Rapid Development of Synaptic Connections and Plasticity Between Sensory Neurons and Motor Neurons of *Aplysia* in Cell Culture: Implications for Learning and Regulation of Synaptic Strength

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Coulson, Rosalind L. and Marc Klein. Rapid development of synaptic connections and plasticity between sensory neurons and motor neurons of *Aplysia* in cell culture: implications for learning and regulation of synaptic strength. *J. Neurophysiol.* 77: 2316–2327, 1997. We describe here the time course of functional synapse formation and of the development of short-term synaptic plasticity at *Aplysia* sensorimotor synapses in cell culture, as well as the effects of blocking protein synthesis or postsynaptic receptors on the development of synaptic transmission and plasticity. We find that synaptic responses can be elicited in 50% of sensory neuron–motor neuron pairs by 1 h after cell contact and that short-term homosynaptic depression and synaptic augmentation and restoration by the endogenous facilitatory transmitter serotonin are present at the earliest stages of synapse formation. Neither block of protein synthesis with anisomycin nor block of two types of postsynaptic glutamate receptor has any effect on the development of synaptic transmission or synaptic plasticity. The rapidity of synapse formation and maturation and their independence of protein synthesis suggest that changes in the number of functional synapses could contribute to short- and intermediate-term forms of synaptic plasticity and learning.

**INTRODUCTION**

Synaptogenesis occurs during the initial wiring of the nervous system and with recovery from neuronal injury. Formation of new synapses also contributes to long-term, protein-synthesis-dependent forms of learning in the intact adult nervous system (Bailey and Chen 1988a,b; Chang and Greenough 1984; Chang et al. 1991; Glanzman et al. 1990), and both up- and downregulation of synapse number occurs with exposure to different environmental stimuli (Black et al. 1990; Schacher and Montarolo 1991; Turner and Greenough 1985). However, it is not known whether the same signaling pathways are used in all these forms of regulation of synapse number.

The relations among several mechanisms for generating synapses (Haydon and Drapeau 1995) would determine whether different types of synaptic plasticity can be mobilized independently or whether they might interfere with, or reinforce, each other. For, example, there may exist critical periods for synaptic modifiability such that newly formed synapses might be uniquely susceptible to—or uniquely protected from—specific kinds of modulation.

The aims of the present study are twofold: first, to describe in broad terms the phenomenology of functional synapse formation between neurons from an adult CNS; and second, to examine the influence of synapse age on the expression of short-term synaptic modulation. More generally, we were interested in examining the relationships among synapse formation in regenerating adult neurons, in development, and in learning.

In the first part of our study, we examined the time course of the appearance of functional synapses and of the increase in excitatory postsynaptic potential (EPSP) amplitude after pairing of sensory neurons and motor neurons of *Aplysia* in cell culture. In addition, we tested the effects of inhibiting protein synthesis and of blocking postsynaptic receptors on the development of synaptic connections and plasticity.

In the second part of our study, we examined the relationship between the age of synapses and their potential for synaptic modulation. Synaptic depression and facilitation at sensorimotor synapses of *Aplysia* contribute to a number of forms of short- and long-term learning (Byrne et al. 1993). We examined three forms of short-term synaptic plasticity at synapses of different ages: homosynaptic depression, synaptic augmentation of depressed synapses by the endogenous modulatory transmitter serotonin (5-HT) (Braha et al. 1990; Ghirardi et al. 1992; Sacktor et al. 1990; Sugita et al. 1992), and synaptic augmentation of rested synapses by 5-HT (Ghirardi et al. 1992; Klein 1993).

Our results indicate that synapses form and mature rapidly after cell contact, and that synapse formation and maturation occur despite the block of postsynaptic receptors. In contrast to long-term learning, synaptogenesis triggered by cell contact does not depend on the synthesis of new protein. In contrast to the situation in developing *Aplysia* (Nolen and Carew 1988; Rankin and Carew 1987, 1988; Rayport and Camardo 1984; Rayport and Kandel 1986), all three forms of synaptic plasticity that we examined appear intact at the earliest stages of synapse formation during regeneration. The rapidity of synapse formation and maturation and their independence of new protein synthesis suggest that changes in the number of functional synapses could in principle contribute not only to long-term synaptic modulation, but also to short- and intermediate-term synaptic plasticity and learning.

**METHODS**

**Cell culture**

To be able to trigger synapse formation in a temporally precise manner, we used a preparation of “soma-to-soma” synapses in
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which neurons are paired only after completely absorbing their neurites (Klein 1994). Such synapses have no apparent morphological or functional differences from those formed in conventional culture conditions or those found in intact ganglia (Klein 1993, 1994). Pairs were tested for synaptic connections at 1–10 h, 17–33 h, or 40–72 h after cell contact; we refer to these as “early” (or “newly formed”), “intermediate,” and “late” synapses, respectively.

Adult Aplysia californica (75–150 g; Marine Specimens Unlimited, Pacific Palisades, CA or Alacrity Marine Biological Services, Redondo Beach, CA) were anesthetized by injection of 60–120 ml of isotonic MgCl₂, and tail sensory neurons and siphon (LFS) motor neurons were isolated and maintained in culture as previously described (Klein 1993, 1994). Sensory and motor neurons were maintained in separate petri dishes at room temperature (21–24°C) in 10% Aplysia hemolymph in Leibovitz L15 culture medium (Gibco BRL, Grand Island, NY) supplemented with salts (Klein 1993). Under these conditions, the neurons retrace their processes and become spherical in shape. After 3 days, the LFS motor neurons were transferred into fresh medium. A single sensory neuron was manipulated into contact with each motor neuron and the pairs were left to incubate between 45 min and 3 days before intracellular recording.

For synapse formation in the presence of postsynaptic receptor antagonists or anisomycin, media were prepared containing the test solution, or the same volume of vehicle, in 10% hemolymph/L15. Unpaired neurons were transferred into the prepared medium, one sensory cell was manipulated into contact with each LFS neuron, and the pairs were incubated at room temperature for 1–3 days. Half of the cells from each animal were paired in test medium and half in control medium containing the vehicle alone. 6-Cyano-7-nitroquinoxaline-2,3-dione (CNQX, Tocris Cookson, St. Louis, MO) was used at a final concentration of 100 μM in 0.5% dimethyl sulfoxide; dl-2-amino-5-phosphonovaleric acid (APV; Sigma) was used at a final concentration of 100 μM in the incubation medium; and anisomycin (Sigma) was used at a final concentration of 20 μM in 0.1% ethanol. Protein synthesis inhibition by anisomycin was tested by monitoring the incorporation of 35S-methionine into protein in desheathed pleural ganglia of Aplysia. Incorporation was reduced by >90% when ganglia were incubated in anisomycin.

When the cell pairs had been incubated in CNQX, APV, or anisomycin-containing medium, they were transferred in 3 μl of medium to a petri dish containing 3 ml of fresh L15 10–20 min before recording. The cells were further washed with 5 ml of artificial seawater between the first action potential and the start of the train of stimuli.

Electrophysiological recording

An Axoclamp 2A amplifier (Axon Instruments) and borosilicate glass micropipettes (tip resistance 10–20 MΩ) filled with 2 M potassium acetate (pH 7.5) were used for intracellular recordings. In some experiments, the postsynaptic electrode was filled with cesium acetate (Eliot et al. 1994b). Recordings were carried out in artificial seawater (composition, in mM: 460 NaCl, 10 KCl, 11 CaCl₂, 55 MgCl₂, and 10 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.5). In a typical recording, the neuron type was confirmed by the response to release of hyperpolarizing current (Eliot et al. 1994a), pre- and postsynaptic resting potential and membrane resistance were noted, and the current required to fire the sensory neuron was determined. The postsynaptic response to the first action potential, referred to as the “test EPSP,” was then recorded. The cells were allowed to rest for 5 min before a train of stimuli was started. The experiment was continued regardless of the presence or absence of EPSPs. Throughout the experiment the sensory neuron was held at −50 mV and the LFS motor neuron was hyperpolarized to −80 mV in current clamp mode.

After a predetermined number of stimuli, 5-HT (creatine sulfate; Sigma) was added in the interval between stimuli. Application was with a hand-held Pipetman directly into the bath; the concentration bathing the cells was estimated from separate dye dilution experiments to be ~25 μM.

Chemical EPSPs were distinguished from electrical coupling by the longer latency, faster rise time, unidirectional nature, and abolition by CNQX (100 μM) or cadmium (50 or 250 μM). Responses were considered to be failures only if there was no positive deflection of the postsynaptic voltage within 50 ms of the peak of the presynaptic action potential. Although this is almost certainly an underestimate of the true failures, it is also probable that some of the smallest responses went undetected and were counted as failures because our detection limit was ~100 μV. We detected spontaneous miniature synaptic potentials in 11 experiments. The mean amplitude for individual experiments ranged from 0.51 to 3.14 mV, with an overall average of 1.04 ± 0.82 (SD) mV. The average coefficient of variation of the miniature potentials was 0.28 ± 0.07.

Data acquisition and analysis

Experiments were recorded continuously on VHS tape and individual episodes were stored on Syquest hard disks with the use of the Spike electrophysiology analysis program (Hilal Associates, Englewood Cliffs, NJ). Records of some experiments were filtered at 300 Hz. Amplitudes and rise times of EPSPs were measured with the use of the Spike program. Records were excluded if the EPSP caused an immediate action potential. In some cases an electrical component, which remained in the presence of CNQX and cadmium, was subtracted from the postsynaptic potential. These were rare and of small magnitude.

Augmentation by 5-HT was calculated relative to the average of the two stimuli before 5-HT. Restoration was calculated relative to the average of the first 5 stimuli of the experiment or to the average of all 15 stimuli before 5-HT; both calculations gave similar results. Results >5 SD different from the mean (1 relative depression and 1 relative restoration) were excluded from the averages. Data are presented as means ± SE, and statistical comparisons were made with the use of Student’s two-tailed t-test. Exponentials were fit to the data with the use of the Prism graphing program (GraphPad Software, San Diego, CA).

RESULTS

Synapses form rapidly between sensory neurons and LFS motor neurons

After ≥1 day in culture, healthy Aplysia neurons adhere on contact. The neurons then rapidly grow together until the junction between the two cells is difficult to define visually. Single sensory cells were manipulated into contact with single LFS motor neurons and intracellular recordings were made from both cells at varying times after pairing. At 1 h postpairing, an EPSP could be evoked in the LFS neuron in response to a sensory neuron action potential in 25% of pairs. The proportion of sensory-LFS pairs that had functional synapses increased with a half-time of 3.6 h to a plateau of 82% in these experiments (Fig. 1A).

Even when no EPSP was detected initially, the experiment was continued with a preplanned train of stimuli and then addition of 5-HT to examine synaptic restoration or augmentation. Synaptic facilitation was induced by adding...
FIG. 1. Time course of synapse formation. A: percentage of sensory neuron–motor neuron pairs with detectable synaptic connections at different times after pairing. First bar: 0–2.5 h after pairing; subsequent bars: 5-h bins. Line: single exponential with time constant of 5.26 h and maximum of 82.11%. B1: amplitude of the excitatory postsynaptic potential (EPSP) response to the test stimulus at different times after pairing (note log scale). Each point represents the response of 1 cell pair to the test stimulus (n = 179); pairs with no response to the test stimulus are not shown. Exponential fit (line) has a time constant of 6.67 h and a maximum of 12.43 mV. B2: data from 0–10 h on an expanded time scale.

5-HT after 2 additional sensory neuron action potentials for augmentation, and after 15 additional action potentials for restoration. In some cases, evoked EPSPs were observed only after the addition of 5-HT, but not before (Fig. 2). When these pairs are included, the percentage of connections at 1 h increases to 50%, which is ~60% of the final plateau value. Synaptic connections recruited by 5-HT were more common between 1 and 10 h after pairing (14 of 130 pairs) than between 17 and 35 h (2 of 61 pairs) or >40 h (3 of 53 pairs) after pairing, but this difference was not statistically significant (P = 0.092, 1- to 10-h pairs vs. >17 h pairs, Fisher’s exact test). There was no difference in the appearance of pairs that formed synapses and those that did not, nor was there any significant difference in resting membrane potential, peak voltage of the action potential, or the current or voltage level at which an action potential was triggered.

Functional chemical synapses formed between sensory and LFS motor neurons, but not between pairs of sensory neurons or LFS neurons. Of 244 pairs that were identified as sensory-LFS pairs, 158 (65%) had chemical synapses (including those recruited by 5-HT). In contrast, only 5 (4%) of 129 pairs that appeared to be sensory-sensory or LFS-LFS had chemical synapses. No synapses were found in which the LFS motor neuron was presynaptic to a sensory neuron. Pairs of the same type of neuron almost always showed strong electrical coupling, which was rare between sensory and LFS neurons.

**EPSP amplitude increases with time after cell pairing**

There was a progressive increase in the amplitude of the test EPSP with time after pairing (Fig. 1B). The mean amplitude of the test EPSP of newly formed synapses (5.35 ± 0.09 mV; n = 70) was significantly smaller than that of intermediate (11.01 ± 1.62 mV; n = 58; t = 3.18, P < 0.002) or late synapses (14.00 ± 2.30 mV; n = 35; t = 4.19, P < 0.0001), which were not significantly different from each other (t = 1.09, P = 0.2). The increase in EPSP amplitude with time after pairing is not explained by changes
FIG. 2. Recruitment of low-probability synapses by serotonin (5-HT). A: 10 consecutive stimuli at 30-s intervals failed to elicit a detectable synaptic response at a sensory neuron–motor neuron pair 2 h after pairing; application of 5-HT after the 10th stimulus caused the appearance of responses to the next 6 stimuli. B: similar experiment with a different pair at 27 h postpairing gave a single, apparently unitary, response to the 7th stimulus before 5-HT (shown in isolation in the inset for clarity) and 5 consecutive larger responses after 5-HT application.

Transmission at newly formed synapses resembles that at mature synapses

The mean rise time of the EPSP was the same at newly formed synapses (10.35 ± 1.40 ms; n = 14) as at intermediate (10.50 ± 0.86 ms; n = 13) or late synapses (12.83 ± 2.15 ms; n = 9). There was also no difference in the pharmacological properties of the postsynaptic receptors at newly formed synapses and later synapses. In all cases, transmission was blocked by the α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA)-type glutamate receptor antagonist CNQX (100 μM; results not shown), as reported for other sensory neuron–motor neuron synapses in Aplysia (Dale and Kandel 1993; Trudeau and Castellucci 1993). We were unable to detect any effect of the N-methyl-D-aspartate (NMDA)-type receptor antagonist APV on the shape or amplitude of the EPSPs from synapses of any age at either hyperpolarized or depolarized potentials, despite the reported efficacy of APV in

in the electrical characteristics of the LFS neuron or of the sensory neuron. There is no correlation between the amplitude of the test EPSP and the input resistance of the LFS neuron (r = 0.01, n = 109) or the amplitude of the sensory neuron action potential (r = 0.002, n = 170), nor was the EPSP amplitude correlated with the resting membrane potential of either cell, the action potential threshold, or the current required to trigger an action potential.

There was considerable variability in EPSP amplitude at all ages. This variability was not reduced by correcting for the membrane resistance of the cells or by comparing cell pairs from the same animal, nor was there any seasonal variation. The single test action potential in the sensory neuron that was evoked at the beginning of each experiment sometimes failed to evoke an EPSP. The probability of such transmission failure at the first stimulus was inversely related to the average EPSP amplitude (r = −0.47, n = 47, P < 0.01) and was the same at different ages for synapses with EPSPs of similar amplitude.
blocking the induction of long-term potentiation at these synapses (Lin and Glanzman 1994) (results not shown). Glanzman (1994) has reported an APV-sensitive component of the EPSP in low-magnesium medium, but we did not attempt to replicate this finding.

*Homosynaptic depression is similar in newly formed and older synapses*

We compared both the magnitude and the kinetics of homosynaptic depression at synapses of different ages, and found no significant difference with age. When synapses were depressed by presenting 15 stimuli at 30-s intervals, the amplitude of the EPSP decayed exponentially with similar time constants at all ages, and the amplitude of the 10th–15th EPSPs was reduced to ~40% of the average of the first 5 EPSPs in all cases (Fig. 3). Although the overall amplitude of the EPSP declines monotonically when averaged across experiments, sequential EPSPs in individual experiments fluctuate in their amplitude (Fig. 3A), reflecting in part the probabilistic nature of quantal transmitter release. At all ages, the probability of failure of the EPSP increased with progressive stimulation, in parallel with the progressive decline in the average amplitude. The higher frequency of failures in the earliest synapses is likely to be a consequence of the fact that the average EPSP is smaller, because there is a good correlation between the number of failures and the amplitude of the EPSP when examined over all the experiments ($r = -0.43$; $n = 40$; $P = 0.0062$).

*Synaptic restoration and augmentation by 5-HT are similar at newly formed and older synapses*

To investigate facilitation at depressed synapses, termed restoration (Klein 1993), synapses were depressed by 15 stimuli at 30-s intervals, and then, after a 5-min rest and 2 additional stimuli, 5-HT was applied to the cells. The average pattern of restoration of groups of synapses at different times after cell pairing is shown in Fig. 4A. When all values are normalized to the mean of the first 15 EPSPs, the difference between restoration at newly formed synapses and at intermediate or late synapses is not statistically significant. To determine whether the tendency for greater restoration in newly formed synapses might be a consequence of the smaller average EPSP amplitude in this group, newly formed synapses were compared with intermediate or late synapses with EPSPs of similar ampli-

![Fig. 3. Homosynaptic depression at synapses at different times after pairing. A: EPSPs 1, 3, 5, 8, 11, and 14 from series of stimuli at 30-s intervals in 3 different cell pairs at 5.5 h (Early), 21 h (Intermediate), and >40 h (Late) postpairing. B: summary data with EPSP amplitudes normalized to the average of all 15 stimuli in each experiment. Single-exponential fits (—) have time constants of 2.0, 2.7, and 1.6 for early, intermediate, and late synapses, respectively; each of these falls within the 95% confidence limits of the others.](image)
FIG. 4. Synaptic restoration by 5-HT at different times after pairing. A: 15 EPSPs were elicited at 30-s intervals, stimulation was interrupted for 5 min, and then stimulation was continued at the original frequency. After the 1st 2 stimuli in the 2nd round, 5-HT was added (↑) to induce synaptic restoration. Amplitudes shown are normalized to the average of all 15 EPSPs before 5-HT. Values for the 1st point after 5-HT application are 8.57 ± 3.57 (SE; early), 4.38 ± 1.54 (SE; intermediate), and 3.73 ± 0.79 (SE; late); neither of the values for the later synapses is significantly different from the early value (P = 0.3 for both comparisons). B: comparison of restoration at synapses with EPSPs of similar amplitudes at different ages. Relative restoration is expressed as the average of the 1st 5 EPSPs in 5-HT divided by the average of the 1st 5 EPSPs of the experiment.

A second form of synaptic facilitation induced by 5-HT is synaptic augmentation, the facilitation of transmission at nondepressed synapses (Klein 1993). Augmentation was tested by giving two stimuli with an interstimulus interval of 1 min to establish a baseline, and then adding 5-HT. Relative augmentation was not significantly different in newly formed, intermediate, and late synapses (Fig. 5A). When synapses were divided into groups of similar amplitude, the increase in EPSP amplitude after 5-HT tended to be smaller with larger EPSPs, but was similar at newly formed, intermediate, and late synapses (Fig. 5B). Some synapses were observed at which EPSPs were detected only after 5-HT application. For these synapses, augmentation is expressed as the mean increase in EPSP amplitude (mV) and compared with synapses at which the average initial EPSP amplitude was between 0 and 1 mV (Fig. 5C).

Inhibition of protein synthesis does not interfere with synapse formation or the development of synaptic plasticity

In view of the necessity for macromolecular synthesis that has been reported for the expression of long-term forms of learning-related synaptic plasticity (Bailey et al. 1992a,b; Barzilai et al. 1989; Bergold et al. 1990; Frey et al. 1993; Ghirardi et al. 1995; Nguyen and Atwood 1990), we tested whether synapse formation itself or the development of short-term synaptic modulation requires the synthesis of new protein. We paired cells in 20 μM anisomycin and left them in the presence of the drug overnight. Pairs were transferred to recording medium without anisomycin immediately before testing. EPSPs were then elicited with presynaptic action potentials, and homosynaptic depression and 5-HT–mediated synaptic restoration were examined. Anisomycin had no effect on any of the synaptic phenomena that we examined (Fig. 6). The efficacy of this batch of anisomycin in blocking protein synthesis was confirmed in separate ex-
FIG. 5. Synaptic augmentation by 5-HT at different times after pairing. A: summary data, normalized to the average of the 2 EPSPs before 5-HT application (†). Relative augmentation, expressed as the average of the 2 EPSPs after 5-HT divided by the average of the 2 EPSPs before 5-HT is 6.47 ± 1.57 (SE; early), 4.92 ± 0.87 (SE; intermediate), and 4.27 ± 0.92 (SE; late). Neither intermediate value nor late value is different from early ($P = 0.4$ for intermediate vs. early and $P = 0.3$ for late vs. early). B: comparison of augmentation at synapses with EPSPs of similar amplitude at different ages. C: augmentation at early synapses with no EPSP before 5-HT (1–8 h, 0 mV) and at early (1–8 h, <1 mV) and intermediate (17–35, <1 mV) synapses with baseline EPSPs between 0 and 1 mV.
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Fig. 6. Synaptic transmission and restoration at synapses formed in the presence of anisomycin. A: EPSPs in control and in anisomycin-incubated synapses elicited at 30-s intervals before and after 5-HT application. B: summary data for amplitude of the test EPSP (left) and for synaptic restoration by 5-HT (right) in control and anisomycin-incubated cultures. Numbers in parentheses: number of cell pairs in each group.

periments by examining incorporation of radioactively labeled amino acids into protein; protein synthesis was blocked by >90% (see METHODS).

Block of postsynaptic receptors does not interfere with synapse formation or the development of homosynaptic depression or 5-HT-induced synaptic restoration

It has been suggested that transmitter released from the presynaptic cell may be one signal in the communication between pre- and postsynaptic cells leading to synaptogenesis (Young and Poo 1983). We therefore tested the effects of blocking postsynaptic receptors on synapse formation and on the development of synaptic plasticity.

Evidence from three laboratories indicates that the transmitter at the sensory neuron–motor neuron synapses of Aplysia is either glutamate or a closely related substance (Dale and Kandel 1993; Lin and Glanzman 1994; Trudeau and Castellucci 1993). We therefore examined the effects of blocking two different types of glutamate receptor at these synapses. We paired cells in the AMPA-type receptor synaptic responses could be elicited from 50% of pairs within 1 h. Rapid formation of synapses has also been reported by others (Buchanan et al. 1989; Haydon 1988; Haydon and Drapeau 1995; Haydon and Zoran 1989). The rapidity of synapse formation in our experiments and its insensi-
FIG. 7. Synaptic transmission and restoration by 5-HT at synapses formed in the presence of 6-cyano-7-nitroquinolinol-w2,3-dione (CNQX) or dl-2-amino-5-phosphonovaleric acid (APV). Stimulation protocol and EPSP normalization as in Fig. 4: 5-HT applied at arrows.

Synaptic strength and the role of second messengers in synaptogenesis

At synapses of all ages, homosynaptic depression was similar and involved both a decrease in mean EPSP amplitude and an increase in the incidence of failures. Exposure to 5-HT induced similar relative facilitation at newly formed synapses as at intermediate or late synapses. These results support the idea that synapses at all ages are composed of similar functional units, but that older synapses contain a greater number of units than newly formed ones.

There was a tendency for larger-amplitude EPSPs to show less restoration and augmentation by 5-HT at all ages, although this difference did not reach statistical significance (Figs. 4 and 5). Correcting for nonlinear summation of EPSPs on the basis of an equilibrium potential of 0 mV (Dale and Kandel 1993) had only a small effect on the

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2. Synapses formed in CNQX (n=6)

3. Synapses formed in APV (n=7)

4. EPSP amplitude increases with time, but other synaptic properties do not change

During the first hours of cell contact, the amplitude of EPSPs increases. The simplest explanation for the increase in strength with time is that new synapses continue to be added, with the additional possibility that synapses may change their properties after being formed. In our experiments, the EPSP amplitude reached 90% of the final plateau by 15 h. The fact that the percentage of pairs with synaptic connections reached 90% of the plateau at a similar time (12 h) suggests that the signal for initiation of synapse formation and the signal for the increase in amplitude may be the same. After 15 h, either the signal ceases to act or a steady state is reached in the turnover of synapses.

The synapses studied here function like mature synapses as early as 45 min after cell contact, the earliest time at which we recorded. The similarity in the rise times of EPSPs at early and late synapses suggests that both the release apparatus and the postsynaptic receptor configuration mature quickly. In cultures of Helisoma neurons also (Haydon and Zoran 1989), the time course of EPSPs evoked within 1 min of contact between pre- and postsynaptic neurons is similar to that of EPSPs at older synapses (Haydon 1988). These findings contrast with findings at the vertebrate neuromuscular junction, where the rise time of spontaneous EPSPs decreases with maturation as the density of acetylcholine receptors increases (Kidokoro et al. 1980). Nonetheless, the possibility cannot be ruled out that changes of this type may be occurring before the earliest recordings in our study.

Modulation of synaptic strength and the role of second messengers in synaptogenesis

At synapses of all ages, homosynaptic depression was similar and involved both a decrease in mean EPSP amplitude and an increase in the incidence of failures. Exposure to 5-HT induced similar relative facilitation at newly formed synapses as at intermediate or late synapses. These results support the idea that synapses at all ages are composed of similar functional units, but that older synapses contain a greater number of units than newly formed ones.

There was a tendency for larger-amplitude EPSPs to show less restoration and augmentation by 5-HT at all ages, although this difference did not reach statistical significance (Figs. 4 and 5). Correcting for nonlinear summation of EPSPs on the basis of an equilibrium potential of 0 mV (Dale and Kandel 1993) had only a small effect on the
values of both types of synaptic facilitation (not shown).
Nonetheless, the fact that this difference in synaptic facilita-
tion did not vary with age is consistent with the general
conclusion of this study, that the properties of newly formed
and older synapses are not fundamentally different.

The similarity of restoration and augmentation at synapses
differences suggests that the second messengers in-
volved in synapse formation are not the same as those in-
volved in 5-HT−induced facilitation at these synapses. If
the same second messengers were already activated during
dynasotogenesis, one would expect occlusion at newly formed
synapses of synaptic augmentation or restoration. Fur-
more, activation of protein kinase A by 5-HT is known to
lower the threshold for initiation of action potentials in the
sensory neurons (Klein et al. 1986), whereas we saw no
difference in action potential threshold in sensory neurons
at early and late synapses or in pairs that did not form syn-
apses at all. It is possible, however, that the same messengers
are only transiently activated on cell contact, and initiate a
train of events that results in synapse formation.

The concomitant appearance of mature forms of synaptic
plasticity at the earliest times in our experiments contrasts
with the progressive development of these forms of plasticity
in the maturing nervous system (Nolen and Carew 1988;
Rankin and Carew 1987, 1988; Rayport and Camardo 1984;
Rayport and Kandel 1986). The differences in plasticity at
synapses between adult neurons and between neurons in the
developing nervous system indicate either that there is a
fundamental difference between mature and immature neu-
rons in the process of synapse formation itself, or else that
the ancillary systems needed for synapse modulation—such
as appropriate circuitry, receptors, receptor-linked adenylate
cyclase or phospholipase C, or the various protein kinases—are
integrated into the developing nervous system only after
the synaptogenic apparatus is already functional.

Synapse recruitment by 5-HT

In some of our pairs there was no detectable EPSP before
the addition of 5-HT, but clear evoked release afterward
(Fig. 2A). In these experiments, 5-HT either caused the
insertion or assembly of additional synaptic structures, or
else activated synapses that were structurally present but that
released with a low probability (>0) or were completely silent ($P = 0$).
Experiments like the one shown in Fig. 2B indicate that some synapses release with a low probability
without being completely silent; the absence of a synaptic
response in a relatively small number of trials, therefore,
cannot be taken as evidence for completely silent synapses.

It is possible that we sometimes did not detect very small
EPSPs that were elicited before the application of 5-HT in
these experiments. However, in several of the experiments
we were able to detect spontaneous miniature EPSPs, and
the amplitudes of the evoked EPSPs both before and after
5-HT appeared to be multiples of the average amplitude of
the spontaneous events. In the experiment of Fig. 2B, for
example, we detected 16 spontaneous miniature EPSPs, with
a mean amplitude of 0.53 ± 0.12 (SD) mV, whereas the
single evoked EPSP detected before the addition of 5-HT
(Fig. 2B, inset) was ~0.5 mV in amplitude. The amplitudes
of the EPSPs evoked in the presence of 5-HT are consistent
with a similar unit amplitude. Taking the miniature potential
amplitude as the quantal size and using the largest response
in 5-HT to estimate the minimum number of release sites
(4 in this case), the pre-5-HT probability of release in this
pair was ~0.025. In the experiment of Fig. 2A, in contrast,
no spontaneous miniature events were detected, indicating
either that none occurred or that those that did occur were
too small for us to distinguish from the noise.

Activation of previously silent synapses has been pro-
posed as a mechanism of long-term potentiation in hippo-
campus (Isaac et al. 1995; Liao et al. 1995; Wang et al.
1996) and of frequency facilitation and long-term facilitation
at the crayfish neuromuscular junction (Wojtowicz et al.
1991). Our results do not allow us to distinguish between
the activation of synapses that are truly silent and an increase
in the probability of release at synapses with a low initial
value.

Implications of rapid synapse mobilization for plasticity

Our results suggest that formation of new synapses can
occur rapidly enough to contribute to short- and interme-
diate-term forms of synaptic plasticity. On the basis of our
work and that of others, we suggest that synapses assemble
rapidly and require new protein synthesis only for their main-
tenance and for the induction of changes that occur with
long-term forms of synaptic plasticity such as long-term fa-
cilitation, potentiation, and depression. The idea that synap-
togenesis may be involved in short- and intermediate-term
plasticity is supported by findings that intermediate-term fa-
cilitation in Aplysia involves the formation of new vari-
cosities (Wu et al. 1995). The fact that synthesis of new protein
is required for both the increase in the number of variocities
that occurs with long-term facilitation and the decrease in
the number of variocities that accompanies long-term de-
pression (Bailey et al. 1992b) suggests that it is likely to be
the synthesis of regulatory molecules, rather than of synaptic
components, that is critical for the induction of these long-
term processes (Ambron et al. 1985). This idea is consistent
with findings that protein synthesis is required for the down-
regulation of the regulatory subunit of protein kinase A (Ber-
gold et al. 1990, 1992; Greenberg et al. 1987), the endocyto-
sis of neural adhesion molecules (Bailey et al. 1992a), and
the persistent activation of protein kinase C (Sossin et al.
1994) that accompany long-term facilitation by 5-HT in
Aplysia.

Neurons anticipate synaptogenesis with the prior synthesis
of synaptic proteins that are then assembled and inserted
into the membrane or activated in response to cell contact.
Endogenous neuronal activity or the activation of receptors
by neurotransmitters, hormones, or growth factors could
both modify existing synapses and cause the rapid insertion
or removal of functional synaptic modules in the membrane.
Changes in synapse number could thus play a role in the
short- and intermediate-term regulation of synaptic efficacy.

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