Synaptic plasticity in rat hippocampal slice cultures: Local "Hebbian" conjunction of pre- and postsynaptic stimulation leads to distributed synaptic enhancement

(long-term potentiation/organotypic slice cultures/optical recording)

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A central theme in neurobiology is the search ABSTRACT for the mechanisms underlying learning and memory. Since the seminal work, first of Cajal and later of Hebb, the synapse is thought to be the basic "storing unit." Hebb proposed that information is stored by correlation: synapses between neurons, which are often coactive, are enhanced. Several recent findings suggest that such a mechanism is indeed operative in the central nervous system. Pairing of activity on presynaptic fibers with strong postsynaptic depolarization results in synaptic enhancement. While there is substantial evidence in favor of a postsynaptic locus for detection of the synchronous preand postsynaptic event and subsequent initiation of synaptic enhancement, the locus of this enhancement and its ensuing persistence is still disputed: both pre- and postsynaptic contributions have been suggested. In all previous studies, the enhancement was presumed to be specific to the synapses where synchronous pre- and postsynaptic stimulation was applied. We report here that two recording techniques-optical recording, using voltage-sensitive dyes, and double intracellular recordings-reveal that synaptic enhancement is not restricted to the stimulated cell. Although we paired single afferent volleys with intracellular stimulation confined to one postsynaptic cell, we found that strengthening also occurred on synapses between the stimulated presynaptic fibers and neighboring cells. This suggests that synaptic enhancement by the "paired-stimulation paradigm" is not local on the presynaptic axons and that, in fact, the synapses of many neighboring postsynaptic cells are enhanced.

It has long been speculated that synaptic enhancement might be controlled by concurrent pre- and postsynaptic activity (1, 2). Although there is a wealth of circumstantial evidence (3-7) for this hypothesis, direct evidence was obtained only recently. Several investigators (8–10) showed that synapses can be strengthened by frequent simultaneous pre- and postsynaptic stimulation. Once this result had been obtained, an obvious question was whether the enhancement is specific to the synapses that received the concurrent stimulation or whether it is a more global phenomenon. Gustafsson et al. (8) showed that in the hippocampus the enhancement is specific on the dendrites. That is to say (cf. Fig. 1), if a synapse (A) between an input fiber (a) and a dendrite is strengthened by simultaneous pre- and postsynaptic activation, the strength of a neighboring synapse (B) between a different fiber (b) and the same dendrite is not changed. One question, however, remained unanswered: what happens to synapse C between the same input fiber and a different neuron? Is this synapse unaffected, like B, or does it, like A, undergo enhancement? To assess this question, one has to measure the strength of synapses lying on the same presynaptic fiber but on different





FIG. 1. Specificity of synaptic enhancement. When synapse A is enhanced by simultaneous stimulation of an input fiber (a) and a postsynaptic neuron, is the efficacy of neighboring synapse B on the same dendrite affected or not? Gustafsson *et al.* (8) answered this question: the strength of synapse B is not affected. The question of axonal specificity of synaptic enhancement, however, remained unanswered: is the strength of synapse C between the same presynaptic fiber (a) and a different postsynaptic neuron also unaffected, or is its efficacy altered? se, Stimulating electrode; ie, intracellular electrode.

postsynaptic neurons. It is thus crucial to be able to record from two (or preferably more) neurons simultaneously. In our experiments, we took advantage of the fact that this is relatively easy in hippocampal slice cultures (11–13) with both optical (14) and conventional electrophysiological techniques. Thus, after establishing that synapses in hippocampal slice cultures do show plasticity (unpublished data), we chose this preparation for investigating whether synaptic enhancement is local on the presynaptic fibers.

MATERIALS AND METHODS

Slice Cultures. Details of the slice culture technique have been described by Gähwiler (11–13). In short, hippocampal slice cultures were prepared from 3- to 7-day-old rats. After decapitation and removal of the hippocampus, the tissue was cut into 350- μ m-thick sections with a McIlwain tissue chopper, which were pasted onto glass coverslips with 15 μ l of plasma (TC chicken plasma, Difco) coagulated by 15 μ l of a thrombin solution. Once the plasma clot had hardened, the coverslips were transferred into culture tubes containing 750 μ l of culture medium [49% (vol/vol) Hanks' basal medium/ 25% (vol/vol) horse serum/24.5% (vol/vol) Hanks' balanced salt solution/0.5% 200 mM L-glutamine (all from Gibco)/1% 2.8 M D-glucose] and put into a roller drum incubator at 34°C. Within 1–2 weeks, the slices flattened to monolayer thickness

Abbreviations: EPSP, excitatory postsynaptic potential; ISI, interstimulus interval; LTP, long-term potentiation.

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and were suitable for our experiments. We were able to keep these cultures viable for up to 6 months.

Pairing Experiments. A stimulating electrode was placed in the Schaffer collaterals of the CA3 or CA1 region (cf. Fig. 2). For intracellular recordings, we impaled neurons in the pyramidal layer of the CA1 region (electrodes filled with 3 M KCl; resistance, 60–100 M Ω). For the double intracellular recordings, we impaled two neurons lying close together in the CA1 region of the hippocampal slice culture (distance, 25–60 μ m). The strength of the test stimulus (duration, 50 μ s) to the Schaffer collaterals was set so that we could record stable excitatory postsynaptic potentials (EPSPs) in the postsynaptic neurons or so that the neurons fired only occasionally. After the responses to a single afferent volley as test stimulus were stable, we applied a pairing paradigm similar to the one developed by Gustafsson et al. (8): concurrent to the presynaptic volley, we depolarized one of the intracellularly recorded neurons by injecting a current pulse, usually of 5 nA (duration, 100 ms); the test stimulus was delivered to the presynaptic fibers 10 ms after the onset of the depolarizing pulse. In each case, we ensured that the membrane potential of the other neuron was not affected by this depolarization. After 25-30 such pairings [interstimulus interval (ISI) usually 4 s], we ceased the depolarization procedure and recorded the response of the neurons to only the test stimulus again. In some cases (e.g., see Fig. 5), we used a modified pairing procedure in which we alternated pairing pre- and postsynaptic stimulation with test stimuli alone. This enabled us to investigate the temporal development of synaptic enhancement for the paired cell as well, by recording the responses to the test stimuli between individual pairing trials.

Optical Recording. Voltage-sensitive dyes were used for these experiments (for details regarding the optical recording technique, see ref. 14). The coverslip with the slice culture lay in a recording chamber perfused at 30 ml/hr with a modified Hanks' balanced salt solution (containing 3.2 mM CaCl₂). The temperature of the solution was kept at $33 \pm 1^{\circ}$ C. The chamber was mounted on an inverted microscope (Zeiss, IM 35) equipped with an epifluorescence filter set (Zeiss, BP 546, FT 580, LP 590). The slice culture was illuminated by a mercury lamp with a stabilized power supply (Siemens). The image of the slice culture was projected with a microscope



FIG. 2. (A) Schematic drawing of the CA1 and CA3 regions of the hippocampus (neurons are not in scale). Open arrowhead, the extracellular electrode (se) with which test stimuli were delivered to the Schaffer collaterals (sc); solid arrowheads, the two intracellular electrodes (ie_1, ie_2; cf. Fig. 4). Superimposed dotted matrix depicts the photodiode array (cf. Fig. 3). (B) Spatial resolution of the optical recording. A part of the 12 \times 12 photodiode array was projected onto a hippocampal slice culture with the proper magnification. For reasons of clarity, a Nissl-stained culture was used.

objective (Zeiss, $\times 63$; 1.25 oil immersion) onto a 12×12 photodiode array (Centronics, M144-5) mounted on the TV stage of the microscope. The photodiodes transformed the fluorescence signals into electrical signals. These signals were amplified, multiplexed, and fed into a microcomputer (DEC LSI 11/73) with direct memory access. The data were transferred to a VAX 750 (DEC) for further signal processing.

We used the styryl dyes RH-237 (15) and RH-414 (16) for our experiments. The medium in the chamber was exchanged for 1–1.5 ml of the voltage-sensitive dye; perfusion was then stopped for 30 min. After recommencing perfusion, we waited 10–20 min to ensure that the dye not bound to cell membranes was washed out. To minimize photodynamic damage, we only recorded fluorescence signals every 30 s. Every third sweep, a stimulus was delivered (i.e., ISI of 90 s); the remaining two sweeps were used for signal correction purposes (14). The pairing procedures were the same as described above.

RESULTS

The goal of our experiments was to test whether synaptic enhancement, induced by simultaneous pre- and postsynaptic stimulation, is spatially well restricted on the presynaptic axons as it is on the postsynaptic dendrite (8). To this end, we placed an extracellular electrode stimulating the Schaffer collaterals and impaled a postsynaptic neuron in the CA1



FIG. 3. Optical recording of the activity of hippocampal tissue stained with the voltage-sensitive dye RH-237 (15). Each trace displays the activity recorded by the photodiode from the corresponding location of the tissue. Vertical scale bar indicates the relative fluorescence change $\Delta F/F$; horizontal scale bar shows the time in each of the traces. The photodiodes of the array (projected onto the tissue) were 23 μ m apart. The recording was performed in the CA1 region of a hippocampal slice culture (cf. Fig. 2). The thin (lower) curves represent an average of two responses (presumably EPSPs) to a stimulus delivered in the Schaffer collaterals. After these two test stimuli (ISI, 90 s), we applied paired stimulation to a neuron that lay under the diode (*). The neuron is outlined schematically in the figure. After 30 pairings, the response of the neurons was tested again. This is displayed in the thick (upper) curves in the figure: they represent the average response of 10 trials during the first 15 min after synaptic enhancement. Hatched area in every signal marks the difference in response before and after pairing. The responses not only of the cell under the diode marked with an asterisk but also of the surrounding neurons are clearly enhanced. This could also be seen on single sweeps; averaging was performed only for the sake of clarity.

region of the hippocampal slice culture (Fig. 2). The actual enhancement was performed as described by Gustafsson etal. (8): single presynaptic volleys were paired with depolarizing current pulses to the postsynaptic cell. Thus the "Hebbian condition" for synaptic enhancement was fulfilled: simultaneous pre- and postsynaptic activity should lead to enhancement of the synapses.

After we were able to show that this paired-stimulation paradigm also leads to synaptic strengthening in hippocampal slice cultures (unpublished data), we addressed the question of axonal specificity of that mechanism in this system.

To simultaneously monitor the strength of synapses to different postsynaptic neurons, we used optical recording with voltage-sensitive dyes. We measured the responses of all the neurons in a field of view of $280 \times 280 \mu m$, as shown in Fig. 2. Before synaptic enhancement, the responses of all these neurons to the presynaptic volley were measured. Then enhancement was accomplished, as described above, by repetitively pairing a postsynaptic stimulus to a single neuron with a single presynaptic stimulus to the Schaffer collaterals. Thereafter, the responses of the neurons were tested again.

The outcome of such an experiment is shown in Fig. 3. The thin curves in the figure represent the averaged responses of the neurons before and the thick curves show the responses after enhancement of the synapses. The postsynaptically stimulated neuron is under the photodiode marked with an asterisk. It is clearly visible that the responses of not only this one neuron but also of neighboring neurons, were enhanced.

It should be stressed that this spread cannot be attributed to poor spatial resolution (e.g., the signal from one neuron being recorded by different photodiodes). We have shown elsewhere (14) that the optical recording procedure used here permits single-cell resolution. Thus, the result in Fig. 3 indicates that not only the synapses that actually received the paired stimulation but also other synapses on the same presynaptic axons are enhanced.[†]

We performed 26 of these experiments; in 11 experiments, enhancement could be induced and measured optically. In each of these 11 cases, we found a spread of the effect that amounted to at least some 150 μ m around the depolarized neuron. In Fig. 3, one can observe a slight decay of the amount of enhancement toward the left edge of the photodiode array. This result, however, should not be overinterpreted, since inhomogeneous illumination of the tissue causes the light intensity at the border of the field of view to be low. Thus, the signals recorded with the photodiodes become less reliable toward the edges of the array.

To confirm this finding obtained with optical methods by conventional electrophysiology, we also performed double intracellular recordings. The stimulating electrode was again placed in the Schaffer collaterals in the CA3 region. We impaled two closely adjacent cells with intracellular electrodes (Fig. 2, ie₁ and ie₂) and recorded from both of them simultaneously. Once the recordings appeared to be stable (spike amplitudes, 60-75 mV; resting potentials, at least -60 mV), test stimuli were delivered. After recording the responses to the test stimulus, we paired 30 depolarizing current pulses in cell 1 with the extracellular stimulation of the Schaffer collaterals. No current was injected into cell 2. We examined the responses of both cells before (Fig. 4 a and f), during (Fig. 4 b and g), and 1 min (Fig. 4 c and h), 10 min (Fig. 4 d and i), and 30 min (Fig. 4 e and j) after the pairing. Surprisingly, but consistent with the results from the optical recording experiments, both the cell that received the postsynaptic depolarization and the other cell show enhanced responses to the original test stimulus (Fig. 4 c and h). The initial slopes of the EPSPs in both cells are steeper, in turn causing the action potentials to occur earlier and more reliably with respect to the stimulus. The effect was rather long-lasting: 10 (Fig. 4 d and i) and even 30 (Fig. 4 e and f) min after pairing, enhancement was still clearly visible in both cells.

Here too, it holds true that in all our experiments in which enhancement of the response of cell 1 (n = 6 of $n_{tot} = 9$) was observed, it was accompanied by an increased response in cell 2. In other words, whenever synaptic enhancement could be induced in the paired cell (which was not always the case), we always observed enhanced responses in both cells.

To quantify the amount of synaptic enhancement and its time course, we chose the latency of the spike as a measure of synaptic strength. For another experiment, we divided the



FIG. 4. Simultaneous intracellular recordings from two CA1 neurons in a hippocampal slice culture. Two closely adjacent cells (distance, $\approx 30 \ \mu m$) were impaled (cf. Fig. 2). We recorded the responses of the two neurons to an extracellular test stimulus that was applied to the Schaffer collaterals with a frequency of 0.25 Hz. The responses of both cells are rather irregular (a and f): sometimes an action potential is elicited, and sometimes only an EPSP can be seen. In every response, the initial slope of the EPSP (which can be taken as relative measure for synaptic strength) is very small. The two panels labeled "during" (b and g) show the membrane potentials of the two neurons during synaptic enhancement by simultaneous pre- and postsynaptic stimulation. The steep increase in the membrane potential of cell 1 is caused by the strong depolarizing current. The current pulses in cell 1 do not cause any significant depolarization or additional action potentials in cell 2. Thus, enhancement of synapses of cell 2 cannot be attributed to this effect. However, observing the cells 1 min (c and h), 10 min (d and i), and 30 min (e and j) after the pairing reveals a long-lasting enhancement of the synapses of both cells: the initial slope of the EPSPs is clearly steeper than before the pairing, which in turn causes the action potentials of both cells to occur more reliably and earlier with respect to the stimulus.

[†]One does not see the enhancement occurring only in a narrow band perpendicular to the dendrites, as might be expected in view of the typical hippocampal architecture. However, hippocampal slice cultures have a somewhat broadened pyramidal layer so that all photodiodes of the array look onto pyramidal cell bodies. Neurons in the pyramidal cell layer lying above and below the postsynaptically stimulated cell also show enhanced responses because their synapses to the Schaffer collaterals lie on the same input fibers as the ones of the postsynaptically stimulated neuron. A spread of enhancement along the axons, thus, causes responses of the neurons to be larger not only along one axis but also perpendicular to it.

data for both neurons into sets of five responses and calculated the average value and the standard deviation of the spike latency for each set. Values for both cells are shown in Fig 5. Again, although only neuron 1 received the postsynaptic, and thus concurrent, stimulation, the data show that, starting with the onset of pairing, the spike latency declines in both cells and reaches a stable minimum after pairing is stopped. In parallel, one observes a similar decrease in the standard deviation, confirming the increase in reliability of firing previously described.

The spike latency is not a suitable criterion for synaptic enhancement in all cases. For instance, in two experiments, the response of one of the cells changed from pure EPSPs with no or only an occasional spike before pairing to action potentials riding on larger EPSPs after pairing (in one experiment, the fraction of the responses with at least one spike increased from 8% to 85%; in the other experiment, it increased from 0% to 48%). In both cases, we counted this as a successful enhancement, although spike latency analysis could, of course, not be applied in either of the two. For those



FIG. 5. Temporal development of synaptic enhancement. Two closely adjacent cells in the CA1 region were simultaneously recorded with intracellular electrodes; similar to the experiment in Fig. 4, pairing was again applied only to cell 1. Single data points represent averages of the spike latencies of five subsequent responses to the test stimulus (ISI, 5 s; interval between adjacent data points thus corresponds to an interval of 25 s); bars denote standard deviations. The period during which pairing with a depolarizing current of 1 nA was applied to cell 1 is indicated with a solid bar; cell 2 did not receive postsynaptic current injection. Starting with the onset of pairing, the spike latency for both cell 1 and cell 2 gradually declines, to reach a stable minimum upon termination of the pairing. Note that in both graphs the decrease of spike latency is accompanied by a reduction of the standard deviation, indicating that the action potentials of both cells occur not only earlier but also more reliably with respect to the stimulus.

experiments for which the spike latency was an appropriate measure, we performed a one-way analysis of variance on the data. In every case that we judged successful on visual inspection and in which the measure of latency was applicable (n = 4), the spike latencies for pre- as opposed to postpairing were found to differ significantly in both cells [(P < 0.0001 in three cases; P < 0.01 in one case, in which for technical reasons we had a very limited set of data points (n = 18)]. In the only case in which spikes were recorded and which we judged unsuccessful, the latency differences proved to be insignificant (P > 0.05).

We performed several control experiments to rule out the possibility of the observed spatial spread of synaptic enhancement being caused by some unspecific effect related to the pre- or postsynaptic stimulation but not to the conjunctive event.

The first and most obvious of these experiments was to test whether repeated (ISI, 4 s) presynaptic stimulation with a single afferent volley as a test stimulus per se would suffice to induce the observed enhancement. Since double intracellular recordings are difficult, we addressed this issue with single-electrode recordings. We measured the intra- and extracellular responses of CA1 pyramidal neurons to a presynaptic stimulus of 0.25 Hz applied over up to 20 min. To obtain a quantitative measure of the effect of presynaptic stimulation alone, we plotted (in a fashion similar to that in Fig. 5) the spike latencies of the responses over time and calculated the regression line through these data points. Its slope β is a measure for the change of latency over time. Our aim was to show that it was small or zero. In each of the cases studied for this test (n = 3), the fit of a regression line was statistically significant (P < 0.0005) and the slope was always very small ($\beta_1 = 0.26$, $\beta_2 = -0.12$, $\beta_3 = 0.06$; all in ms of spike latency per min).

This means that over this range of time, we could not observe significant enhancement of the cells' responses in any of the three cases. This possibility of enhancement being due to presynaptic stimulation alone seemed very unlikely to begin with, since we found a very marked correlation between the commencement of paired stimulation and the enhanced responses of the cells in all our pairing experiments (cf. Fig. 5).

In additional experiments, we verified that postsynaptic stimulation alone was not sufficient to induce the observed effect.

The experiments with double intracellular recordings also make it highly unlikely that the unspecific strengthening is caused by gap junctions or by synapses on local collaterals. Before and after each experiment, we tested for connections between the two cells by firing either one of them by intracellular current injection. We very rarely (at least in the cell body) observed coupling between the two recorded neurons. The only pair of cells that was found to be interconnected was not considered in the analysis.

DISCUSSION

Our results show that enhancement of synapses by the paired-stimulation paradigm is not specific to synapses for which there is paired stimulation in the strict sense: synapses whose neurons did not receive the postsynaptic stimulation are also enhanced. This "synaptic recruitment" extends over at least 150 μ m of axon around the primarily enhanced synapse.

This finding was quite a surprise to us. It had previously been shown (8, 9, 17) that synaptic enhancement is specific on dendrites—i.e., if some synapses on a dendrite are enhanced, neighboring synapses on the same dendrite are not. This finding seemed to imply that the mechanism underlying this effect is indeed specific. Yet, as pointed out in Fig. 1, our results, although unexpected, are not at variance with these results. Whereas those experiments show that synaptic strengthening can be specific on a dendrite, our results indicate that this enhancement seems not to be well restricted on the presynaptic axon.

We observed that the spread of synaptic enhancement amounts to at least some 150 μ m on the presynaptic axon. It is, however, unlikely that this enhancement extends over the whole axon: it has been demonstrated that long-term potentiation (LTP) of an ipsilateral pathway to the hippocampus does not cause enhancement of the contralateral synapses of the same fibers (18). These results, however, apply to synapses lying several millimeters apart; they do not allow one to infer that synaptic enhancement on axons is spatially well confined.

On the contrary, some reports on LTP make it quite plausible that synaptic enhancement is not specific on axons. Sastry et al. (19) suggested that LTP is produced by hyperpolarization of the axon terminals, leading to an increase in the size of the action potential, which in turn increases the amount of transmitter released, thus producing a larger response of the postsynaptic neurons. Indeed, this fits well with our results, since one would expect such a hyperpolarization to spread over at least part of the axon. It would thus lead to a larger action potential in a substantial area around the primarily reinforced synapse(s) and result in an increased response of many neurons surrounding the intracellularly stimulated one. Also, if one imagines the synaptic strengthening being induced by a second messenger presynaptically, it is quite likely that this substance spreads within the presynaptic fiber, thereby causing strengthening to occur in a relatively large area around the primarily reinforced synapse.

Our experiments are, of course, strongly related to the question of whether synaptic enhancement is performed preor postsynaptically. There are several reports in the literature, some favoring a presynaptic (20-22) and some favoring a postsynaptic mechanism (23-25). None of these experiments, however, is fully conclusive. The results from our experiments, indicating a presynaptic spread of the enhancement, seem to support the notion of a strong presynaptic component in the mechanism underlying synaptic enhancement, as was also suggested by several groups working on LTP (19, 20, 26).

This is not in conflict with the idea that the postsynaptically located N-methyl-D-aspartate receptor is the device detecting the simultaneity of pre- and postsynaptic events (27, 28). It rather emphasizes the need to distinguish between the mechanism detecting the condition under which enhancement is to be initiated and the mechanism that actually performs the enhancement (29, 30). It is conceivable that the first is located on the postsynaptic side, whereas the latter is presynaptic.

Recent data (31) suggest that synaptic enhancement in LTP is mediated by an early presynaptic component (lasting \approx 30 min) and a late postsynaptic component (which starts after \approx 30 min). Also, our results indicate that the presynaptic spread and thus the presynaptic component of enhancement can last up to 30 min (cf. Fig. 4). Our finding of axonal spread of synaptic enhancement, taken together with the observed temporal distinctness of pre- and postsynaptic mechanisms (31), raises the intriguing question of whether the presumed late postsynaptic component accepts the whole multitude of presynaptically enhanced synapses or whether it recognizes only those synapses that were stimulated in a truly Hebbian manner.

Our findings do not conform with the Hebb rule in the strict sense: synaptic strengthening does require Hebbian conjunction of pre- and postsynaptic stimulation; it is, however, at least for the first 30 min, not restricted to the synapses of neurons that have actually received the concurrent stimulation. This decrease in specificity of presynaptic enhancement does admittedly lead to certain saturation problems (in due course, all available synapses might turn out to become enhanced). Yet, at the same time, "synaptic recruitment" might well have profitable consequences: it could serve as an amplification mechanism, causing coincidences between few neurons to be sufficient to enhance connections within a considerably larger group of neurons. The extent of "synaptic recruitment" may be viewed as the solution to an optimization problem created by the opposing drives of saturation and amplification. The extent to which the solution we observed for the hippocampus (i.e., synaptic recruitment over at least 150 μ m of axon) applies to other brain regions remains to be investigated in more detail.

At least preliminary results (A. Kossel, T.B., and J. Bolz, unpublished data) seem to indicate that a similar phenomenon can be observed in conventional slices of rat neocortex.

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