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Rapid Increase in Clusters of Presynaptic Proteins at Onset of Long-Lasting Potentiation

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A change in the efficiency of synaptic communication between neurons is thought to underlie learning. Consistent with recent studies of such changes, we find that long-lasting potentiation of synaptic transmission between cultured hippocampal neurons is accompanied by an increase in the number of clusters of postsynaptic glutamate receptors containing the subunit GluR1. In addition, potentiation is accompanied by a rapid and long-lasting increase in the number of clusters of the presynaptic protein synaptophysin and the number of sites at which synaptophysin and GluR1 are colocalized. These results suggest that potentiation involves rapid coordinate changes in the distribution of proteins in the presynaptic neuron as well as the postsynaptic neuron.

Although there is consensus that the induction of plasticity in the CA1 region of the hippocampus usually involves Ca^{2+} influx through postsynaptic *N*-methyl-D-aspartate (NMDA) receptor channels, there is less agreement about the expression of the plasticity (*1–3*). Recent studies have provided strong support for postsynaptic mechanisms (*4*), including changes in the surface membrane expression of α-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) glutamate receptors in synaptic clusters or "puncta" that label for GluR1 (*4–7*). However, there is also evidence that long-lasting potentiation may involve presynaptic mechanisms as well (*1–3*). To investigate such presynaptic changes, we examined immunoreactivity for GluR1 and a presynaptic vesicle-associated protein, synaptophysin, in cultured hippocampal neurons.

Hippocampal neurons in culture can undergo activity-dependent long-lasting synaptic plasticity with many of the key features of long-term potentiation (LTP) or long-term depression (LTD) in the CA1 region of hippocampus in slices or in vivo (*5, 8–11*). Application of glutamate to the cultures can also produce long-lasting potentiation or depression (*6, 12*). Brief (~1 min) application of glutamate in 0 Mg^{2+} saline (to allow opening of NMDA receptor channels) produced a rapid and long-lasting increase in the amplitude of excitatory postsynaptic currents (EPSCs) at synapses between pairs of hippocampal neurons in dissociated cell culture (*13*) (Fig. 1A). Glutamate also produced a rapid and long-lasting increase in the frequency of spontaneous miniature EPSCs (mEPSCs) (Fig. 1B) with no change in their amplitude (average 5 to 30 min after glutamate, 102 ± 6% before glutamate; average before glutamate, 8.8 ± 0.3 pA). Furthermore, the glutamate-induced increase in mEPSC frequency was blocked when the bath solution contained the NMDA receptor antagonist D-aminophosphovalerate (D-APV), consistent with the idea that glutamate acts by stimulating postsynaptic NMDA receptors.

Brief application of glutamate also produced an increase in the number of GluR1-immunoreactive (IR) puncta in cultures fixed 5 min after the glutamate application, compared to control cultures from the same batch that were fixed 5 min after saline application (average from all such experiments, 166 ± 18% of control, n = 26 and 25 dishes, t(49) = 3.45, $P < 0.01$) [*S1* (*14*)] (Fig. 2, A and B). Although the size and fluorescence intensity of the GluR1 puncta varied considerably, there was no difference in these parameters between control and glutamate-treated cultures (intensity, 96 ± 2% of control; size, 101 ± 2%, control average = 4.2 ± 0.2 μm^2), suggesting that the increase in number was not due to an overall shift in size or intensity that made more puncta detectable.

We double-labeled the cultures with an antibody against synaptophysin, a vesicle-associated protein used as a presynaptic marker. In control cultures, some but not all (32.7 ± 2.2%) of the synaptophysin puncta colocalized with GluR1 puncta (appearing as yellow or, more often, as adjacent red and green puncta) and therefore might participate in functional glutamatergic synapses. The remaining synaptophysin-IR puncta may form synapses with other types of receptors or may be nonsynaptic. As for GluR1-puncta, brief exposure to glutamate produced increases in the number of synaptophysin-IR puncta [204 ± 17%, t(49) = 5.93, $P < 0.001$] and sites where synaptophysin and GluR1 were colocalized [282 ± 46%, t(49) = 3.81, $P < 0.001$]. Moreover, for both GluR1-IR and synaptophysin-IR puncta, glutamate produced a greater increase in the number of puncta that were colocalized than puncta that were not [GluR1, 275 ± 44% versus 143 ± 15%, t(25) = 3.99, $P < 0.001$; synaptophysin, 288 ± 46% versus 174 ± 11%, t(25) = 2.84, $P < 0.01$] (*S2*). Like GluR1-IR, there was no change in the intensity (97 ± 2% of control) or size (104 ± 3%, control average = 4.5 ± 0.2 μm^2) of the synaptophysin-IR puncta.

The glutamate-induced increases in the number of synaptophysin-IR puncta, GluR1-IR puncta, and sites where they were colocalized were all blocked by the NMDA antagonists D-APV (Fig. 2, A and B) or MK801 [100 μM; synaptophysin, 159 ± 13% versus 99 ± 7%, n = 5 and 6, t(9) = 4.43, $P < 0.01$; GluR1, 126 ± 34% versus 89 ± 10%; colocalized, 163 ± 24% versus 91 ± 16%, t(9) = 2.60, $P < 0.05$]. These results suggest that, like the potentiation of mEPSC frequency, the increase in synaptophysin-IR puncta may depend on activation of postsynaptic NMDA receptors. Furthermore, like the potentiation, the increase in the number of synaptophysin-IR puncta was maintained at nearly the same level 30 min after the glutamate application [192 ± 23% of control, n = 6 and 5, t(9) = 2.98, $P < 0.05$], consistent with a role in maintenance of the potentiation (*S3*).

To verify that these changes were not particular to the pre- and postsynaptic markers examined, we also demonstrated that brief application of glutamate produced increases in the number of puncta that labeled for the presynaptic proteins synapsin I (Fig. 2, C and D) and

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synuclein (not shown) (*15*), and for the postsynaptic protein, PSD95, as well as sites where synapsin I and PSD95 were colocalized (Fig. 2, C and D). On the other hand, glutamate produced no change in the number of puncta that labeled for the NR1 subunit of postsynaptic NMDA glutamate receptors (102 ± 17%, n = 6 and 6), as previously shown for long-term depression in culture (*5, 6*), suggesting that there is no general change such as overall growth. The increase in number of PSD95 puncta was accompanied by an offsetting decrease in their average size (Fig. 2E), suggesting that the new PSD95-IR puncta could arise from splitting of preexisting puncta (*4*). By contrast, the average size of the synapsin-IR puncta, like that of the synaptophysin-IR and GluR1-IR puncta did not change.

Where then do the new synaptophysin-IR puncta come from? New protein synthesis is not required; brief exposure to glutamate still produced increases in the number of synaptophysin-IR puncta, GluR1-IR puncta, and sites where they were colocalized in cultures treated with the protein synthesis inhibitor anisomycin (Fig. 3, A and B). We then tested for the involvement of cytoskeleton-mediated clustering of existing synaptophysin within presynaptic processes, as may occur for GluR1 postsynaptically (*4–7*). Such clustering would likely require cytoskeletal proteins such as actin, which colocalizes with presynaptic proteins as well as with GluR1 (*16, 17*). We therefore examined the effect of an inhibitor of actin polymerization, cytochalasin D, and found that the glutamate-induced increases in the number of synaptophysin-IR puncta, GluR1-IR puncta, and colocalization sites were all blocked in cultures that had been treated with cytochalasin D (Fig. 3, A and C). Thus, actin plays an important role in the increases in number of puncta, although we cannot distinguish between an active transport role or a more permissive scaffolding role (*S4*). Like the early phase of LTP in hippocampal slices (*18–20*), the glutamate induced increase in mEPSC frequency in culture was similarly unaffected by the protein synthesis inhibitor anisomycin, but inhibited by the actin polymerization inhibitor cytochalasin D (*S5*), suggesting that the actin-dependent changes in synaptophysin-IR and GluR1-IR puncta could contribute to the potentiation of mEPSC frequency.

To verify our immunocytochemical results in individual neurons over time, we expressed a synaptophysin-GFP (green fluorescent protein) fusion protein in cultured hippocampal neurons by infection with recombinant adenovirus (*S6, S7*). Ten minutes after brief exposure to saline (control), most synaptophysin-GFP puncta remained stable, but there were also both losses of preexisting puncta and gains of new puncta that approximately canceled each other out (Fig. 4, A and C). Ten minutes after brief exposure to glutamate, more puncta were gained and fewer puncta were lost than in the control group, resulting in a net increase in the number of puncta. This increase was maintained at least 30 min, and was blocked by D-APV (Fig. 4B). These results provide an independent replication of the results obtained with immunocytochemistry (*S8*). In addition, we examined the onset of the effect and found no glutamate-induced increase in the number of puncta 1 min after the glutamate application, indicating that the induction of the increase takes between 1 and 10 min.

We next examined the effects of glutamate on subthreshold changes in fluorescence, which might provide information about the origin of the new puncta (Fig. 4D). We saw no evidence for splitting of presynaptic terminals into two (*4*). We did, however, see three other types of changes: (i) Some puncta appeared to move along a process (Fig. 4D1). As previously described (*21*), these puncta are prefabricated packets of synaptic proteins that are being transported to their final destination. Their movement would not contribute to the increase in total number, and was not affected by glutamate (3.8 ± 0.7% versus 5.5 ± 1.4% of puncta). (ii) Some subthreshold puncta stayed in the same location and became more intense (orange) (Fig. 4D2). (iii) Some relatively diffuse regions of fluorescent material (green areas) appeared to aggregate into nearby suprathreshold puncta (red or orange spots) (Fig. 4D3). Such aggregation could contribute to the appearance of truly new puncta, as well as to intensification of preexisting puncta.

To quantify aggregation of puncta, we measured the changes in total fluorescence of the puncta and the surrounding area within 5 μm of the puncta (*22*) (Fig. 4E). For puncta that were gained (Post only), the increase in fluorescence of the puncta was accompanied

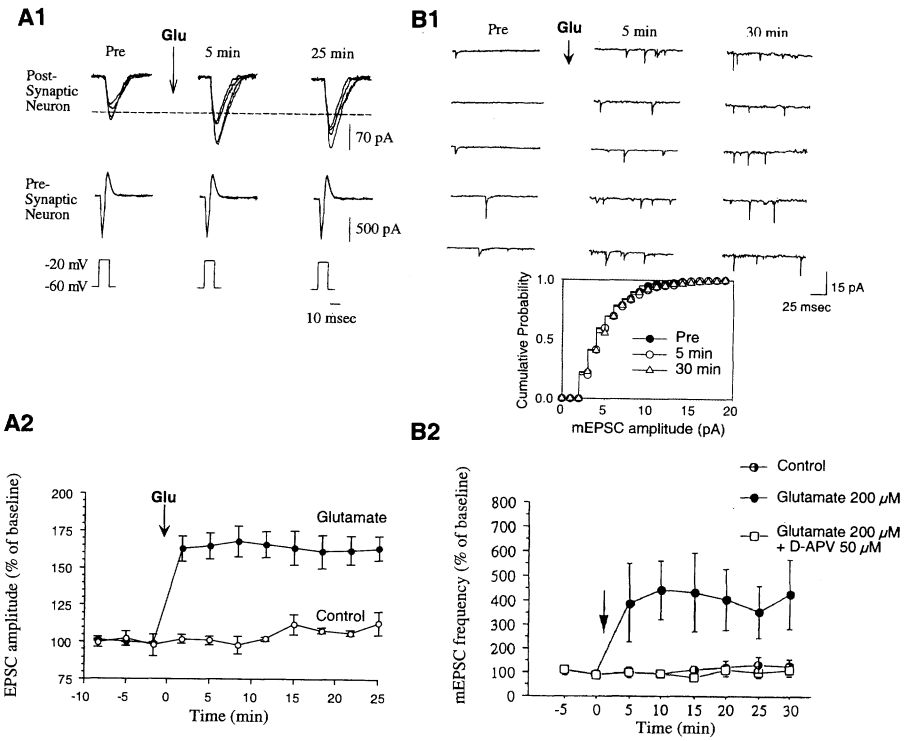

Fig. 1. Glutamate produces increases in EPSC amplitude and mEPSC frequency. (**A1**) Examples of evoked EPSCs produced in a postsynaptic neuron by step depolarization that elicited an inward current in the presynaptic neuron before (Pre), and 5 and 25 min after, brief application of 200 μM glutamate to the dish. (**A2**) The average change (mean ± SEM) in EPSC amplitude following brief application of glutamate or saline (control). There was a significant overall difference between the two groups in a two-way analysis of variance (ANOVA) with one repeated measure [$F(1,6) = 51.84$, $P < 0.01$], and planned comparisons showed that the groups were significantly different at each time point after the glutamate application ($P < 0.01$ in each case). Each point represents the average of 20 successive trials, normalized to the average value during the 10 min before glutamate application (baseline) in each experiment. The average baseline values were 59 ± 23 pA, n = 4 (glutamate) and 99 ± 41 pA, n = 4 (control), not significantly different. (**B1**) Examples of spontaneous mEPSCs before (Pre), and 5 and 30 min after, brief application of glutamate to the dish. (Inset) The amplitude distributions of the mEPSCs at those times. (**B2**) The average change in mEPSC frequency following brief application of glutamate, saline (control), or glutamate and D-APV (50 μM). There was a significant overall difference between the three groups [$F(2,20) = 4.51$, $P < 0.05$], with the glutamate group significantly different from both the control group and the glutamate + D-APV group at each time point ($P < 0.05$ in each case). Data were normalized to the average value during the 10 min before glutamate application (baseline) in each experiment. The average baseline values were 41 ± 10 min^{-1}, n = 11 (control), 38 ± 12 min^{-1}, n = 8 (glutamate), and 36 ± 10 min^{-1}, n = 4 (glutamate + D-APV), not significantly different by a one-way ANOVA.

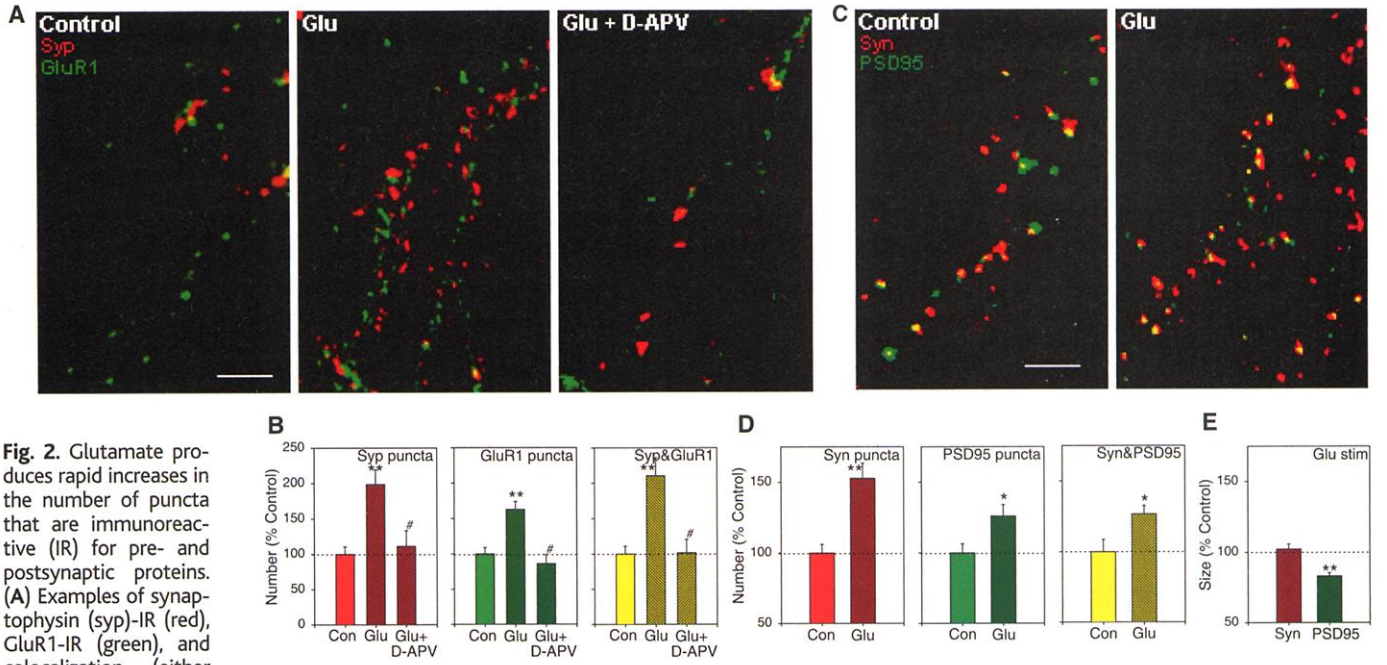

Fig. 2. Glutamate produces rapid increases in the number of puncta that are immunoreactive (IR) for pre- and postsynaptic proteins. (**A**) Examples of synaptophysin (syp)-IR (red), GluR1-IR (green), and colocalization (either yellow or adjacent red and green) in a control dish (left), a dish fixed 5 min after brief application of 200 μM glutamate (middle), and a dish fixed after application of glutamate in the presence of 200 μM D-APV (right). Scale bar, 5 μm. (**B**) Average results from experiments like the one shown in (A) (n = 6 dishes in each group). In this and subsequent figures, ** = $P < 0.01$, * = $P < 0.05$ compared to control, and # = $P < 0.05$ compared to glutamate. The number of puncta in a representative field (94 μm by 142 μm) have been normalized to the number in comparable fields in control dishes from the same culture batch. The average control values (innumber of puncta) were 47 ± 8 (syp), 75 ± 10 (GluR1), and 20 ± 3 (colocalized). (**C**) Examples of synapsin I (syn)-IR, PSD95-IR, and colocalization in a control dish (left) and a dish fixed 5 min after brief application of glutamate (right). (**D**) Average results from experiments like the one shown in (C) (n = 6 and 6). The average control values were 112 ± 8 (syn), 90 ± 7 (PSD95), and 58 ± 6 (colocalized). (**E**) Glutamate also produced a rapid decrease in the size of the PSD95-IR puncta. The average control values were 4.7 ± 0.2 μm^2 (syn) and 3.9 ± 0.2 (PSD95).

by a decrease in fluorescence of the surrounding area, consistent with aggregation into the puncta. Conversely, for puncta that were lost (Pre only), the decrease in the fluorescence of the puncta was accompanied by an increase in fluorescence of the surrounding area, consistent with disaggregation. There was no significant difference in these effects between the cultures treated with saline, glutamate, or glutamate and D-APV, and the results have been pooled. These results suggest that in control conditions the puncta undergo continual aggregation and disaggregation that are in approximate equilibrium, and that glutamate temporarily shifts the balance so that a greater number of puncta aggregate.

There is an increase in the number of synaptophysin-IR puncta during protein synthesis-dependent late-phase LTP lasting more than 2 hours (*23*), when there is actual growth of new synapses (*4*). Our results now demonstrate that the number of synaptophysin puncta increases as early as 5 to 10 min after stimulation, before any synaptic growth has been thought to occur. However, because growth of new postsynaptic filopodia and spines can occur within 10 to 20 min after the induction of LTP (*24, 25*), we examined neurons transfected with GFP instead of synaptophysin-GFP, but did not see any consistent changes in their mophology during the first

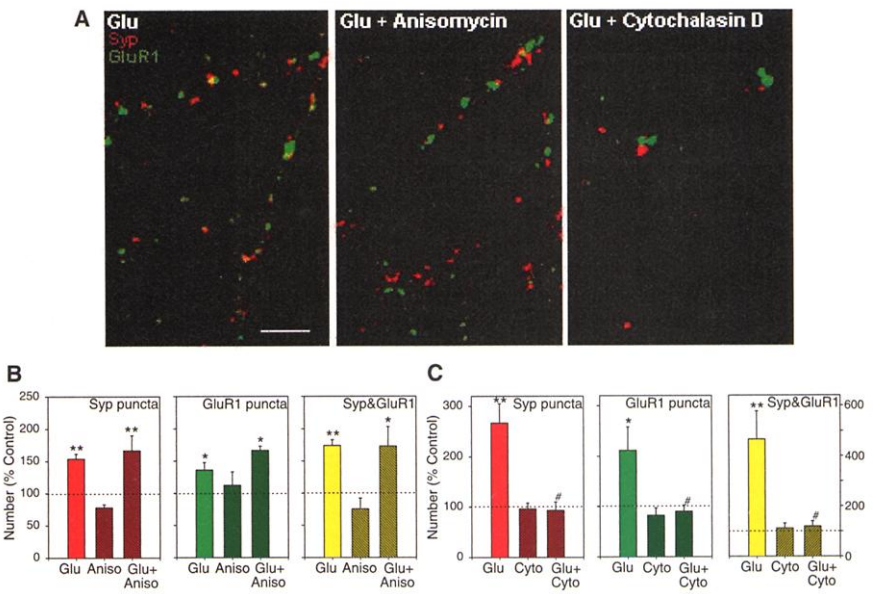

Fig. 3. The glutamate-induced increases in puncta are not blocked by an inhibitor of protein synthesis (anisomycin), but are blocked by an inhibitor of actin polymerization (cytochalasin D). (**A**) Examples of synaptophysin-IR, gluR1-IR, and colocalization in dishes fixed 5 min after brief application of either glutamate (left), glutamate following treatment with 30 μM anisomycin for 1 hour (middle), or glutamate following treatment with 30 μM cytochalasin D for 15 hours (right). (**B**) Lack of effect of anisomycin on the glutamate-induced increase in puncta (n = 6 in each group). The average control values (in number of puncta) were 42 ± 8 (synaptophysin), 42 ± 6 (GluR1), and 12 ± 3 (colocalized). (**C**) Effect of cytochalsin D on the glutamate-induced increase in puncta (n = 9, 6 and 6). The average control values were 30 ± 5 (synaptophysin), 44 ± 7 (GluR1), and 7 ± 1 (colocalized).

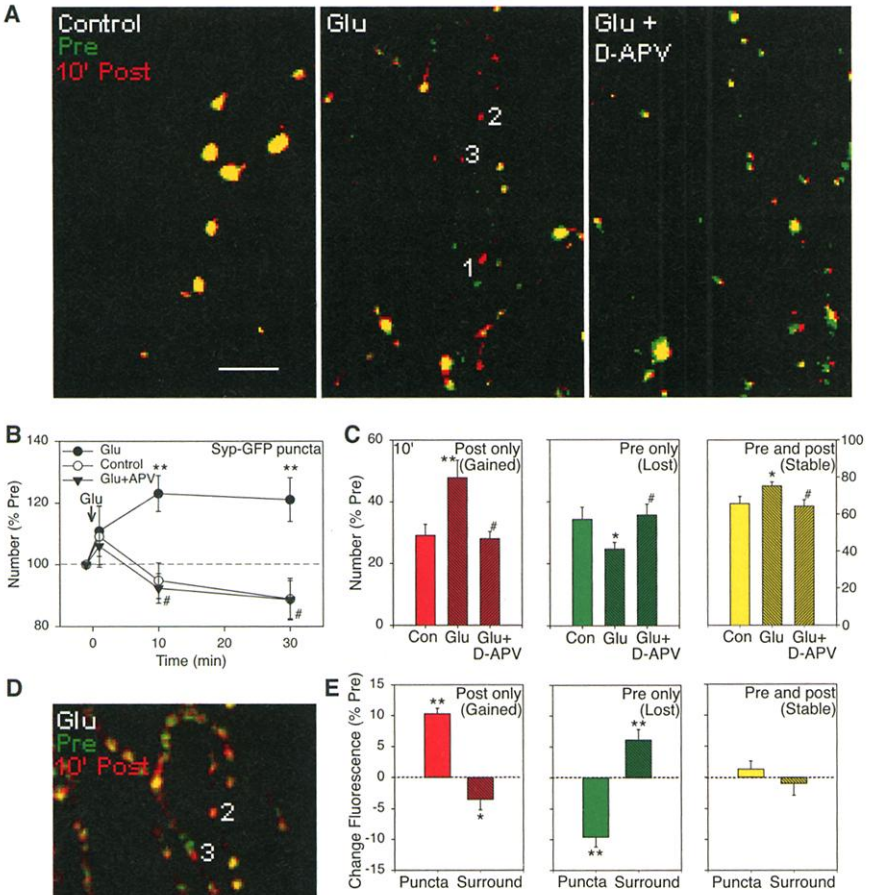

Fig. 4. Glutamate produces a rapid increase in the number of synaptophysin-GFP puncta. (**A**) Examples of synaptophysin-GFP fluorescent puncta before (Pre, green) superimposed on those 10 min after (Post, red) brief application of saline (control, left), glutamate (middle), or glutamate in the presence of 200 μM D-APV (right). Puncta that were present in the posttest only appear red, those that were present in the pretest only appear green, and those that were present at both times appear yellow. Scale bar, 10 μm. (**B**) Average time course of changes in the total number of synaptophysin-GFP fluorescent puncta following brief exposure to glutamate, saline (control), or glutamate and D-APV. There was a significant overall difference in number of puncta between the three groups in a two-way ANOVA [$F(2,48) = 6.06$, $P < 0.01$], with the glutamate group being significantly different from the control group and the glutamate + D-APV group at both 10 min and 30 min ($P < 0.01$ in each case). (**C**) Average number of synaptophysin-GFP puncta present in the 10-min posttest only (left), the pretest only (middle), or both tests (right) in each of the experimental groups. The number of puncta in the field (235 μm by 355 μm) have been normalized to the total number on the pretest in each experiment. The average pretest values were 102 ± 14, $n = 18$ (control), 108 ± 13, $n = 24$ (glutamate), and 172 ± 33, $n = 9$ (glutamate + D-APV). (**D**) Examples of changes in subthreshold fluorescence signals. The image is the same as the middle panel of (A) (which was generated with an intensity threshold to facilitate recognition and counting of puncta) but without the threshold. (**E**) The average changes in fluorescence of the puncta and the surrounding area within 5 μm for puncta that were counted in the posttest only (left), the pretest only (middle), or both the pre- and posttest (right). The changes in fluorescence have been normalized to the total fluorescence of the puncta and surround on the pretest in each experiment.

30 min following glutamate application. Thus, the new glutamate-induced synaptophysin puncta that we have observed probably do not reflect the growth of new terminals, although some might constitute a reserve pool of material for new terminals that have not yet formed (*21*). Alternatively, the new puncta may be associated with the conversion of "silent" presynaptic terminals to functional ones, as several previous studies have found an increase in functional terminals (indicated by vesicle cycling) during early as well as late-phase LTP (*26–29*). In either case, our results support the emerging view that even the early stages of long-lasting plasticity involve microstructural changes and show that those changes can occur presynaptically as well as postsynaptically in a coordinated fashion, as occurs during synaptic development (*30*).

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