses, it is not known whether these synapses can detect and be persistently modified by precisely coincident pre- and postsynaptic activity.

At many excitatory synapses, coincident pre- and postsynaptic spiking of different temporal patterns has been shown to result in either LTP or LTD (Bi and Poo, 1998, 2001; Debanne et al., 1998; Feldman, 2000; Froemke and Dan, 2002; Magee and Johnston, 1997; Markram et al., 1997; Zhang et al., 1998); excitatory inputs which are repetitively activated before postsynaptic spiking become strengthened, while those activated after postsynaptic spiking become weakened. This allows for associative modification of coincident excitatory inputs, as suggested by Hebb’s postulate of learning (Hebb, 1949). Furthermore, this precise timing requirement reflects a dynamic interplay of membrane depolarization and Ca²⁺ influx through the NMDA receptor as well as voltage-gated Ca²⁺ channels (Bi and Poo, 1998; Koester and Sakmann, 1998) and argues for the incorporation of temporal specificity into the concept of coincidence detection at excitatory synapses (Abbott, 2000; Bi and Poo, 2001). Whether similar coincidence detection mechanisms also exist at inhibitory synapses is unclear. The precision with which GABAergic synapses can detect coincidence will be of particular interest in light of findings that spike timing may be used to encode information in neural networks (Hopfield, 1995).

In the present work, we have examined whether inhibitory synapses can be associated with modified postsynaptic spiking and further explored the cellular basis of coincidence detection at GABAergic synapses. Our findings suggest that coincident pre- and postsynaptic spiking alters the activity of KCC2, a K⁺-Cl⁻ cotransporter, resulting in changes in the reversal potential of GABAergic synaptic currents (E_{GABA}) and modulation of the strength of GABAergic inhibition. This novel form of postsynaptic modulation of GABAergic synapses allows coordinated changes in excitatory and inhibitory inputs to the postsynaptic neuron in response to neuronal activity.

Results

Coincident Pre- and Postsynaptic Activation Modifies GABAergic Synapses

We first examined whether the timing of postsynaptic spiking relative to presynaptic stimulation affects the efficacy of GABAergic synapses. Whole-cell perforated patch-clamp recordings with gramicidin (25 µg/ml) were made from pairs of interconnected neurons in low-density rat hippocampal cultures. Gramicidin forms pores which are permeable to monovalent cations and small uncharged molecules but not to Cl⁻, permitting reliable recordings of GABAergic currents (Kyrozis and Reichling, 1995; Owens et al., 1996). GABAergic postsynaptic currents (GPsCs) were identified by the time course, reversal potential (−50 to −90 mV), and sensitivity to blockade by bicuculline (10 µM), a GABA_{A} receptor antagonist. Current pulses were injected into the postsynaptic neuron (current clamped at −70 mV) to fire action potentials in synchrony with repetitive presynaptic stimulation (5 Hz, 30 s), with the peak of postsynaptic spiking...
relative to presynaptic activation varied from 0 to ±90 ms (Figure 1A, inset). We found a persistent increase in the amplitude of GPSCs (voltage clamped between −65 to −85 mV) when repetitive postsynaptic spiking occurred within 20 ms before or after the onset of the GABAergic postsynaptic potentials (GPSPs) (Figures 1A and 1B). The changes in the GPSC amplitude induced by coincident activity averaged 24.2% ± 4.0%, a significant difference compared to baseline control values (p < 0.001, unpaired two-tailed Student’s t test; n = 6; Figure 1B). Experiments where no spike-timing induction protocol was given showed no significant change in GPSC amplitude over the same duration (p = 0.13, n = 8; Figure 1B). Due to the difficulty in obtaining stable access resistance during long-term recordings with gramicidin, further experiments were performed with amphotericin B perforation (150–220 μg/ml). Since amphotericin B is partially permeable to Cl−, experiments began only when control recordings ensured a steady-state [Cl−] was established. In the latter experiments, coincident pre- and postsynaptic spiking within ±20 ms also produced a significant persistent increase in the GPSC amplitude (26.4% ± 5.1%; p < 0.001, n = 24; Figure 1B). No significant differences were observed between results obtained using either amphotericin B or gramicidin (p = 0.3, unpaired two-tailed Student’s t test). Furthermore, coincident pre- and postsynaptic activation also induced similar significant increases in
GPSPs or postsynaptic potentials (Potentials notion for by effect min post- not the the coincident to in from with repetitive or Positive found protocols not postsynaptic in inducing the 1989b). or obvious GPSC in postsynaptic involving the GPSCs and the slope conductance of GPSCs at various times before and after repetitive stimulation (Figure 3). We found that the voltage dependence of GPSCs and the slope conductance did not change during the control period. However, after coincident pre- and postsynaptic activity, E_{\text{GPSC}} shifted toward more depolarizing values, consistent with the increase in the GPSC amplitude, with no obvious change in slope conductance (Figures 3A, 3A, and 3C). This shift in E_{\text{GPSC}} was statistically significant when compared to the shift in E_{\text{GPSC}} for noncoincident, presynaptic-only, or postsynaptic-only protocols (p = 0.002, one-way ANOVA followed by Scheffe pairwise comparison).

Since GPSC amplitudes during the control period were monitored with the postsynaptic neuron voltage clamped below the E_{\text{GPSC}}, the shift toward more positive levels following coincident activity led to an increase in the driving force for synaptic currents and, thus, an increase in the GPSC amplitude.

In contrast to the above observations, we found no significant change in either E_{\text{GPSC}} or the slope conductance when the interval between pre- and postsynaptic spiking was outside the interval of ±50 ms (p > 0.05; Figures 3B, 3B, and 3C). Furthermore, repetitive spiking of either the pre- or postsynaptic neuron alone (at 5 Hz, 30 s) had no significant effect on E_{\text{GPSC}} (p > 0.05; Figure 3C). Interestingly, repetitive presynaptic stimulation alone resulted in a significant reduction of the slope conductance when compared to coincident pre- and postsynaptic activity (p = 0.01, Figure 3C), which could account for the persistent reduction in the GPSC amplitude. In all experiments involving coincident activity, both the total conductance and the rise time of GPSCs were unchanged. The 10%–90% rise time during the control period (2.6 ± 0.9 ms; n = 9) and that found 10–20 min after stimulation were not significantly different (99.7% ± 5.1% of control values, n = 9), suggesting no obvious alteration in the space clamp of the recording. Taken together, our results support the notion that there was a true shift in the E_{\text{GPSC}} triggered only by coincident pre- and postsynaptic activity.

To determine whether GABAergic synaptic transmission, in addition to the coincident pre- and postsynaptic activity, is required for the increase in GPSC amplitude, experiments were performed with bicuculline (10 μM)
Figure 3. Mechanisms Underlying Changes in the GPSC Amplitude

(A1 and B1) Example recordings of changes in the GPSC amplitude induced by repetitive coincident (+5 ms, [A1]) and noncoincident (+75 ms, [B1]) activity, using gramicidin perforated patch. Traces below depict sample GPSCs before (1) and after (2) repetitive stimulation (scales: 100 pA, 20 ms).

(A2 and B2) GPSC amplitudes were measured at different clamping voltage and plotted against the holding voltage before (diamonds, solid line) and 10 min after (circles, dashed line) repetitive stimulation. The line represents the best linear fit; its abscissa intercept determines the $E_{\text{GPSC}}$ and its slope is taken as the synaptic conductance.

(C) Summary of changes in $E_{\text{GPSC}}$ and synaptic conductance under various stimulation conditions. Data represent average changes (±SEM) in $E_{\text{GPSC}}$ and slope conductance at 10-20 min after repetitive coincident activity relative to that observed during the control period. Change in $E_{\text{GPSC}}$ induced by coincident activity (spike-timing interval within ±20 ms) is significantly different from that found for all other conditions ($p < 0.002$), including noncoincident activity (spike-timing interval beyond ±50 ms). The change in slope conductance for presynaptic stimulation only is significantly different from that found for coincident activity ($p < 0.01$). The number refers to the number of experiments.

(D) Examples of recordings performed with picrotoxin (top panel; 50 μM; black bar) or bicuculline (bottom panel; 10 μM; black bar) present during the coincident stimulation protocol (+5 ms; arrow), using gramicidin perforated patch.
or picrotoxin (50 μM), both GABA<sub>a</sub> antagonists, present during the coincident stimulation. No apparent increase in the GPSC amplitude was induced by the stimulation protocol, as indicated by the measurements done after the antagonists were completely washed out (bicuculline, n = 5; picrotoxin, n = 3; Figure 3D). Thus, GABAergic synaptic transmission is required for the associative modification of GPSCs.

To further examine potential presynaptic changes in transmitter release after coincident activation, we measured the paired-pulse ratio of GPSCs, which normally reflects the release probability of the presynaptic neuron. Paired-pulse ratios (PPRs) were obtained by comparing the amplitude of GPSCs produced by stimulating the presynaptic neuron with a pair of pulse stimuli. At an interval of 50 ms, the amplitude of the second GPSC was found to be the same as the first GPSC, resulting in an average PPR of 1.0 ± 0.06 (n = 4) during the control period. At 10–20 min following coincident spiking, the PPR was 1.0 ± 0.08 (n = 4). The absence of any significant change in PPR does not support presynaptic changes in transmitter release probability as an underlying mechanism of synaptic modification by coincident activity.

**The Role of K<sup>+</sup>-Cl<sup>-</sup> Cotransporter Activity**

An obvious cellular mechanism mediating acute activity-induced changes in [Cl<sup>-</sup>]<sup>i</sup> is the modulation of various Cl<sup>-</sup> cotransporters (Jarolimek et al., 1999; Misgeld et al., 1986; Payne et al., 1996; Rivera et al., 1999; Rohrbough and Spitzer, 1996; Sun and Muralis, 1999) and channels (Smith et al., 1995) that are known to regulate [Cl<sup>-</sup>]<sup>i</sup>. Members of the K<sup>+</sup>/Cl<sup>-</sup> cotransport gene family expressed in hippocampal neurons include the Na<sup>+</sup>/K<sup>+</sup>/2Cl<sup>-</sup> (NKCC) cotransporter, which normally raises [Cl<sup>-</sup>]<sup>i</sup> (Sun and Muralis, 1999), and KCC2, which normally lowers it (Payne et al., 1996; Rivera et al., 1999). To examine the mechanism underlying the coincident activity-induced shift of E<sub>GPSC</sub> toward more positive levels, we bath applied furosemide (100 μM), a loop diuretic that inhibits cation chloride cotransport (Jarolimek et al., 1999; Payne et al., 1996; Thompson and Gahwiler, 1989b). We found that in most neurons examined (7/9) the furosemide treatment resulted in an increase in GPSC amplitude due to a shift in E<sub>GABA</sub> toward positive levels (from −66.2 ± 2.1 to −57.1 ± 4.4 mV; n = 7), and subsequent coincident pre- and postsynaptic activity became ineffective in modifying the GPSC amplitudes (Figures 4A, top panel, and 4C).

Two types of hippocampal neurons have been distinguished after 27–31 days in vitro under experimental [K<sup>+</sup>]<sub>j</sub> and [Cl<sup>-</sup>]<sub>j</sub> conditions: cells that show either similar or different dendritic and somatic GABA-induced currents, based on differences in the driving force of these currents (Kelsch et al., 2001). In cells that showed differences in dendritic versus somatic currents, furosemide abolished the differences, suggesting furosemide-sensitive KCC2 may generate a somatodendritic [Cl<sup>-</sup>]<sup>i</sup> gradient. Consistent with this idea, we found that in furosemide-insensitive neurons, which were encountered infrequently in our cultures, coincident activity was ineffective in altering GPSCs (n = 2; Figure 4A, middle panel). Furthermore, neurons that exhibited an activity-induced increase in GPSC amplitudes also showed furosemide sensitivity when tested with the drug at the end of the experiment (n = 9; Figure 4A, bottom panel). We note that these experiments were performed in HCO<sub>3</sub>-free extracellular solution, thus GABA<sub>a</sub> receptor activation-dependent HCO<sub>3</sub>- efflux (Frech et al., 1999) did not contribute to the increase in [Cl<sup>-</sup>]<sup>i</sup> observed.

Furosemide may also inhibit the NKCC cotransporter (Cabanchik and Greger, 1992), which differs from KCC2 by being insensitive to extracellular K<sup>+</sup> (Thompson and Gahwiler, 1989b). However, we found that in these hippocampal neurons E<sub>GPSC</sub> was sensitive to external K<sup>+</sup> and the sensitivity was blocked in the presence of furosemide (Figure 4B), consistent with the specific effect of furosemide on KCC2. Figure 4C summarizes the effects of furosemide treatment on activity-induced changes in the GPSC amplitude after coincident and noncoincident activity. The elevation of GPSC amplitude induced by coincident activity was totally abolished by the furosemide treatment. Taken together, these results support the notion that changes in the GPSC amplitude by coincident activity result from an activity-induced inhibition of KCC2 and a shift of E<sub>GPSC</sub> toward more positive levels.

**Somadendritic Differences in [Cl<sup>-</sup>]<sup>i</sup>**

To further examine whether differential KCC2 activity in hippocampal neurons is capable of creating a local change in [Cl<sup>-</sup>]<sup>i</sup>, we compared the difference between synaptic E<sub>GPSC</sub> and the reversal potential of GABA-induced currents (E<sub>GABA</sub>) measured at the soma. The synaptic E<sub>GPSC</sub> was determined by stimulating the presynaptic neuron and recording the resulting current that passed when the postsynaptic neuron was held at varying holding potentials (−90 to −50 mV, 5 mV increments). The reversal potential of E<sub>GABA</sub> was determined by puffing pulses of GABA (20 ms) onto the soma, in the absence of presynaptic stimulation. The average E<sub>GABA</sub> at the soma was −60.0 ± 2.5 mV (n = 9), which was significantly more positive than that of E<sub>GPSC</sub> observed at the synapse in the same neurons (−65.3 ± 3.3 mV, p = 0.0002, paired two-tailed Student’s t test). Moreover, the difference between E<sub>GABA</sub> at the soma and E<sub>GPSC</sub> became insignificant after repetitive coincident stimulation (−61.9 ± 2.7 and −61.5 ± 3.1 mV, respectively, p = 0.5). These results are consistent with the notion of a higher KCC2 activity at the dendrite and the subsequent reduction of KCC2 activity induced by coincident activity, which eliminated the somadendritic difference.

**The Role of Postsynaptic Ca<sup>2+</sup> Elevation**

Since voltage-gated Ca<sup>2+</sup> channels are likely to be activated by postsynaptic spiking, we further examined the effect of nimodipine, an inhibitor of high-threshold voltage-gated L-type Ca<sup>2+</sup> channels, and 1,2-bis(2-Aminophenoxy)ethane-N,N,N’,N’-tetraacetic acid (BAPTA), a fast [Ca<sup>2+</sup>]<sup>i</sup> buffer, on the increase of the GPSC amplitude resulting from coincident activation. As shown in Figure 5, in the presence of nimodipine (15 μM) or with BAPTA (30 mM) in the postsynaptic whole-cell pipette, there was a gradual reduction in the GPSC amplitude after repetitive coincident stimulation within the interval of ±20 ms (5 Hz, 30 s; Figures 5A and 5B). Furthermore,
we observed that there was no significant change in $E_{\text{GPSC}}$. The decrease in GPSC amplitude was accounted for by the reduction in total synaptic conductance (Figure 5C).

To exclude the possibility that the above result using BAPTA was due to the increased Cl⁻ exchange from the pipette to the cytoplasm by the use of whole-cell recordings, which are required for effective loading of BAPTA, we examined the effects of coincident pre- and postsynaptic activation on GPSCs, using whole-cell recordings in the absence of BAPTA (Figure 5B). We found that changes in the GPSC amplitude induced by coincident activity averaged 47.4% ± 19.1% (n = 4; Figure 5B), which was not significantly different from those observed using either gramicidin or amphotericin B (p = 0.6). Thus, postsynaptic spiking appears to activate a nimodipine-sensitive Ca²⁺ influx, leading to both a shift of $E_{\text{GPSC}}$ and a protection against a decrease in the Cl⁻ conductance due to repetitive synaptic activation.

Synapse Specificity in the Activity-Induced Modification

Is the activity-induced shift in $E_{\text{GPSC}}$ restricted to activated synapses that are exposed to coincident pre- and postsynaptic activation? We address this question of synapse specificity using triple-perforated patch-clamp recordings from neurons making divergent or converging GABAergic connections. For the GABAergic presynaptic neuron making diverging connections with two postsynaptic cells, we found that repetitive coincident stimulation of one pair of pre- and postsynaptic neurons resulted in a significant elevation of the GPSC amplitude for the synaptic connection between them, without affecting the efficacy of the connection made on to the other postsynaptic neuron (Figure 6A; n = 5), which was voltage clamped at −70 mV during the period of coincident stimulation. Repetitive coincident stimulation produced a 4.2 ± 0.8 mV depolarizing shift in $E_{\text{GPSC}}$ at the stimulated input, while $E_{\text{GPSC}}$ at the unstimulated input also shifted in the depolarizing direction but to a much lesser extent (0.9 ± 0.3 mV).

Seven triplets with convergent GABAergic inputs onto a single postsynaptic neuron were also examined. In 5/7 cases, we found that the increase in GPSC amplitude induced by coincident pre- and postsynaptic activation resulted in significant increase in the GPSC amplitude at the stimulated input, while the input from the unstimulated neuron remained unchanged or depressed (Fig-
Coincident Spiking Modifies GABAergic Synapses

In the remaining two cases, increases in GPSC amplitude were found at both stimulated and unstimulated inputs; however, the stimulated input always underwent a larger change in GPSC amplitude (Figures 6B and 6D). For the convergent inputs, $E_{\text{GABA}}$ underwent on average a $4.9 \pm 1.1 \text{ mV (n = 7)}$ shift toward more positive levels at the stimulated input, while the unstimulated input also underwent a shift in the same direction but to a lesser extent ($2.3 \pm 1.2 \text{ mV, n = 7}$). These results suggest that activity-induced regulation of KCC2 and the consequent changes in dendritic $[\text{Cl}^-]$ are largely synapse specific for converging inputs, although substantial spread to the unstimulated input could occur under some circumstances, a variability that may result from differences in the relative dendritic location of the convergent inputs.

Coincident Activity Modifies GABAergic Synapses in Hippocampal Slices

In order to confirm that modification of GABAergic synapses by coincident pre- and postsynaptic activity is not a phenomenon occurring only in the culture preparation, experiments were performed in acute rat hippocampal slices (postnatal days 12 to 19). Presynaptic GABAergic neurons were stimulated with a bipolar stimulating electrode placed in S. radiatum, while gramicidin-perforated patch-clamp recordings were made from CA1 pyramidal neurons. All experiments were performed in the presence of CNQX (15 μM) and D-AP5 (25 μM) to block NMDA and AMPA receptors, respectively, in order to reveal GABAergic synaptic currents. The identity of GABAergic currents was confirmed on the basis of their time course, reversal potential ($-50$ to $-90 \text{ mV}$), and their sensitivity to blockade by bicuculline (10 μM). The spike-timing induction protocol involved injecting current pulses into the postsynaptic neuron (current clamped at $-70 \text{ mV}$) to fire action potentials in synchrony with presynaptic activation at a frequency of 5 Hz (30 s). When the peak of the postsynaptic action potential relative to the onset of the GPSPs was set within ±20 ms, a persistent increase in the GPSC amplitude was observed (21.0% ± 6.5%, n = 7; Figures 7A, and 7C). The magnitude of the GPSC increase in response to coincident pre- and postsynaptic activity was not significantly different from that observed in hippocampal cultures described above ($p = 0.63; \text{Figure 7C}$).

Current-voltage plots determined before and after the spike-timing induction protocol indicate that the increase in the GPSC amplitude was accompanied by a shift in $E_{\text{GAPC}}$ to more positive values (3.2 ± 0.5 mV; n = 4; Figures 7A) and 7D), similar to that found in culture experiments. The decrease in slope conductance (3.2% ± 4.1%, n = 4) was also not significantly different from that observed in culture ($p = 0.4$). In contrast, pre- and postsynaptic stimulation with noncoincident spiking intervals (beyond ±50 ms) resulted in a decrease in the GPSC amplitude ($-13.9% ± 12.2%; n = 5$; Figures 6C and 6D).

Figure 5. The Role of L-Type Ca$^{2+}$ Channels and Postsynaptic Ca$^{2+}$ Elevation

(A) Changes in GPSC amplitudes induced by coincident stimulation (5 Hz, 30 s) in the presence of nimodipine (15 μM).
(B) Summary of the effect of BAPTA on coincident stimulation-induced changes in GPSC amplitudes. The recording was performed with breakthrough whole-cell recording in the presence (filled circles) or absence (open circles) of BAPTA (30 mM) in the recording pipette.
(C) Summary of effects of nimodipine and BAPTA on the changes in $E_{\text{GAPC}}$ and synaptic conductance induced by coincident stimulation. The differences in $E_{\text{GAPC}}$ and conductance changes between the control group (coincident stimulation in the absence of the drugs) and the drug-treated groups were significant (nimodipine: $E_{\text{GAPC}}$, $p = 0.0001$; conductance, $p = 0.04$. BAPTA: $E_{\text{GAPC}}$, $p = 0.04$; conductance, $p = 0.0002$).

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**References**

1. **Figure 5.** The Role of L-Type Ca$^{2+}$ Channels and Postsynaptic Ca$^{2+}$ Elevation

2. **Figure 6.** Changes in GPSC amplitudes induced by coincident stimulation (5 Hz, 30 s) in the presence of nimodipine (15 μM).

3. **Figure 7.** Summary of the effect of BAPTA on coincident stimulation-induced changes in GPSC amplitudes. The recording was performed with breakthrough whole-cell recording in the presence (filled circles) or absence (open circles) of BAPTA (30 mM) in the recording pipette.

4. **Figure 8.** Summary of effects of nimodipine and BAPTA on the changes in $E_{\text{GAPC}}$ and synaptic conductance induced by coincident stimulation. The differences in $E_{\text{GAPC}}$ and conductance changes between the control group (coincident stimulation in the absence of the drugs) and the drug-treated groups were significant (nimodipine: $E_{\text{GAPC}}$, $p = 0.0001$; conductance, $p = 0.04$. BAPTA: $E_{\text{GAPC}}$, $p = 0.04$; conductance, $p = 0.0002$).
Figure 6. Synapse Specificity in the Coincident Stimulation-Induced Modification of GABAergic Synapses

(A) Example recording of a triplet with divergent connections made by a GABAergic neuron onto two postsynaptic neurons. Enhancement of GPSC amplitude was observed at the connection exposed to coincident stimulation, but no change was observed at that made onto the (unstimulated, control) neuron.

(B and C) Example recording of two triplets with convergent connections made by two GABAergic neurons onto one postsynaptic neuron. Increase in the GPSC amplitude induced by coincident stimulation resulted in either a similar increase (B) or a reduction (C) in the GPSC amplitude of the other (unstimulated, control) convergent input.

(D) Summary of results on the spread of synaptic modification between divergent GABAergic outputs and converging GABAergic inputs. Data collected from triplets similar to those shown in (A)-(C), and percentage changes in the average GPSC amplitude recorded from the same triplet are connected by the line.

7B, and 7C). The percentage decrease in the GPSC amplitude observed in acute hippocampal slices was not significantly different from the decrease produced by noncoincident activity in culture (p = 0.52; Figure 7C). Similar to the experiments performed in culture, the decrease in GPSC amplitude resulting from coincident activity protocols did not result from a significant change in $E_{\text{GPSC}}$ (1.4 ± 0.2 mV; n = 3, p = 0.75; Figure 7D). The decrease in GPSC amplitude produced by noncoincident activity was accompanied by a 4.1% ± 3.4% change in slope conductance, which was not significantly different from that observed in hippocampal cultures (p = 0.3).

Neuronal excitability and the efficacy of synaptic transmission can depend upon the temperature of the neuronal tissue (Schoepfle and Erlanger, 1941; Takeya et al., 2002). To determine whether the ability of coincident pre- and postsynaptic activity to modify GABAergic synapses was temperature dependent, experiments were also performed on acute hippocampal slices heated to 35°C. At this elevated temperature, coincident pre- and postsynaptic activity produced a 32.0% ± 5.9% (n = 5) increase in GPSC amplitude, while noncoincident activity produced a slight decrease (−12.8% ± 9.8%, n = 4). This was not significantly different from that observed at room temperature (Figure 7C). Similarly, we found at 35°C, there was a similar shift in $E_{\text{GPSC}}$ (4.4 ± 0.5 mV, Figure 7D) following coincident activity, as compared to that observed at room temperature.

Discussion

In the present study, we have demonstrated that coincident pre- and postsynaptic activity within a window of ±20 ms decreases cation chloride cotransporter activity, resulting in a shift in the $E_{\text{GPSC}}$ to more positive values and an increase in the GPSC amplitude when assayed at a level below the reversal potential. Repetitive presynaptic activity alone led to a reduction of the GPSC amplitude, which is mediated by a decrease in the total synaptic conductance. Coincident postsynaptic spiking appears to prevent this decrease in synaptic conductance. Furthermore, the effects of coincident activity,
Coincident spiking modifies GABAergic synapses in acute hippocampal slices. (A1 and B1) Example recordings of changes in the GPSC amplitude induced by repetitive coincident (+5 ms, [A1]) and noncoincident (+75 ms, [B1]) activity, using gramicidin perforated patch. Traces below (left) depict sample GPSCs before (1) and after (2) repetitive stimulation, which are also shown on the right after scaling. Scales: 20 pA, 20 ms.

(A2 and B2) Examples of current-voltage relation of GPSCs measured during experiments shown in (A1) and (B1), before (solid lines, diamonds and squares) and 10 min after (dashed line, circles) the repetitive coincident (A2) and noncoincident (B2) stimulation.

(C) Summary of percent change in the GPSC amplitude in both hippocampal cultures and slices. Data represent average changes in GPSC amplitude (± SEM) 10–20 min after the repetitive coincident and noncoincident stimulation, relative to that observed during the control period. There was no significant difference observed between slice and culture preparations for either coincident or noncoincident stimulation (p = 0.6). There was also no significant difference between experiments performed on slices at room temperature or when heated to 35°C (coincident, p = 0.2; noncoincident, p = 0.9).

(D) Summary of the change in $E_{\text{GPSC}}$ induced by coincident and noncoincident spike-timing intervals for recordings in both hippocampal cultures and slices. There was no significant difference observed between slice and culture preparations for either coincident or noncoincident stimulation (p = 0.2), at room temperature, or at 35°C (coincident, p = 0.2; noncoincident, p = 0.6).

Both the protection from the reduction in synaptic conductance and the positive shift in $E_{\text{GPSC}}$ require postsynaptic high-threshold nimodipine-sensitive Ca$^{2+}$ channels, which are likely to be activated by postsynaptic spiking. Thus, inhibitory inputs can be regulated by changes in either synaptic conductance or reversal potential, thereby allowing for a dynamic control of inhibition.
Coincidence Detection by GABAergic Synapses

The existence of the ±20 ms time window for the modification of GABAergic synapses demonstrates that these synapses are indeed capable of detecting and being modified by coincident pre- and postsynaptic activity, a well-known property of most excitatory glutamatergic synapses. GABAergic inputs typically do not trigger postsynaptic spiking, but other convergent excitatory inputs do. The latter was simulated in the present study by injecting depolarizing currents into the postsynaptic neuron. Thus, our findings suggest that GABAergic inputs can be modified via association with coincident excitatory inputs. Since excitatory inputs that cause repetitive postsynaptic spiking are likely to be potentiated themselves, the plasticity of excitatory inputs would occur in parallel with a depolarizing shift in $E_{\text{RSC}}$ of all temporally coincident GABAergic inputs, resulting in an enhanced net excitation of the postsynaptic neuron. Thus, the associative modification of GABAergic inputs allows the level of inhibition to be modulated in accordance with the temporal pattern of postsynaptic activity, providing a more active role for inhibitory inputs in the plasticity of neuronal networks.

The capacity for coincidence detection is believed to be important for learning and memory functions of the nervous system. For glutamatergic synapses, the NMDA receptor operates as a molecular coincidence detector, since it allows high levels of $\text{Ca}^{2+}$ influx only when the postsynaptic neuron is depolarized in the presence of presynaptic release of glutamate (Bliss and Collingridge, 1993; Malenka and Nicoll, 1993). Furthermore, the coincidence detection machinery at glutamatergic synapses appears to respond differentially to the temporal order of pre- and postsynaptic activity (Bi and Poo, 1998, 2001; Debanne et al., 1998; Markram et al., 1997; Zhang et al., 1998). In contrast, the coincidence detector at GABAergic synapses does not appear to respond to the temporal order of pre- and postsynaptic activity. It does, however, require synaptic GABA$_\alpha$ receptor activation in the presence of postsynaptic spike-induced $\text{Ca}^{2+}$ influx. Spikes initiated at the axonal initial segment are known to propagate backward to the dendritic tree and, thus, may activate high-threshold voltage-gated $\text{Ca}^{2+}$ channels, such as L-type $\text{Ca}^{2+}$ channels at the dendrite (Magee and Johnston, 1997; Stuart et al., 1997). The requirement of the spatial and temporal correlation of the spike-induced $\text{Ca}^{2+}$ influx with components of GABAergic transmission, such as GABA$_\alpha$ receptor activation or $\text{Cl}^-$ fluxes, is likely to underlie the apparent time window for the modification of GABAergic synapses.

Given the rapidity of the effect of coincident activity, alterations in the level of gene transcription or protein synthesis are unlikely. Thus, changes in the level of expression or posttranslational modification of cation chloride cotransporters are likely to account for the decrease in $\text{Cl}^-$ efflux. Recruitment and removal of synaptic AMPA receptors have been reported following the induction of LTP (Hayashi et al., 2000) and LTD (Carroll et al., 1999), respectively, at hippocampal CA1 excitative synapses. The decrease in $\text{Cl}^-$ transporter activity may also result from removal of functional KCC2 from the plasma membrane. The KCC2 protein contains five consensus phosphorylation sites for protein kinase C and one tyrosine protein kinase phosphorylation site (Payne et al., 1996). Serine/threonine phosphorylation has been shown to regulate KCC2 activity in oocytes (Strange et al., 2000), while cytosolic tyrosine kinase activity regulates $K^+ /\text{Cl}^-$ cotransport activity during the maturation of hippocampal neurons (Kelsch et al., 2001). Increased $\text{Ca}^{2+}$ levels associated with spike-induced L-type $\text{Ca}^{2+}$ channel activation, in conjunction with GABA$_\alpha$ receptor activation, may activate intracellular signaling molecules that downregulate KCC2 activity by dephosphorylation, in a manner similar to the dephosphorylation of AMPA receptors following the induction of LTD (Lee et al., 1998).

The percent change in GPSC amplitude in response to coincident pre- and postsynaptic differences, such as the composition of GABA$_\alpha$ receptor activity, varied from −27.4% to 79.8% (Figure 2). While this variability may result from subunits, the magnitude of the $\text{Ca}^{2+}$ rise, and the level of KCC2 expression, it is also likely that the variability was due in part to different types of presynaptic neurons. It is known that presynaptic interneuron identity may influence the properties of postsynaptic currents (Maccabber et al., 2000). Because of the potential existence of a somadendritic gradient of $[\text{Cl}^-]_s$, dendritically projecting interneurons (Callaway and Ross, 1995; Hoffman et al., 1997; Miles et al., 1996) may be more likely to be susceptible to modification of the GPSC amplitude via the mechanism reported here. Further studies that more thoroughly classify GABAergic neurons on the basis of their projections, neurochemical content, and physiological and morphological properties (Klausberger et al., 2003; McBain and Fisahn, 2001) are required to determine cell type dependence of activity-induced modification.

LTD of GABAergic Synapses

The persistent reduction of synaptic conductance following repetitive presynaptic stimulation (without postsynaptic spiking) is similar to LTD of GABAergic responses reported previously in these hippocampal cultures (Fitzsimonds et al., 1997) and hippocampal slices (McLean et al., 1996). The decrease in inhibitory synaptic conductance may serve as a means to regulate the gain of inhibition in accordance to the level and temporal pattern of excitation, in this case, a lack of synchronous postsynaptic excitation. The mechanism underlying this reduction of synaptic conductance—whether it is due to a reduced transmitter release or a reduction of postsynaptic GABA$_\alpha$ channel activity—is presently unknown. Our results suggest that when postsynaptic spiking triggered by convergent excitatory inputs is temporally correlated with presynaptic activation, there is an apparent $\text{Ca}^{2+}$-dependent signal that counteracts against the process underlying the reduction of synaptic conductance. Since the total synaptic conductance of inhibitory synapses is important for shunting inhibition, preventing the decrease in conductance also contributes to the reduction of inhibition for those inhibitory inputs that are temporally correlated with the postsynaptic excitation. However, substantial spontaneous in vivo spiking activity may influence the stability of synaptic modifications (Zhou et al., 2003), and bursting spiking activity is likely to impose additional spiking history-dependent pre- and postsynaptic effects.
that alter the nature of synaptic modification (Froemke and Dan, 2002). Further studies in an in vivo preparation are required to verify the findings reported here.

**Postsynaptic Shifts in $E_{	ext{GABAC}}$ as a Mechanism for GABAergic Synaptic Modification**

Our data showed that coincident spiking led to GPSCs that were more depolarizing. In a few cases, initially hyperpolarizing GPSCs were converted to depolarizing GPSCs following coincident activity. Furthermore, since $E_{	ext{GABAC}}$ often resides near the resting membrane potential, even small changes in $E_{	ext{GABAC}}$ can produce marked effects on the amplitude of GPSCs. The change in the driving force for GPSCs due to the shift in $E_{	ext{GABAC}}$ after coincident activity is sufficient to account for the change in the GPSC amplitude. For example, in the example illustrated in Figure 7A, the 6.8 mV change in $E_{	ext{GABAC}}$ would result in a 57% change in the amplitude of GPSCs, which is close to that observed.

Our finding that coincident activity induces a shift of the reversal potential of GABAergic responses suggests that there is a rapid activity-dependent and persistent modulation of $[\text{Cl}^-]$ at the synapse. Results from triplet recordings on convergent GABAergic inputs (Figure 6) suggest that such modulations of $[\text{Cl}^-]$ can be locally restricted, only affecting synapses undergoing coincident activation. The average measured change in the reversal potential was $4.6 \pm 0.8$ mV ($n = 11$) at 10–20 min after coincident stimulation (Figure 3), which would predict a change in the $[\text{Cl}^-]$ by about 2 mM. Our observed changes are consistent with the previous finding (Collin et al., 1995) that following pairing of sustained postsynaptic depolarization with exogenous GABA application to hippocampal CA1 pyramidal neurons resulted in persistent modification of somatic responses to exogenous GABA. During the coincident pre- and postsynaptic stimulation protocol, postsynaptic stimulation may create a positive driving force for $\text{Cl}^-$ at the synapse, resulting in a local increase in $[\text{Cl}^-]$. A rough calculation based on the average synaptic conductance of $\sim 10$ nS and a dendritic segment volume of 100 $\mu$m$^3$, coincident postsynaptic activity of 150 spikes may induce an increase of $[\text{Cl}^-]$ in the order of 1 mM. However, such transient elevation of $[\text{Cl}^-]$ can not account for the persistent changes in GPSC amplitude, unless the activity of $\text{Cl}^-$ transporter is also persistently reduced. Transient changes in $[\text{Cl}^-]$ in the order of mM, e.g., that resulted from the reduction of $[\text{K}^+]$, should be quickly abolished after the termination of experimental manipulations (see Figure 4B). We hypothesize that increased $[\text{Ca}^{2+}]$, through its downstream effectors, may persistently modify the activity of KCC2 cotransporters at the activated GABAergic synapses. Furthermore, the results from triplet experiments suggest that coincident activity may induce local modification of $\text{Cl}^-$ transporter activity in these neurons. Local regulation of $[\text{Cl}^-]$ has been observed in retinal neurons, where a differential targeting of $\text{Cl}^-$ transporter KCC2 can produce both depolarizing and hyperpolarizing GABA responses in the same cell (Vardi et al., 2000).

**Involvement of KCC2 Activity**

Our results indicate that among the family of $\text{K}^+/\text{Cl}^-$ transporters, KCC2 is responsible for the increase of $[\text{Cl}^-]$ by coincident activity. This conclusion is based on several lines of evidence. First, we showed that $E_{	ext{GABAC}}$ was sensitive to external $\text{K}^-$, a property specific to KCC2, and this sensitivity to $\text{K}^-$ was blocked by furosemide, consistent with a specific effect of furosemide on the KCC2 activity. Second, our experiments were performed at a developmental stage when KCC2 expression is known to be upregulated, while that of NKCC is downregulated (Ganguly et al., 2001; Kakazu et al., 1999; Lu et al., 1999; Plotkin et al., 1997), Third, KCC2 is localized at inhibitory synapses in both mouse spinal cord (Hubner et al., 2001) and rat retina (Vu et al., 2000), suggesting KCC2 is strategically positioned to maintain local postsynaptic $[\text{Cl}^-]$. Finally, KCC2 is known to undergo activity-dependent regulation in cultured hippocampal neurons (Ganguly et al., 2001). Upregulation of KCC2 expression during neonatal development results in a hyperpolarizing shift of $E_{	ext{GABAC}}$, leading to the switch of GABAergic transmission from excitatory to inhibitory (Ganguly et al., 2001; Rivera et al., 1999). Our results suggest that coincident activity acts specifically to decrease KCC2 activity leading to the establishment of a localized change in $[\text{Cl}^-]$. Such bidirectional regulation of GABA synapses via changes in the $\text{Cl}^-$ transporter activity supports the notion that developmental remodeling of synapses and activity-dependent synaptic plasticity share common cellular mechanisms.

**Multiple Functions of GABAergic Inputs**

Inhibitory inputs are usually located in regions of the neuron where they can exert substantial influence on signal integration; small changes in their efficacy can result in large effects on neuronal output. In general, a GABAergic input can be inhibitory through either shunting or hyperpolarization of the membrane (Staley and Mody, 1992). Therefore, changes in either the synaptic conductance or $E_{	ext{GABAC}}$ will affect the characteristics of inhibition. With the shift in $E_{	ext{GABAC}}$ toward positive levels above the resting potential following correlated stimulation, a depolarizing GABAergic input will exert a complicated effect of both shunting inhibition and facilitating excitation depending on the relative timing and spatial distribution of GABAergic and glutamatergic inputs. For example, depolarizing GABAergic responses, while capable of shunting spatially and temporally coincident excitatory inputs, may facilitate distant subthreshold excitatory inputs that arrive on the decay phase of GPSCs (Chen et al., 1996). In addition, depolarizing GABAergic inputs may help to activate voltage-gated calcium channels or even unblock NMDA receptors, thus influencing the function and/or plasticity of other convergent synapses (Bazhenov et al., 1999). Finally, shifts of $E_{	ext{GABAC}}$ of inhibitory inputs may modify the reliability of initiating forward propagating action potentials (Debanne et al., 1998) and subsequent transmitter release from the postsynaptic neuron following activation of inhibitory inputs. Through associative modification based on coincidence with postsynaptic spiking, these multiple functions of GABAergic synapses can be coordinated in accordance with the activity of glutamatergic inputs to the same postsynaptic neuron.
Experimental Procedures

Cell Culture

Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as previously described (Bi and Poo, 1998). In brief, hippocampi were removed from E18–20 embryonic rats and treated with trypsin for 20 min at 37°C, followed by gentle trituration. The dissociated cells were plated at densities of 26,000 to 100,000 cells/ml on poly-L-lysine coated glass coverslips in 35 mm petri dishes. The plating medium was Dulbecco’s minimum essential medium (DMEM; BioWhittaker), supplemented with 10% fetal calf serum (Hyclone), 10% Ham’s F12 with glutamine (BioWhittaker), and 50 U/ml penicillin-streptomycin (Sigma). Twenty-four hours after plating, the culture medium was changed to the above medium containing 20 mM KCl. Both glia and neurons are present under these culture conditions. Cells were recorded from 9 to 14 days in culture.

Hippocampal Slices

Hippocampal slices were prepared from postnatal day 12 to 19 Sprague-Dawley rats. Rats were briefly anesthetized with halothane and decapitated. Brains were removed and submerged in ice-cold sucrose-CSF: 206 mM sucrose, 2.8 mM KCl, 1 mM CaCl2, 1 mM MgCl2, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM NaHCO3, 10 mM D-glucose, 0.4 mM ascorbic acid, pH 7.4 when equilibrated with 95% O2–5% CO2, 295 mOsml. Transverse hippocampal slices (400 μM) were cut using a Vibratome 3000 sectioning system (St. Louis, MO). Slices were transferred to a holding chamber (for a minimum of 1.5 hr) where they were maintained in continually perfused oxygenated (95% O2–5% CO2) artificial CSF: 124 mM NaCl, 2.8 mM KCl, 2 mM MgSO4, 1.25 mM NaH2PO4, 26 mM CaCl2, 26 mM NaHCO3, 10 mM D-glucose, 0.4 mM ascorbic acid, pH 7.4 when equilibrated with 95% O2–5% CO2, 295 mOsml.

Electrophysiology

Hippocampal Cultures

Whole-cell perforated patch recordings were performed on pairs or triplets of interconnected cultured neurons (Bi and Poo, 1998). For perforated patch recordings, either glutamate or amphotericin B was used for perforation. The recording pipettes were made from glass capillaries (VWR), with a resistance of ~2 MΩ. For experiments using amphotericin B, the pipettes were tip filled with internal solution (K-Glutonate, 154 mM; NaCl, 9 mM; MgCl2, 1 mM; HEPES, 1 mM; EGTA, 0.2 mM) and then back filled with internal solution containing amphotericin B (150 to 220 μg/ml, Calbiochem). Experiments using glutamate were tip filled with an internal solution of 150 mM KCl and 10 mM HEPES and back filled with this internal solution containing gluteate (25 μM). For experiments using BAPTA loading into the postsynaptic cell, throughout whole-cell recordings were made. In these experiments, the internal solution was modified (K-Glutonate, 100 mM; BAPTA, 30 mM; MgCl2, 15 mM; NaCl, 5 mM; EGTA, 0.5 mM; HEPES, 10 mM; Mg-ATP, 2 mM). The external bath solution was a HEPES-buffered saline (HBS: NaCl, 150 mM; KCl, 3 mM; HEPES, 10 mM; CaCl2, 3 mM; glucose, 5 mM; MgCl2, 2 mM). The bath was constantly perfused with fresh recording medium at a slow rate throughout the recording, and all experiments were performed at room temperature. The neurons were visualized by phase-contrast optics (Nikon Diaphot).

Recordings were performed with patch clamp amplifiers (Axopatch 200B; Axon Inc.). Signals were filtered at 5 kHz using amplifier circuitry. Data was acquired and analyzed using Axoscope 8.0 (Axon Inc.). Recordings started after the access resistance had dropped to between 20 and 30 MΩ. The series resistance was compensated to 80% throughout the experiment. In addition, series resistance was monitored throughout the experiments and did not change by more than 5% during a typical experiment. For assaying synaptic connectivity in culture, each neuron was stimulated at a low frequency (0.05 Hz) by a 1 ms step depolarization from −70 to +20 mV in voltage-clamp mode. All GPSCs were distinguished from excitatory postsynaptic currents by the following criteria: longer decay times, reversal potentials between −50 and −90 mV, and in some cases, sensitivity to bicuculline methiodide (10 μM; Research Biochemicals International). In most recordings, the postsynaptic neu-ron was voltage clamped at −70 to −90 mV, resulting in inward GPSCs. In a few cases when the reversal potential was around −80 mV, the postsynaptic neuron was clamped at −60 mV, resulting in outward GPSCs. Prior to the induction protocol, a minimum of 10 min of control baseline recording was performed. The induction protocol consisted of repetitive presynaptic stimulation (100 mV step depolarization from V0 of −70 mV, duration 1 ms, 5 Hz for 30 s) each paired with a postsynaptic spike induced by injection of depolarizing current pulses (2 nA, 2 ms). Experiments were continued only when a stable baseline GPSC amplitude (<10% variation) and reversal potential were observed before induction. The change in GPSC amplitude resulting from the spike-timing induction protocol was calculated by comparing the average size of the GPSC amplitude between 10 and 20 min after the induction protocol to the average GPSC amplitude obtained in a 10 min baseline recording made prior to the induction protocol.

The synaptic reversal potential of GPSCs was determined by varying the holding potential of the postsynaptic cell in 5 mV increments (−90 to −30 mV) and measuring the resulting GPSC amplitude. To determine the reversal potential, we used linear regression to calculate a best-fit line for the voltage dependence of GPSCs. The interpolated intercept of this line with the abcissa was taken as the reversal potential. The slope of the same line was then taken as the respective slope conductance. Determination of Erev, at the soma was made in a manner similar to that described for the synapse, except that GABA was puffed onto the cell body in 30 s intervals (100 μM, 20 ms, 5 psi) through a micropipette (~1.5–2 MΩ) with a Picospritzer II (Parker Instrumentation). In the present study, the amplitude of GPSCs was in the range of 50–400 pA. Therefore, we estimate that per presynaptic action potential, release occurred at about 8 to 60 release sites (given a conductance per single release site of about 0.44 ± 0.02 pS; Nusser et al., 1998). D-amin-5-phosphonopentaonic acid (AP5) and 6-cyano-7-nitroquinoxaline-2, 3-dione (CNQX), and nimodipine were purchased from Research Biochemicals International. Amphotericin B was used as the perforating agent for experiments performed with AP5 and CNQX. Furosemide was purchased from Sigma. All Student’s t tests performed were two tailed. The use of either unequal or paired Student’s t tests is noted in the text. One-way ANOVAs followed by Scheffe pairwise comparisons were used to test for significance between multiple comparisons.

Acute Hippocampal Slices

A bipolar stimulating electrode placed in the S. radiatum was used to activate interneurons (100 μs, 5 V, 0.05 Hz). Whole-cell perforated patch recordings using glutamic acid were made from CA1 pyramidal neurons (glutamic acid and internal solution were prepared as described). Recordings were made with Kimax-51 electrodes with a tip resistance of ~6 MΩ. The external bath solution was the same as the aCSF that the slices had been maintained in, with the addition of 10 μM CNQX and 25 μM D-AP5. The bath solution was oxygenated with 95% O2–5% CO2 and continually perfused at a rate of 2 ml/min and, in some experiments, heated to 35°C with an automatic temperature controller (Warner Instruments). The neurons were visualized with a CCD camera (Hamamatsu). Recordings were performed with patch clamp amplifiers (Axopatch 200B; Axon Inc.), and data were collected and analyzed in similar manner as described above for hippocampal cultures.

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