Distributed synaptic modification in neural networks induced by patterned stimulation

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Activity-dependent changes in synaptic efficacy or connectivity are critical for the development[1], signal processing[2] and learning and memory functions[3–6] of the nervous system. Repetitive correlated spiking of pre- and postsynaptic neurons can induce a persistent increase or decrease in synaptic strength, depending on the timing of the pre- and postsynaptic excitation[7–13]. Previous studies on such synaptic modifications have focused on synapses made by the stimulated neuron. Here we examine, in networks of cultured hippocampal neurons, whether and how localized stimulation can modify synapses that are remote from the stimulated neuron. We found that repetitive paired-pulse stimulation of a single neuron for brief periods induces persistent strengthening or weakening of specific polysynaptic pathways in a manner that depends on the interpulse interval. These changes can be accounted for by correlated pre- and postsynaptic excitation at distant synaptic sites, resulting from different transmission delays along separate pathways. Thus, through such a 'delay-line' mechanism, temporal information coded in the timing of individual spikes[14–17] can be converted into and stored as spatially distributed patterns of persistent synaptic modifications in a neural network.

Cultures of dissociated rat hippocampal neurons were used to study functional networks consisting of both glutamate- and γ-aminobutyric acid (GABA)-mediated synapses[13]. Perforated whole-cell recordings of synaptic currents were made from 2–3 neurons in networks of about 10–20 neurons. The evoked postsynaptic currents (PSCs) elicited by a brief depolarizing stimulus (+100 mV, 1 ms) applied to another neuron, or to the recorded neuron itself, usually exhibited complex but reproducible patterns with distinct components (Fig. 1a). Each PSC component corresponds to a polysynaptic pathway with a specific transmission delay, which is due to synaptic delays and the time required for the initiation and conduction of action potentials, estimated to be in the range of 4–10 ms per synapse (see Methods). Under low-frequency test stimulation, the average probability of occurrence (P) for each PSC component remained relatively constant throughout the recording period (Fig. 1b). The PSC profile thus provides a reliable means for monitoring the status of synaptic efficacy along multiple polysynaptic pathways in the network.

To examine the effect of repetitive local stimulation on remote synapses in the polysynaptic pathway, we applied a train of paired-pulse stimuli (PPS) with a defined interpulse interval (IPI) to a single neuron in the network (voltage clamp $V_c = -70$ mV). Paired pulses were chosen because of their simple temporal structure. After 20 paired pulses at 1 Hz, we frequently observed changes in the PSC profile in either the stimulated neuron (Fig. 2a, c–f) or a different neuron in the network (Fig. 2b). These changes included the appearance of new components (Fig. 2a, d) and the disappearance or change in P of pre-existing components (Fig. 2b–d), reflecting remodelling of polysynaptic pathways in the network. Such pathway remodelling was persistent, lasting for as long as stable recording was made (up to 1.5 h), indicating that long-term changes had occurred in specific pathways between the stimulated and recorded neurons. In Fig. 2d, repetitive PPS induced the appearance of a new component 3, and an increase and decrease in P for components 2 and 1, respectively; thus, the same pattern of stimuli can induce opposite changes along different pathways. In most cases, changes in the PSC profile involved alterations in P for particular components without significant changes in the amplitude. Thus, the patterned stimulation had apparently resulted in changes at remote synaptic connections, thereby changing the probability of successful transmission along different pathways leading to the recorded neuron. Such distributed changes are consistent with the notion of distributed representation and storage of information in neural networks[3,4].

When multiple episodes of repetitive PPS (20 pairs, 1 Hz) were applied to the same neuron, pathway remodelling depended on the

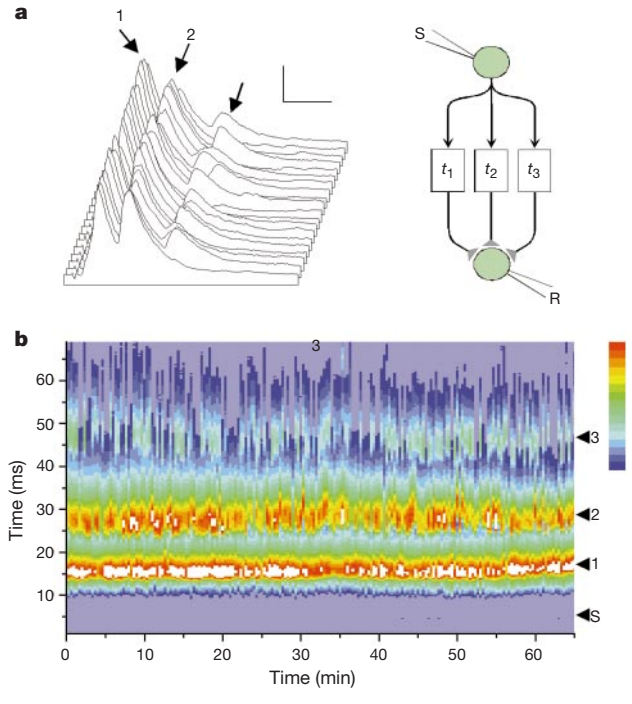

Figure 1 Polysynaptic pathways in a network of cultured hippocampal neurons. **a**, Traces depict 20 consecutive postsynaptic currents (PSCs) (inward currents are shown upwards) recorded from a hippocampal neuron in response to test stimulation (0.05 Hz) of a nearby neuron (see Methods). The diagram on the right depicts three hypothetical polysynaptic pathways leading to the recorded neuron with transmission delays of t_1, t_2 and t_3, respectively, corresponding to the onset latencies of the three distinct PSC components. Scales: 20 ms, 500 pA. **b**, The stability of polysynaptic pathways. Shown are 195 consecutive PSCs, each triggered by a test stimulus and represented by a single vertical line, with current magnitude coded by colour. Colour code: linear scale 150–700 pA; white and background colours represent above- and below-scale values, respectively. Arrowheads indicate the time of stimulation (S) and peaks of identified PSC components (1–3).

IPI of the paired pulses. In the experiment shown in Fig. 2e, no persistent change in PSCs was induced by paired pulses with 60 ms IPI, but paired pulses of 40 ms IPI induced two new PSC components with a relatively high *P*, which persisted for more than 1 h. In another experiment (Fig. 2f), the first episode of stimulation (IPI, 100 ms) had no significant effect, but the second episode with 50 ms IPI resulted in an increased *P* for two components, one of which was reduced by further stimulation with a third episode (IPI 20 ms). In a third experiment (see Supplementary Information), four separate episodes were applied to the network to show further the specificity of the IPI in inducing pathway remodelling. Therefore, pathway remodelling is highly dependent on the precise temporal pattern of the repetitive localized stimulation.

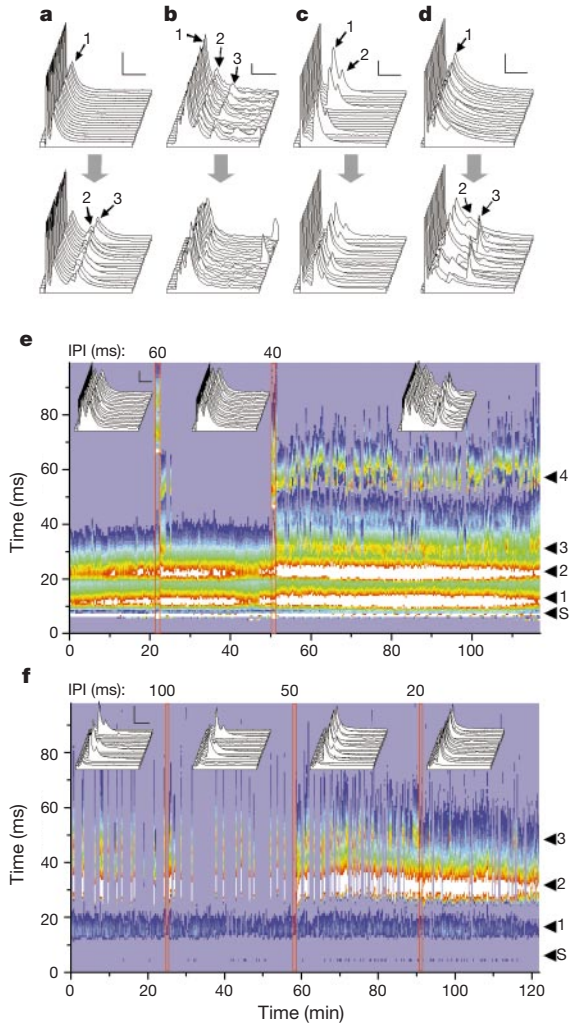

Figure 2 Pathway remodelling induced by repetitive paired-pulse stimulation (PPS). **a**–**d**, Four experiments showing different types of change in the PSC profile. For each experiment, consecutive PSCs elicited by test stimuli before (top) and 10 min after (bottom) PPS (20 pairs, 1 Hz) with interpulse interval (IPI) of 50 (**a**), 30 (**b**), 50 (**c**) and 100 ms (**d**) are shown. PSCs were recorded either in the stimulated neuron (**a**, **c**, **d**: note the large stimulus artifact) or in a nearby neuron (**b**). Scales: 20 ms, 1 nA (**a**, **b**); 20 ms, 500 pA (**c**, **d**). **e**, **f**, Changes in the PSC profile depend on the IPI of the paired pulses. **e**, Two episodes of PPS (marked by vertical red lines) applied to the recorded neuron; insets, 20 consecutive PSCs at 10–16, 40–46, and 80–86 min. Scales: 20 ms, 500 pA. Colour code as in Fig. 1b: 0.2–1.2 nA, linear scale. **f**, PSCs elicited by stimulating a different neuron in the network (test frequency 0.07 Hz); insets, samples of PSCs at 10–14, 40–44, 80–84 and 110–114 min. Scale: 20 ms, 400 pA. colour code: 50–400 pA, linear scale.

Repetitive correlated pre- and postsynaptic excitation can induce long-term synaptic modification, and the direction of synaptic changes depends on the temporal order of pre- and postsynaptic activation[7–13]. In the same hippocampal cultures[13], postsynaptic spiking within 20 ms after presynaptic activation (positively correlated

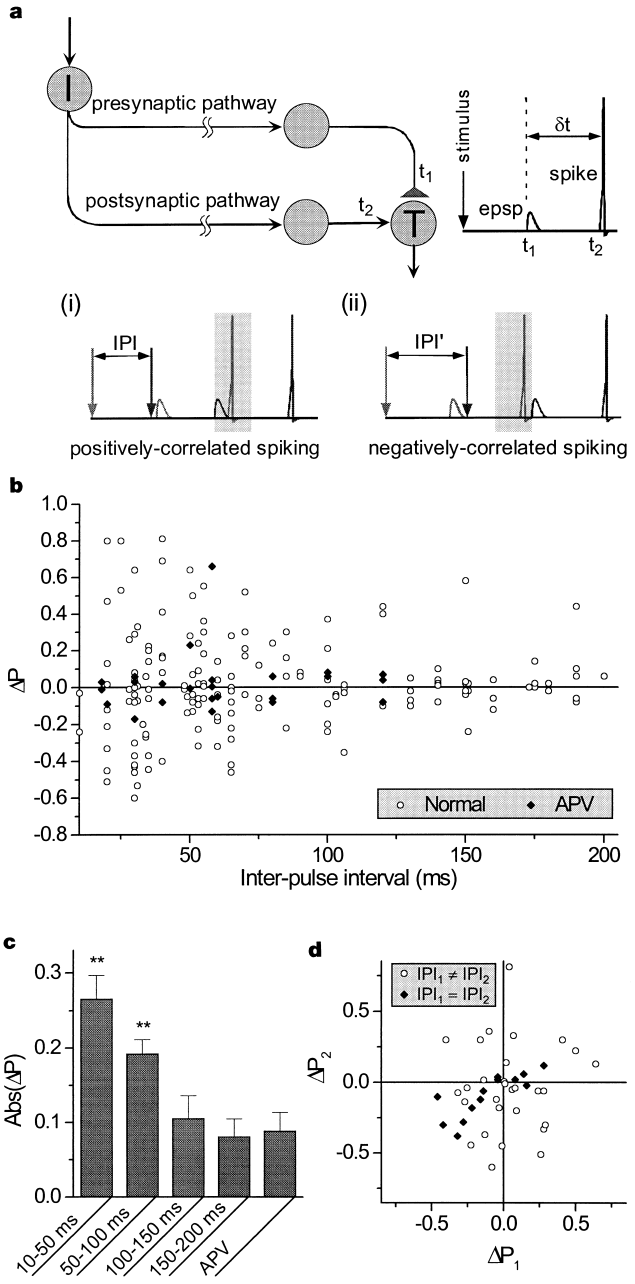

Figure 3 Pathway remodelling induced by correlated excitation through transmission delay. **a**, Correlated pre- and postsynaptic excitation at a 'remote' synapse (triangle) by stimulation transmitted along two converging pathways (see text). The 'postsynaptic' synapse (with respect to the remote synapse) can trigger spikes at the target neuron. Shaded areas indicate effective time windows (~20 ms) for synaptic modifications[13]. **b**, **c**, Summary of pathway remodelling induced by PPS (20 pairs, 1 Hz) of various IPIs. Each point represents the result for one identified PSC component from a single experiment without (Normal) or with 50 μM D-AP-5 (APV). Columns in **c** represent averages of absolute values of ΔP (*y* axis) from experiments with different ranges of IPIs (*x* axis). Two asterisks, significantly different from the APV group ($P < 0.005$, *t*-test). **d**, Correlation between changes in probability of occurrence (ΔP_1 and ΔP_2) after two consecutive episodes of PPS with the same or different IPIs. Correlation coefficients: $\rho = 0.058$ for $IPI_1 \neq IPI_2$ ($P > 0.25$, *t*-test); $\rho = 0.81$ for $IPI_1 = IPI_2$ ($P < 0.001$, *t*-test).

spiking) leads to long-term potentiation (LTP), whereas post-synaptic spiking within 20 ms before presynaptic activation (negatively correlated spiking) leads to long-term depression (LTD). Thus, paired-pulse facilitation or temporal integration of the evoked postsynaptic potentials (EPSPs) at the remote synapses might generate positively correlated spiking at these synapses, resulting in pathway remodelling by homosynaptic potentiation. However, the prevailing paired-pulse depression in these cultures (see Supplementary Information) makes such a process unlikely. An alternative mechanism that might generate correlated spiking at remote synapses is based on different transmission delays along converging pathways. As shown in Fig. 3a, localized stimulation of an input neuron (I) results in activation of two separate pathways that converge on a target neuron (T) with a difference in the transmission delay of $t_2 - t_1$ (or 'δt'). For the case of $t_2 > t_1$, repetitive PPS with IPI $< \delta t$ may result in positively correlated spiking, and hence LTP at the remote synapse made by the presynaptic pathway onto neuron T; whereas stimulation with

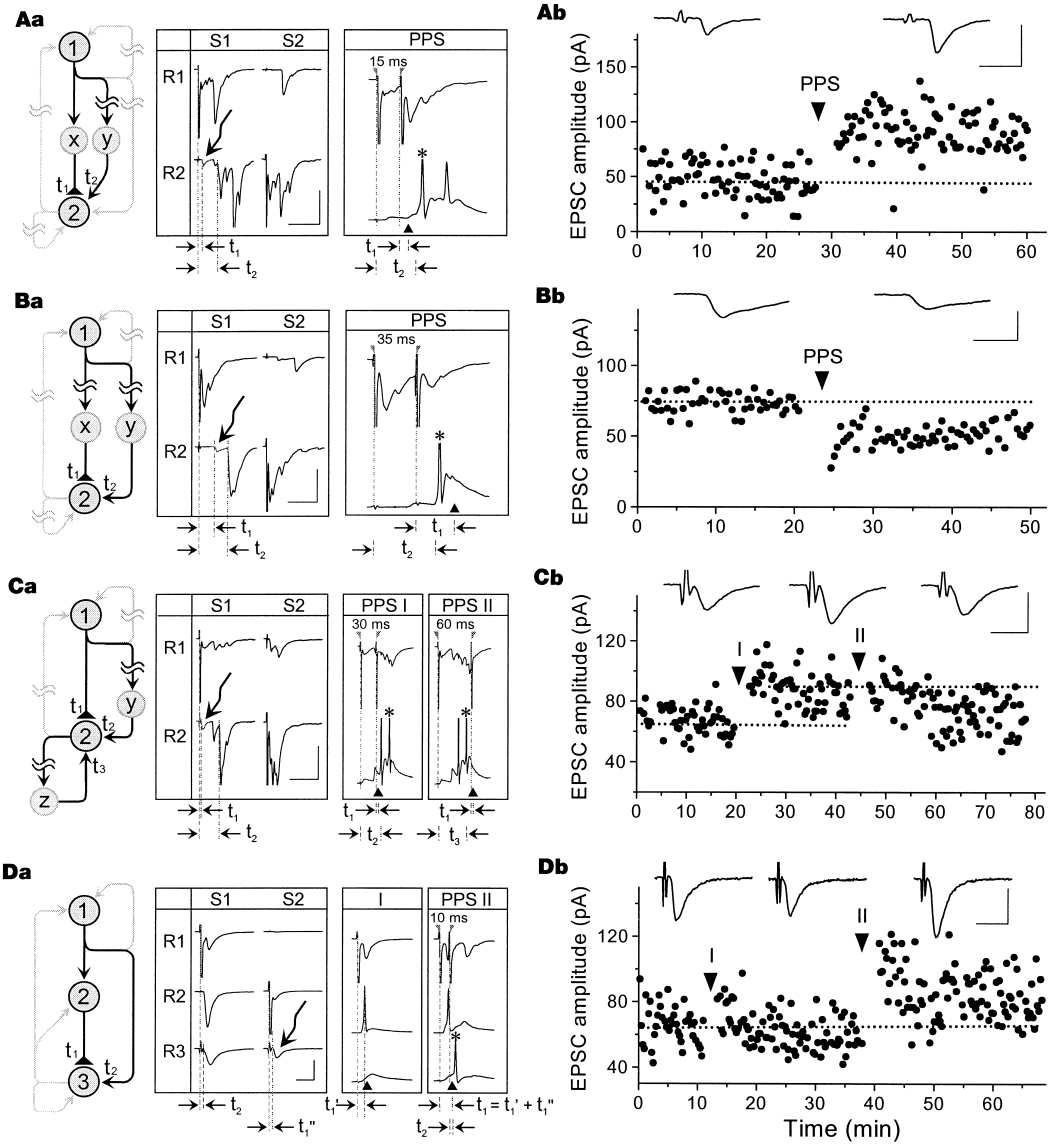

Figure 4 Modifications of remote excitatory synapses by correlated spiking. **Aa**, Synaptic connections between two recorded neurons (left panel). Dark lines, two convergent pathways of interest (transmission delays: $t_1 = 6$ ms; $t_2 = 27$ ms); grey lines, other detected connections; cells x, y, unrecorded neurons upstream of cell 2; triangle, the 'remote' synapse ($E_{x\rightarrow 2}$) under examination; ≈ denotes polysynaptic connections. PSCs were recorded from cells 1 and 2 (R1 and R2) when they were stimulated (S1 and S2) separately (middle panel). Arrow points to the PSC component corresponding to $E_{x\rightarrow 2}$. Synaptic responses recorded from cells 1 (top) and 2 (bottom) during repetitive PPS (1 Hz, 50 s) of cell 1 while cell 2 was current-clamped (right panel). Note expanded timescale. Scales: 40 ms, 500 pA for EPSCs in S1, S2; 20 ms, 50 mV for EPSPs in PPS. **Ab**, LTP at $E_{x\rightarrow 2}$ resulting from positively correlated spiking during PPS for the circuit shown in **Aa**. Test stimuli (0.05 Hz) were applied to cell 1. Insets, sample EPSCs (mean of 10 consecutive events) 10 min before and 20 min after PPS. Scales: 10 ms, 100 pA. **B**, LTD at $E_{x\rightarrow 2}$ ($t_1 = 28$ ms) by negatively correlated spiking through $E_{y\rightarrow 2}$ ($t_2 = 51$ ms). Scales: 50 ms, 500 pA for EPSCs, 25 ms, 50 mV for EPSPs (**Ba**), 10 ms, 100 pA (**Bb**). **C**, LTP and LTD at $E_{1\rightarrow 2}$ (monosynaptic, $t_1 = 3$ ms) by correlated spiking through $E_{y\rightarrow 2}$ and $E_{z\rightarrow 2}$ ($t_2 = 36$ ms; $t_3 = 50$ ms). During the two episodes of PPS, recurrent excitation (through $E_{z\rightarrow 2}$) caused a second spike at cell 2. Scales: 50 ms, 500 pA for EPSCs, 50 ms, 50 mV for EPSPs (**Ca**); 10 ms, 100 pA (**Cb**). **D**, A serially connected triplet with all neurons recorded. Synapse $E_{1\rightarrow 2}$ was suprathreshold. Transmission delay of the 1⟶2⟶3 pathway ($t_1 = 12$ ms) was due to the latency of synaptic delay and spike initiation at cell 2 ($t_1' = 8$ ms), and the synaptic delay at $E_{2\rightarrow 3}$ ($t_1'' = 4$ ms). Two episodes of repetitive stimulation (I, 50 single pulses; II, 50 paired pulses; IPI 10 ms, both at 1 Hz) were applied to cell 1 while cell 2 and 3 were under current clamp. Scales: 20 ms, 200 pA for EPSCs, 10 ms, 50 mV for EPSPs (**Da**); 20 ms, 50 pA (**Db**).

IPI $> \delta t$ may result in negatively correlated spiking, and hence LTD at that synapse. Modification of this remote synapse may change the firing probability of neuron T, leading to a change in the corresponding PSC components in downstream neurons. Such a heterosynaptic mechanism is sensitive to the IPI of the paired-pulse stimuli and may thus account for the pathway remodelling described above.

The effects of paired pulses with IPIs of 10–200 ms were examined. In each experiment, we started with an IPI within this range and tested 2–4 episodes of 20 paired pulses (at 1 Hz) of progressively decreasing or increasing IPIs. Before and after each episode, PSCs were monitored by low-frequency test stimuli (at 0.05 Hz) for 20–40 min. When changes in the probability of occurrence (ΔP) of identified PSC components were plotted against the IPIs of the stimuli (Fig. 3b), we found that the changes decreased with increasing IPI, with IPIs below 100 ms being most effective in inducing pathway remodelling (Fig. 3b, c). This is consistent with a decreasing probability of forming converging pathways with longer pathlength differences that are responsible for generating correlated spiking by PPS in the cultured networks.

We further compared the results of applying two consecutive episodes of paired pulses of the same IPI with those of different IPIs. As shown in Fig. 3d, a strong correlation was observed between changes in P (ΔP_1 and ΔP_2) after the first and the second episode of the same IPIs. In contrast, two episodes of IPIs differing by 10–40 ms yielded results that showed little correlation, indicating the network's capability of discriminating a temporal difference of ~10 ms. These results support the heterosynaptic mechanism shown in Fig. 3a.

Long-term modification of many central synapses depends on the activation of *N*-methyl-D-aspartate (NMDA) subtypes of glutamate receptors[5,18,19]. As shown in Fig. 3b, significantly fewer changes in the PSC profile were induced by PPS in the presence of D-2-amino-5-phosphonopentanoic acid (D-AP5; 50 μM), a specific blocker of NMDA receptors. The D-AP5 treatment did not significantly affect the network activity, as shown by comparison of the PSC profile before and after the treatment (data not shown), but the reduction in pathway remodelling in the presence of D-AP5 is consistent with the involvement of NMDA receptor-dependent LTP and LTD[13]. We did observe two cases of significant changes (>20%) in P of PSC components in the presence of D-AP5. This may reflect other NMDA-receptor-independent mechanisms, such as modifications of inhibitory GABA-mediated synapses[19,20] or activity-induced changes in cell excitability[21].

To verify directly that remote synaptic changes can be induced by correlated excitation through converging pathways, we examined modifications of identified PSC components by PPS when the postsynaptic cell was under current clamp. As shown in Fig. 4A–C, the relevant transmission delays (t_1, t_2, t_3) along identified pathways from cell 1 to cell 2 (equivalent to neuron T in Fig. 3a) can be precisely determined from the recorded excitatory synaptic inputs (designated as '$E_{x\rightarrow 2}$', '$E_{y\rightarrow 2}$' and '$E_{z\rightarrow 2}$'). In these experiments, during repetitive PPS, the postsynaptic spike (marked by an asterisk) due to a suprathreshold input ('$E_{y\rightarrow 2}$' or '$E_{z\rightarrow 2}$') triggered by the first stimulus was positively or negatively correlated with the EPSP (onset marked by arrowhead) of the 'remote' synaptic input ($E_{x\rightarrow 2}$ or $E_{1\rightarrow 2}$) triggered by the second stimulus, resulting in LTP or LTD, respectively. Note that the EPSP triggered by the first stimulus fell outside the 20 ms window[13] for synaptic modification by correlated spiking. The specificity of IPIs for modifying the identified synapses is consistent with the heterosynaptic mechanism shown in Fig. 3a. Experiments were also performed on simpler networks using simultaneous whole-cell recordings from three neurons. In the case shown in Fig. 4D, the serially connected triplet has two converging pathways onto neuron 3. Repetitive single-pulse stimulation of cell 1 had no significant effect on synapse $E_{2\rightarrow 3}$, although synapse $E_{1\rightarrow 2}$ was potentiated as expected (data not shown). However, stimulation of neuron 1 with paired pulses (IPI 10 ms) caused spiking at cell 3 due to summation of EPSPs from $E_{1\rightarrow 3}$ and $E_{2\rightarrow 3}$, resulting in LTP at $E_{2\rightarrow 3}$. Together, these experiments confirm that repetitive local stimulation of a neuron can induce LTP and LTD at remote polysynaptic sites depending on the timing of correlated pre- and postsynaptic excitation, which in turn is determined by differential transmission delays along converging pathways.

Tetanic stimulation of afferent fibres to hippocampal CA3 neurons or perforant path (PP) fibres results in long-term activation of latent polysynaptic pathways[20] or potentiation of population spikes at existing polysynaptic pathways[22], respectively. The underlying mechanisms for these phenomena appeared to be a change in inhibitory control[20] or synaptic coupling of tetanizing trains through direct PP–CA3/CA1 synapses with asynchronous polysynaptic volleys occurring in the intrahippocampal circuitry[22]. Our results from dissociated neuronal cultures provide a direct demonstration of a generic property of neural networks: temporal correlation through transmission delays can be used for selective pathway modifications in accordance with the precise temporal structure of the input stimuli. Such a strategy of temporal-to-spatial conversion, different from that using paired-pulse facilitation and slow inhibition for selective activation in the hippocampal circuit[23], is reminiscent of using delay lines for parallel processing of temporally structured information in the auditory system[24,25] and in neural network models[16,26,27]. Here, polysynaptic pathways may be regarded as delay lines with a temporal resolution in the order of milliseconds and a range of delay times proportional to the size of the network. In intact nervous systems, synaptic input from a single neuron is usually not sufficient to trigger postsynaptic spiking. Thus, single neurons serially connected may not form functional polysynaptic pathways. However, the firing of a cultured neuron hyperinnervating others can be considered to be analogous to the *in vivo* situation of synchronous firing of a group of neurons with common targets[15,28]. Polysynaptic transmission pathways in culture therefore simulate *in vivo* pathways which mediate information flow by correlated firing[15,28]. If spike timing encodes neural information[14–17], the delay line architecture combined with spike-timing-based synaptic modifications provide a network mechanism to convert and store temporal information into spatially distributed patterns of synaptic modifications. □

Methods

Cell culture

Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as described[13,29]. Hippocampi were removed from E18–20 embryonic rats and treated with trypsin for 15 min at 37 °C, followed by washing and gentle trituration. The dissociated cells were plated on poly-L-lysine-coated glass coverslips in 35-mm petri dishes at densities of 30,000–90,000 cells per dish. The plating medium was Dulbecco's Minimum Essential Medium (DMEM, BioWhittaker) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone), 10% Ham's F12 with glutamine (BioWhittaker) and 50 U ml^{-1} penicillin-streptomycin (Sigma). Twenty-four hours after plating, one-third of the culture medium was replaced by the same medium supplemented with 20 mM KCl. Cells were used for electrophysiological studies after 10–15 days in culture, when functional neuronal networks had been established. A typical network selected consisted of about 10–20 neurons on a patch of glial cell monolayer of ~1–2 mm^2 in size. Larger networks in older cultures usually exhibit unpredictable bursts of spontaneous activity and complex PSC profiles, and were thus avoided.

Electrophysiology

Whole-cell perforated patch recordings[30] from 2–3 hippocampal neurons were performed simultaneously, using amphotericin B (150 μg ml^{-1}, Calbiochem) for perforation. The micropipettes were made from borosilicate glass capillaries (VWR), with a resistance in the range of 1.5–3 MΩ. The internal solution contained the following (in mM): K-gluconate 136.5, KCl 17.5, NaCl 9, $MgCl_2$ 1, HEPES 10, EGTA 0.2, pH 7.2. The external bath solution was a HEPES-buffered saline (HBS) containing the following (in mM): NaCl 150, KCl 3, $CaCl_2$ 3, $MgCl_2$ 2, HEPES 10, glucose 5, pH 7.3. For blocking NMDA receptors, 50 μM D-AP5 (RPI) was added to HBS. The culture was constantly perfused with fresh external solution at ~1 ml min^{-1} at room temperature throughout the recording period.

The neurons were visualized by phase-contrast microscopy with an inverted microscope (Nikon Diaphot) while recording (in voltage clamp unless otherwise stated ($V_c = -70$ mV) and stimulation (1 ms, +100 mV step depolarization in voltage clamp) was performed using patch-clamp amplifiers (Axopatch 200B, Axon) interfaced with a PC. Signals filtered at 5 kHz using amplifier circuitry were sampled at 10 kHz and analysed using Axoscope software (Axon). Series resistance (10–30 MΩ) was always compensated at 80% (lag 100 μs). For assaying synaptic connectivity, each neuron was stimulated at a low frequency (0.03–0.06 Hz), and the responses from the other neurons as well as autaptic responses in the stimulated neuron itself were recorded (see Fig. 4). Monosynaptic currents had onset latencies <4 ms (ref. 29). Polysynaptic currents had onset latencies ≥6 ms and often exhibited multiple components, with frequent failure for some PSC components to occur during the test stimulation. *P* of an identified component is measured based on its response to 50–100 consecutive test stimuli (0.05 Hz unless otherwise indicated). For polysynaptic pathways in these cultures, each additional synapse usually introduced a delay of 4–10 ms (2–5 ms for synaptic delay and action-potential conduction and 2–5 ms delay for the initiation of an action potential). Neurons in these cultures were either glutamate-mediating or GABA-mediating in nature and could be identified based on the time course, reversal potential and pharmacology of their evoked synaptic currents (EPSCs and IPSCs, respectively)[13,29]. EPSCs were blocked by 10 μM 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX, RBI) whereas IPSCs were blocked by 10 μM bicuculline (RBI). The IPSCs had distinctly longer decay time and more negative reversal potentials (around −50 mV) than EPSCs. In a typical culture, we estimated that less than 20% of the neurons were GABA-mediated.

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Benzodiazepine actions mediated by specific γ-aminobutyric acid$_A$ receptor subtypes

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GABA$_A$ (γ-aminobutyric acid$_A$) receptors are molecular substrates for the regulation of vigilance, anxiety, muscle tension, epileptogenic activity and memory functions, which is evident from the spectrum of actions elicited by clinically effective drugs acting at their modulatory benzodiazepine-binding site. Here we show, by introducing a histidine-to-arginine point mutation at position 101 of the murine α1-subunit gene, that α1-type GABA$_A$ receptors, which are mainly expressed in cortical areas and thalamus[1], are rendered insensitive to allosteric modulation by benzodiazepine-site ligands, whilst regulation by the physiological neurotransmitter γ-aminobutyric acid is preserved. α1(H101R) mice failed to show the sedative, amnesic and partly the anticonvulsant action of diazepam. In contrast, the anxiolytic-like, myorelaxant, motor-impairing and ethanol-potentiating effects were fully retained, and are attributed to the nonmutated GABA$_A$ receptors found in the limbic system (α2, α5), in monoaminergic neurons (α3) and in motoneurons (α2, α5)[1]. Thus, benzodiazepine-induced behavioural responses are mediated by specific GABA$_A$ receptor subtypes in distinct neuronal circuits, which is of interest for drug design.

Fast synaptic inhibition in the mammalian brain is largely mediated by the activation of GABA$_A$ receptors, which are heteromeric GABA-gated chloride channels[2]. Their opening frequency is enhanced by agonists of the benzodiazepine site, which is the basis of their therapeutic effectiveness but also of their undesired side effects. The classical benzodiazepines such as diazepam interact indiscriminately with all benzodiazepine-sensitive GABA$_A$ receptor subtypes (α1, α2, α3 and α5) with comparable affinities[3,4]. These receptors have a histidine at a conserved position (α1-H101, α2-H101, α3-H126 and α5-H105). In contrast, the benzodiazepine-insensitive receptor subtypes in the brain (α4 and α6) have an arginine in the corresponding position.

Recombinant diazepam-sensitive receptors can be rendered diazepam-insensitive by replacing this histidine by arginine without altering the GABA sensitivity, as shown for the α1 subunit[5,6] and the α2, α3 and α5 subunits[7]. The pharmacological significance of the predominant GABA$_A$ receptor subtype in the brain, which contains