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Anterograde and retrograde effects of synapse formation on calcium currents and neurite outgrowth in cultured leech neurons

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SUMMARY

The aim of our experiments has been to analyse how formation of chemical synapses affects the distribution of calcium (Ca^{2+}) currents and neurite outgrowth of leech Retzius cells. Previous results showed that Ca^{2+} currents measured in the initial process or 'stump' of postsynaptic cells were significantly smaller than those in corresponding sites on presynaptic neurons. In the present experiments, neurons were plated together in close apposition as pairs or as triads, with the tip of one Retzius cell touching the soma of another. Ca^{2+} currents from selected areas of the neuronal surfaces were measured by loose-patch recording before and after the formation of chemically mediated synaptic connections, which developed in about 8 h. With three cells arranged in a row, the last of the series, which was purely postsynaptic (i.e. with no target), also showed a dramatic reduction in Ca^{2+} currents in its initial segment, compared with the currents seen in either the first cell (purely presynaptic) or the second cell of the chain (which was both postsynaptic to the first cell and presynaptic to the third). This suggests that retrograde as well as anterograde effects on Ca^{2+} currents occurred as a result of synapse formation: the Ca^{2+} currents in the middle cell did not decrease although a synapse had been formed on it. To test for additional consequences of synapse formation, neurite outgrowth was measured in postsynaptic cells and in single cells plated on an extract of extracellular matrix containing laminin (ECM-laminin). After 48 h, the total length of neurite outgrowth in postsynaptic cells was only about one third of that in single cells. These results show that the establishment of a chemical synapse between two cells produces characteristic changes in membrane properties and sprouting; the nature of these changes depends on whether the neuron itself has formed synapses on another cell and whether or not synapses have formed upon it.

1. INTRODUCTION

Neurons isolated from the central nervous system (CNS) of the leech grow and form highly specific, chemically mediated synaptic connections in culture. For example, the stump of an isolated Retzius cell forms presynaptic endings upon the soma of another Retzius cell in culture, creating a unidirectional synapse at which 5-HT is the transmitter (Liu & Nicholls 1989). The physiological properties and transmission at such synapses are strikingly similar to those in the CNS of the animal. In culture, with well-defined synaptic sites, it becomes possible with loose-patch recording technique (Stühmer *et al.* 1983) to measure the distribution of ionic currents in various regions of the cells before and after synapse formation and to determine how they change (García *et al.* 1990; Fernández-de-Miguel *et al.* 1992). Of particular interest are Ca^{2+} currents. Although these are small and difficult to isolate, tail currents that are seen at the end of depolarizing pulses in patches held at the potassium equilibrium potential (E_K) are carried almost entirely by Ca^{2+} with negligible contamination by other ions (Fernández-de-Miguel *et al.* 1992). Earlier studies (García *et al.* 1990) showed that Ca^{2+} currents at the stump of a single Retzius cell in culture were larger than those at the soma. With paired Retzius cells that had formed chemical synapses, Ca^{2+} currents were larger in the stump of the presynaptic neuron compared with those recorded in the same region of the postsynaptic cell (Fernández-de-Miguel *et al.* 1992).

The present experiments were done on single cells, pairs of cells and on triads of Retzius cells arranged in a row. Recordings were made 24 h after placing cells together in culture when chemical synapses form and become stronger, and before an electrical component develops (Liu & Nicholls 1989). With three cells arranged in a row, the first Retzius cell is purely presynaptic, the middle is both post- and presynaptic, and the third cell is purely postsynaptic. One question that has been analysed is whether differences in Ca^{2+}

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currents that develop progressively in stumps after synapse formation arise from up-regulation at presynaptic sites or down-regulation at postsynaptic sites. A second problem concerns the role of postsynaptic targets in influencing Ca^{2+} currents within presynaptic neurons. By comparing triads with single cells and pairs of cells in culture it becomes possible to analyse the role of pre- and postsynaptic elements in producing changes in Ca^{2+} currents.

A third problem analysed here concerns the way in which synapse formation regulates neurite outgrowth. Several lines of evidence suggest a role for Ca^{2+} entry in promoting neurite outgrowth (see Kater & Mills 1991). As the purely postsynaptic tip is the region from which growth starts, we compared single with postsynaptic neurons after they had been plated on ECM-laminin.

2. MATERIALS AND METHODS

(*a*) *Preparation*

Leeches were obtained from Ricarimpex (Audenge, France) and used in the months of December through May. Actively swimming leeches of 6–10 cm in length were chosen.

Retzius cells were removed from the CNS of the leech *Hirudo medicinalis* following the procedure described by Dietzel *et al.* (1986). The cells were left for 24 h in Leibovitz 15 medium ((GIBCO) supplemented with: 2% (by volume) heat-inactivated foetal calf serum (FCS); 6 mg ml^{-1} glucose; 2 mmol l^{-1} glutamine and 0.1 mg ml^{-1} garamycin), to allow the release of extracellular matrix from the cell surface.

(*b*) *Tissue culture to record Ca^{2+} tail currents*

Cells were plated in L-15 medium without FCS in microwell dishes precoated with polylysine. FCS was added 2 h after the plating. Cells were plated as singles or in triplets with the stump of one cell touching the soma of the next, to form chemical synapses (Liu & Nicholls 1989).

Intracellular recordings were done in every cell to measure resting potentials and to test synapse formation. Only cells showing resting potentials between −40 mV and −50 mV were chosen for measurement of Ca^{2+} currents.

(*c*) *Pipettes and solutions to record Ca^{2+} tail currents*

Pipettes with resistances between 0.3 MΩ and 0.6 MΩ were pulled in a two-stage protocol using a vertical puller (David Kopf Instruments). The shafts of the pipettes were coated with Sylgard to reduce capacitative artefacts. The pipette was placed over the cell membrane and gentle suction was applied to form a seal. Typical seal resistances were between 4.0 MΩ and 6.0 MΩ.

Ca^{2+} tail currents were recorded by using loose patch technique from patches held at E_K (Fernández-de-Miguel *et al.* 1992). The pipette solution contained (in millimoles per litre): $CaCl_2$ (10) to increase the amplitude of the Ca^{2+} tail currents; KCl (18) to reduce E_K from −85 mV to −50 mV; NaCl (10) to avoid inward Na^+ currents. The solution was buffered with Trizma maleate (10) to pH 7.4, and osmolarity was adjusted to 350 mosm l^{-1}† by the addition of *N*-methyl-D-glutamine chloride. During the recordings, cells were bathed in L-15 medium. The pipette solution produces a depolarization of 5 mV. This, together with the difference between the resting potential of the cell and E_K (−50 mV), was compensated by adjusting the holding potential of the patch.

† One osmole contains one mole of osmotically active particles.

(*d*) *Loose patch clamp set-up*

A modified version of the original circuit proposed by Stühmer *et al.* (1983) was used to record currents (García *et al.* 1990). A modified head stage with an OPA 111 was used, and currents were amplified with an EPC 7 amplifier (List Electronics, Darmstadt). Compensations of electrode series resistance, seal-leak resistance, and transients were done in a separate adaptor box. Remaining uncompensated leak currents were subtracted with a P/3 protocol similar to the one described by Bezanilla & Armstrong (1977). Ca^{2+} currents were activated with 10 ms 100 mV depolarizing pulses, and tail currents were recorded. Data were collected and stored for analysis in a computer via an A/D converter using an acquisition rate of 50 KHz. Averages of three traces were made to reduce noise.

(*e*) *Measurements of neurite outgrowth*

Retzius cells were paired with the stump of one cell touching the soma of the other, in microwell dishes containing L-15 medium. To prevent adhesion of the cells to the bottom of the dish, no substrate was used and the culture medium was supplemented with FCS. After 12 h, the pairs of Retzius cells could be removed without the cells being separated and the synapses being destroyed. Single cells were treated in the same way. Single and paired cells were then transferred to microwell dishes precoated with ECM-laminin. The ECM-laminin extract was prepared by EDTA extraction from leech ganglia capsules, as described by Masuda-Nakagawa *et al.* (1988). Total length of neurite outgrowth was measured by a computer imaging system (Image-1 by Universal Imaging, West Chester, Pennsylvania, U.S.A.) between 12 h and 48 h after the cells were plated on the ECM-laminin extract.

3. RESULTS

(*a*) *Ca^{2+} tail currents in triplets of cells*

Ca^{2+} tail currents from single cells and from triplets of Retzius cells that had formed synapses were recorded from patches held at E_K. The amplitudes of the

Table 1. *Ca^{2+} tail current amplitudes (pA), recorded 24 h after plating from the stumps and somata of Retzius cells that were purely presynaptic (pre), both pre- and postsynaptic (middle) or purely postsynaptic (post)*

Means ± standard errors are tabulated. The currents recorded from the stumps of postsynaptic cells were smaller than those recorded from single cells, presynaptic cells, or middle cells ($p < 0.016$ for all comparisons). The differences between currents recorded from the stumps of single cells and those recorded from presynaptic and middle cells were not significant ($p > 0.15$), nor were the differences between measurements at the somata ($p > 0.04$, any pairing).

location	stump	central soma	polar soma
single ($n = 13$)	159 ± 18	67 ± 6	62 ± 6
pre ($n = 6$)	182 ± 17	60 ± 6	70 ± 9
middle ($n = 6$)	185 ± 32	73 ± 13	68 ± 11
post ($n = 6$)	79 ± 15	56 ± 9	69 ± 12

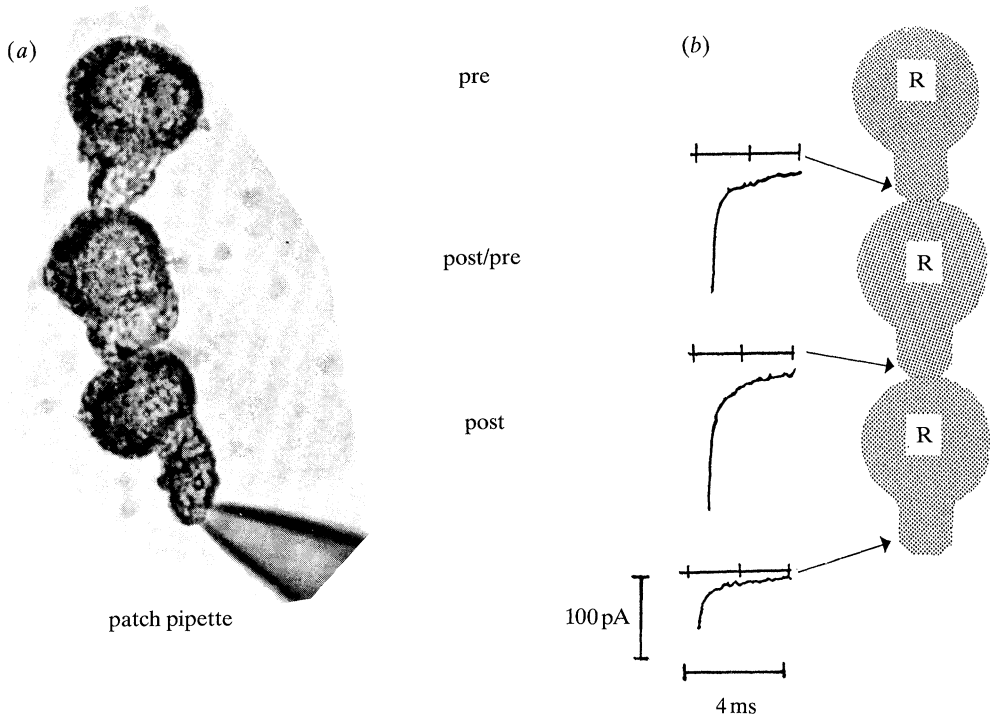

Figure 1. (*a*) Micrograph of three Retzius cells aligned in a row. A loose patch pipette is at the stump of the purely postsynaptic cell (post). The cell at the top is purely presynaptic (pre); the cell in the middle (post/pre) is postsynaptic to the pre cell and presynaptic to the post. Somata diameters are 70–80 μm. (*b*) Ca^{2+} tail currents recorded from the stumps of three Retzius cells that had formed chemical synapses, arranged as in (*a*). The three cells had been plated for 24 h in culture. The amplitudes of the currents in the presynaptic and middle cells were virtually identical, whereas the amplitude of the tail current at the stump of the purely postsynaptic cell was markedly reduced.

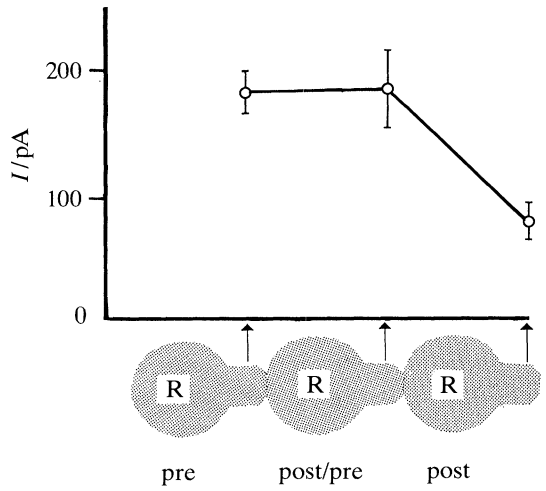

Figure 2. Mean amplitudes of the Ca^{2+} tail currents (±s.e.) recorded at the stumps of six triads of Retzius cells that had previously been shown to form chemical synapses (see text). The same patch pipette was used to record from the three cells of each triplet. Ca^{2+} tail-currents recorded from the three cell bodies did not show comparable changes.

currents recorded in this study varied far less than those obtained in our earlier observations (see Fernández-de-Miguel *et al.* 1992). In addition, the amplitudes of Ca^{2+} tail currents were only about half the amplitude of those reported previously. It may be that the reduced variability and smaller amplitudes were results of the short period of three months (December–February) during which all triplet experiments were done.

Table 2. *Ca^{2+} tail current amplitudes (pA) recorded from the stumps and somata of single Retzius cells as a function of time after plating*

Means ± standard errors are tabulated. The currents recorded from the stumps were always larger than those recorded from the central or polar somata ($p < 0.01$ at all times). The increase in stump current between 4 h and 24 h was not significant ($p \simeq 0.25$). No significant differences were found between currents recorded from the central and polar somata.

time	stump	central soma	polar soma
4 h ($n = 21$)	126 ± 14	64 ± 9	51 ± 4
24 h ($n = 13$)	159 ± 18	67 ± 6	62 ± 6
48 h ($n = 6$)	163 ± 25	46 ± 7	58 ± 14

In six triplets of cells plated in series, synapse formation was demonstrated in all the cells by recording electrically with microelectrodes. Impulses in the first cell gave rise to synaptic potentials in the middle cell; stimulation of this cell gave rise to synaptic potentials in the third cell, which was purely postsynaptic. Measurements of Ca^{2+} tail currents were made from the stumps, somata and polar region of the somata (this is the region opposite to the stump) of each of the three cells in all the triplets after 24 h in culture (Fernández-de-Miguel *et al.* 1992). When currents in the three cells were compared, we observed that the amplitudes of Ca^{2+} tail currents in the stumps of presynaptic and middle cells were nearly identical. The mean tail-current amplitude in the purely pre-

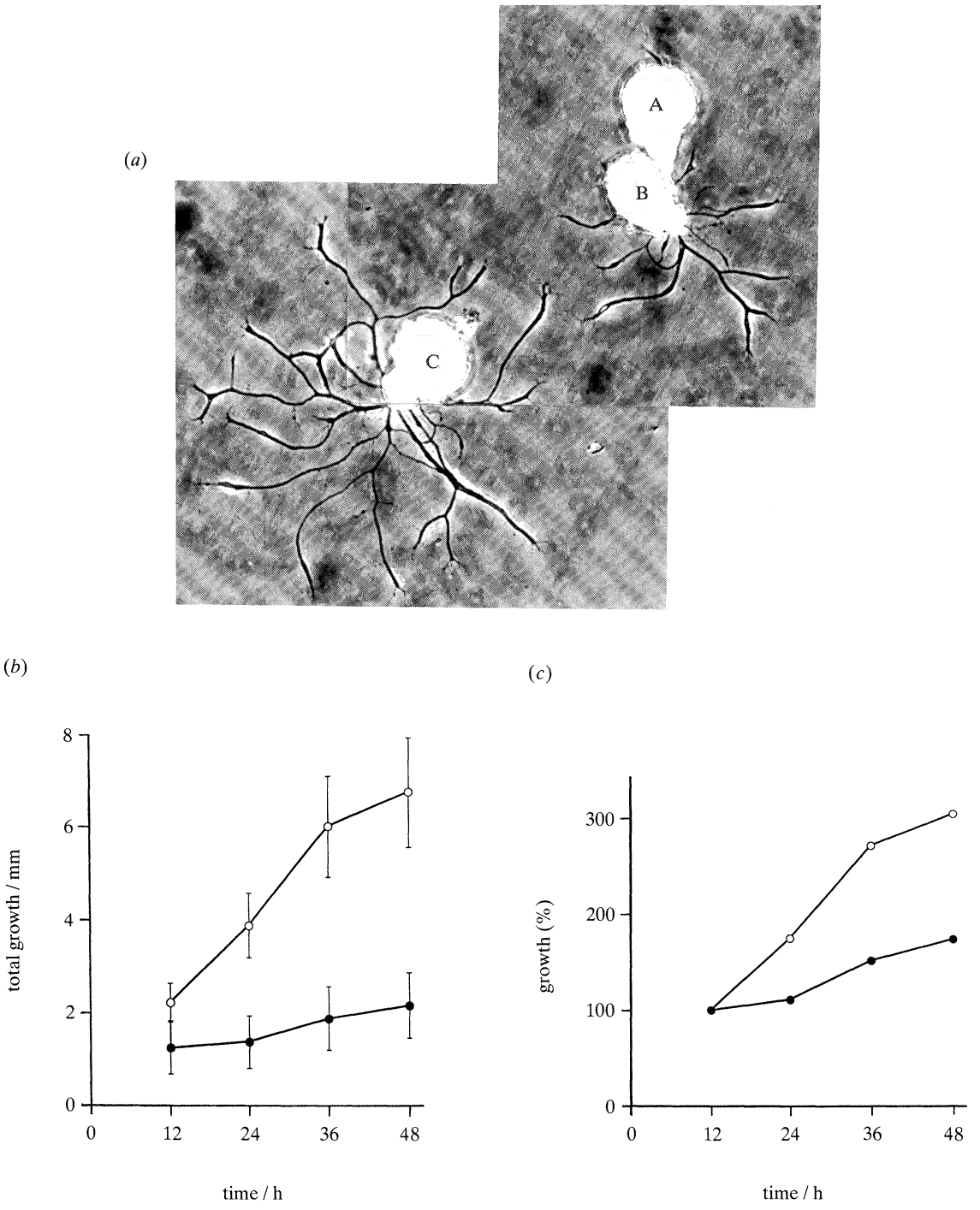

Figure 3. Decreased neurite outgrowth from postsynaptic compared with single cells plated on ECM-laminin substrate. (*a*) Outgrowth from a pair of cells and a single cell after 24 h. Although the patterns were similar, the total length of the processes was less in cell B (which was postsynaptic to cell A), than in the single cell C. Outgrowth of the presynaptic cell A occurs over the soma of B rather than on the substrate. (*b*) Mean values (±s.e.) of neurite outgrowth (in millimetres) of single cells (open circles) and postsynaptic cells (filled circles). All values after 12 h were significantly different ($p < 0.05$) (*c*) To compare rates of outgrowth, neurite length was normalized to values observed at 12 h. In postsynaptic cells, the rate as well as the amount of growth was reduced. Somata diameters are 70–80 μm.

synaptic cells was 182 ± 17 pA, whereas in the middle cells the mean amplitude was 185 ± 32 pA (figure 1, table 1). In contrast, the tail currents recorded from the stumps of purely postsynaptic cells were smaller (79 ± 15 pA). This represented a 60% reduction compared with presynaptic or middle cells ($p < 0.016$). Preliminary observations suggested that, in cases in which synapses were not formed but the cells were in close apposition, no changes in Ca^{2+} currents occurred on the stumps. No significant differences were noted between the amplitudes of the tail currents at the central and polar regions of the soma of any of the cells forming a triplet.

In addition to the Ca^{2+} tail currents, K^+ currents were measured in the stumps during the depolarizing pulse; no significant differences were obtained between

the amplitudes of the outward K^+ currents in the stumps of cells forming a triplet, measured 2 ms, 4 ms or 10 ms after the onset of the depolarizing pulse (e.g. at 10 ms means $\pm$ s.e. for presynaptic cells 2.1 ± 0.4 nA, middle cells 1.9 ± 0.3 nA, postsynaptic cells 1.4 ± 0.3 nA).

(*b*) *Ca^{2+} tail currents in single cells*

In principle, the smaller amplitudes of Ca^{2+} currents in postsynaptic cells after synapse formation could be caused by either a decrease or a failure to increase. To distinguish between these possibilities, Ca^{2+} tail currents from single Retzius cells were measured over a period of 48 h after the cells had been plated (see table 2). In some experiments, the same cells were recorded from time to time over the 48 h; in others, different cells were recorded from on each occasion, with similar results.

As in our earlier experiments, the largest Ca^{2+} currents occurred in the stumps of single Retzius cells, compared with the polar or central regions of the soma. This pattern remained similar for at least 48 h (table 2). Between 2 h and 6 h after the single cells had been plated, the amplitudes of the Ca^{2+} tail currents at the stumps were 126 ± 14 pA ($n = 21$ cells). The currents after 24 h in culture were slightly but not significantly larger (159 ± 18 pA, $n = 13$, $p \approx 0.25$) and then remained constant over at least the next 24 h (163 ± 25 pA, $n = 6$, at 48 h). The amplitudes of the Ca^{2+} tail currents in the stumps of single Retzius cells after 2–6 h and 24 h were not different from those recorded in stumps of purely presynaptic and middle cells of triads (table 1). A comparison of the tail currents on the stumps of single cells, presynaptic cells and postsynaptic cells (table 1) shows that the previously observed differences between pre- and postsynaptic cells arises from a reduction in the amplitudes of Ca^{2+} tail currents in postsynaptic cells.

(*c*) *Effects of synapse formation on neurite outgrowth*

Quantitative measurements of neurite outgrowth were made in single cells and in postsynaptic Retzius cells to determine what other consequences might result from the formation of a synapse upon a cell. For these experiments, cells were paired for 12 h in L-15 medium without substrate and then transferred to a substrate of ECM-laminin. Retzius cells plated on ECM-laminin showed a characteristic pattern of neurite outgrowth with straight and slender processes and a low density of branching points. The pattern and rate of neurite outgrowth measured were similar to those already described for Retzius cells (Chiquet & Acklin 1986; Grumbacher-Reinert 1989). When the extent of neurite outgrowth was compared in single and postsynaptic cells, a remarkable reduction was observed in the postsynaptic cells (figure 3*a*). After having been plated for 12 h on ECM-laminin, neurite growth was not significantly different in single (2.2 ± 0.40 mm) and postsynaptic (1.2 ± 0.6 mm) cells. By 24 h, a significant difference was detected ($p < 0.034$; two-tailed Student's T test) between single (3.8 ± 0.7 mm) and postsynaptic (1.4 ± 0.6 mm) cells (figure 3*b*). After 48 h, single cells had produced an outgrowth of 21.5 ± 0.7 mm total length, whereas that of postsynaptic cells was 6.8 ± 1.2 mm ($p < 0.02$). By normalizing neurite lengths to values at 12 h, the calculated increases in growth at 24 h, 36 h and 48 h were 74 %, 171 % and 204 % for single cells, and 11 %, 51 % and 73 % for postsynaptic cells (figure 3*c*). No differences were detected between the thicknesses of neurites or the branching pattern of single cells and postsynaptic cells (figure 3*a*).

4. DISCUSSION

From previous experiments, it is known that synapse formation has anterograde effects on Ca^{2+} currents recorded from the stump of the cell that is postsynaptic (Fernández-de-Miguel *et al.* 1992). Here we show an effect in the reverse direction: a postsynaptic target cell can also affect presynaptic Ca^{2+} currents in its partner. Thus, in triads, the decrease in Ca^{2+} currents was prevented in the middle Retzius cell, even though it was postsynaptic to the first cell. Our results suggest the development of retrograde signalling, without providing clues as to the mechanism. The retrograde signalling prevents the anterograde mediated decrease in Ca^{2+} currents. One difference between the third 'target' cell of the triad and the other two is that its growth cones come into immediate contact with the substrate (polylysine or ECM-laminin). By contrast, the first and second cells send their processes over the surface of the next cell in line.

By loose patch clamp recording, we were unable to determine whether the decrease in Ca^{2+} current densities at the stump of purely postsynaptic Retzius cells arose from modulation of the channels (Reuter 1983; Strong *et al.* 1987; Plummer *et al.* 1991), or from changes in channel density. Because no obvious differences occurred in the outward potassium currents recorded from the stumps of pre- or postsynaptic cells, it is unlikely that changes in Ca^{2+} current density simply reflect differences in membrane folding. Although in earlier experiments it appeared that Ca^{2+} tail currents were reduced in presynaptic cells at the polar region of the soma (Fernández-de-Miguel *et al.* 1992), no such changes occurred in the present study. We do not know the reason for this discrepancy, which may have been caused by the same factors that reduced variability of the tail-current amplitudes at the stumps.

In view of the reduced Ca^{2+} currents observed at the region of the postsynaptic cells from which growth starts (the stump), it was of considerable interest that synapse formation also influenced neurite outgrowth. A possible functional implication could be that, during development and regeneration, synaptic interactions inform cells about the connectivity of a circuit. It is not known whether a retrograde mechanism of growth control exists, similar to that shown here for Ca^{2+} channels. Nor is it known whether the decreased Ca^{2+} currents participate in this effect. An attractive speculation could be that once synapses form on a neuron during development and regeneration, its outgrowth becomes transynaptically modulated.

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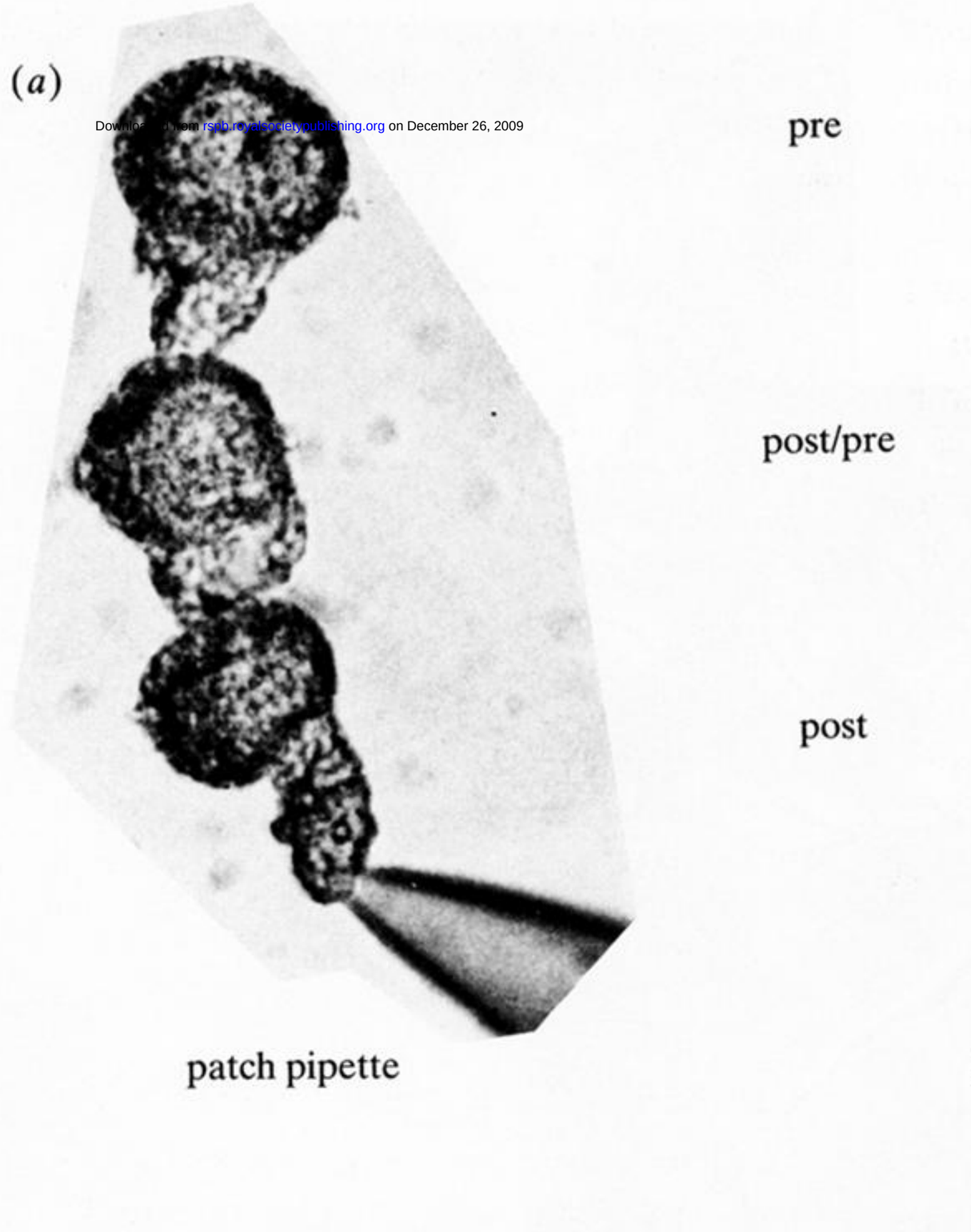

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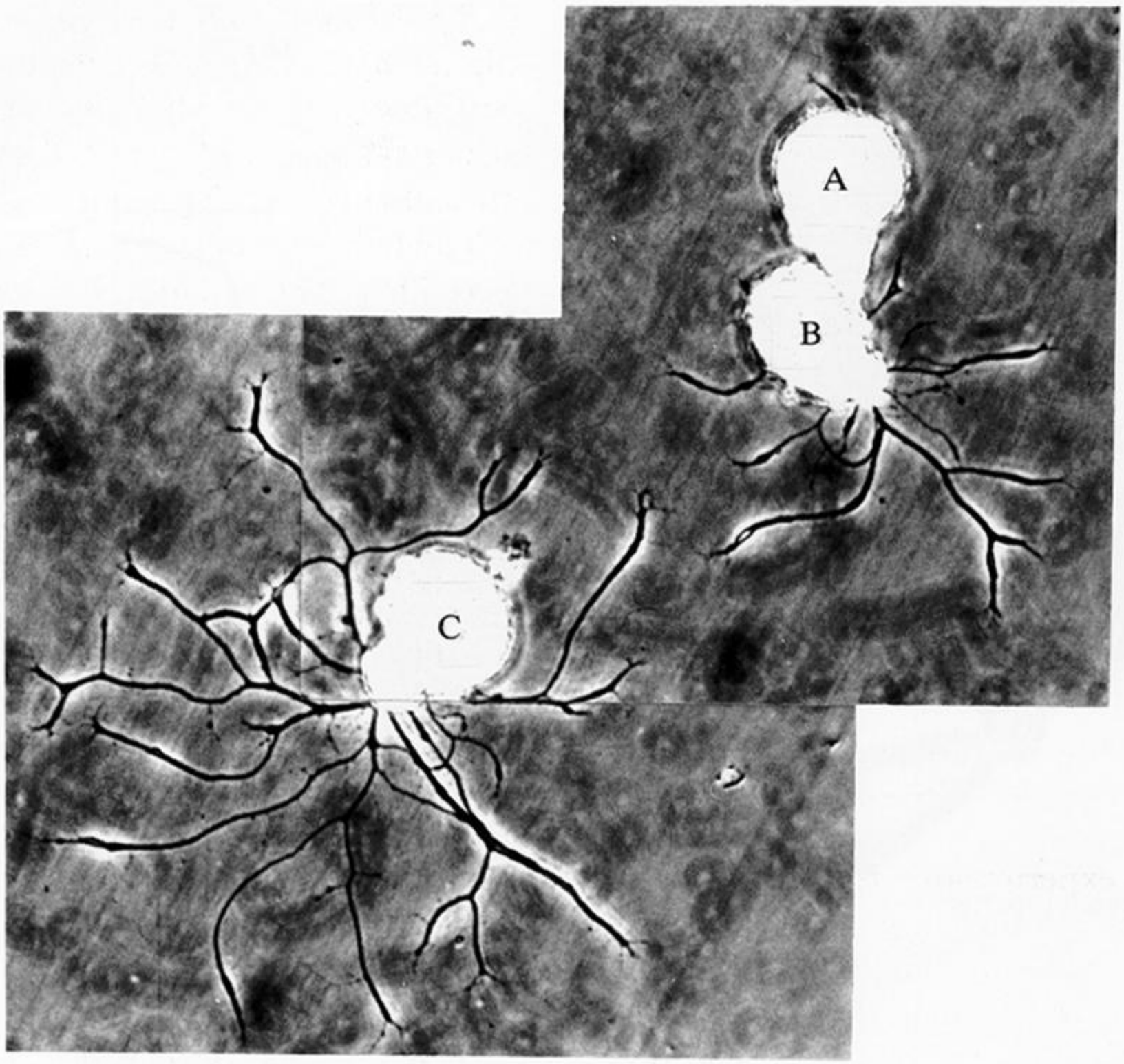

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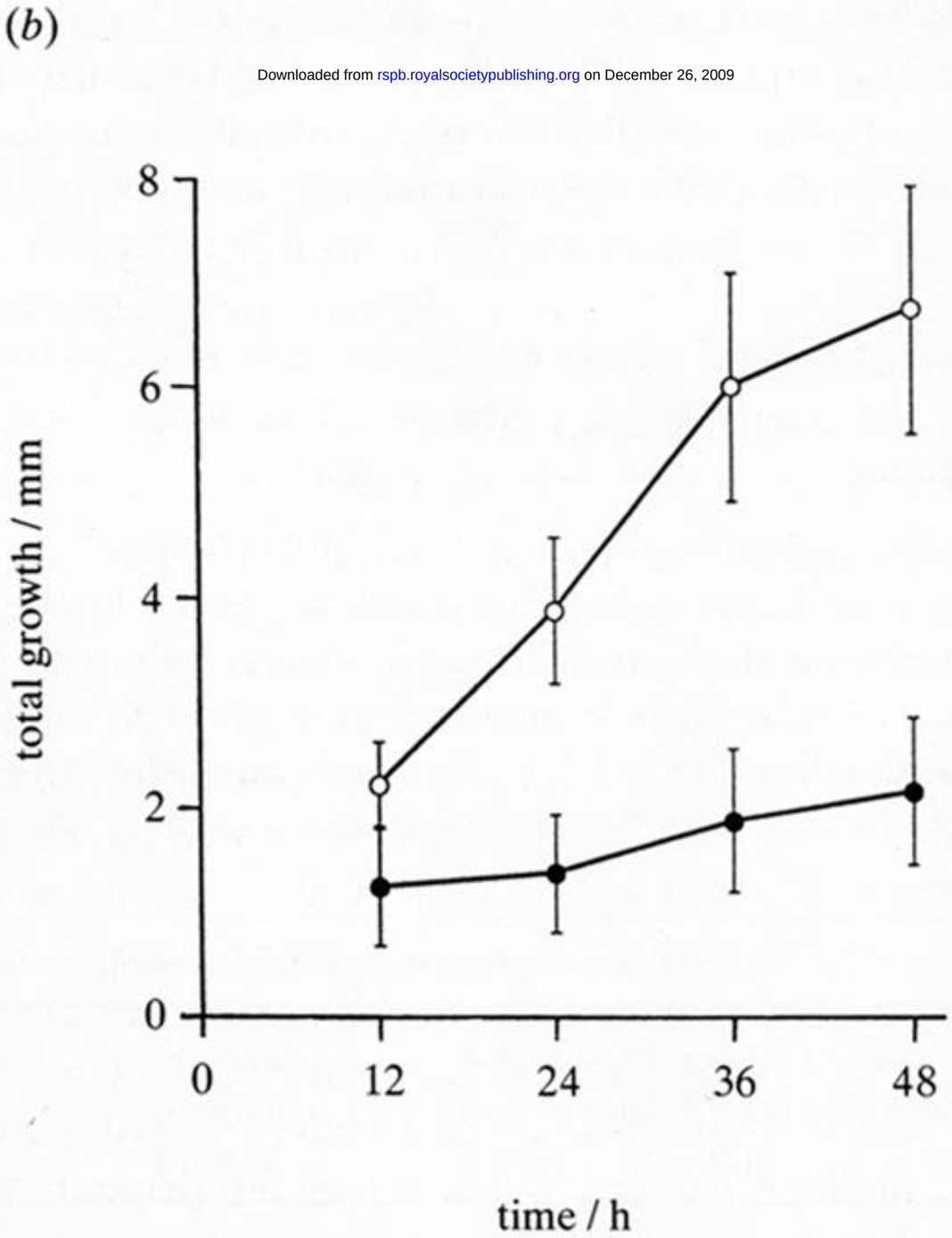

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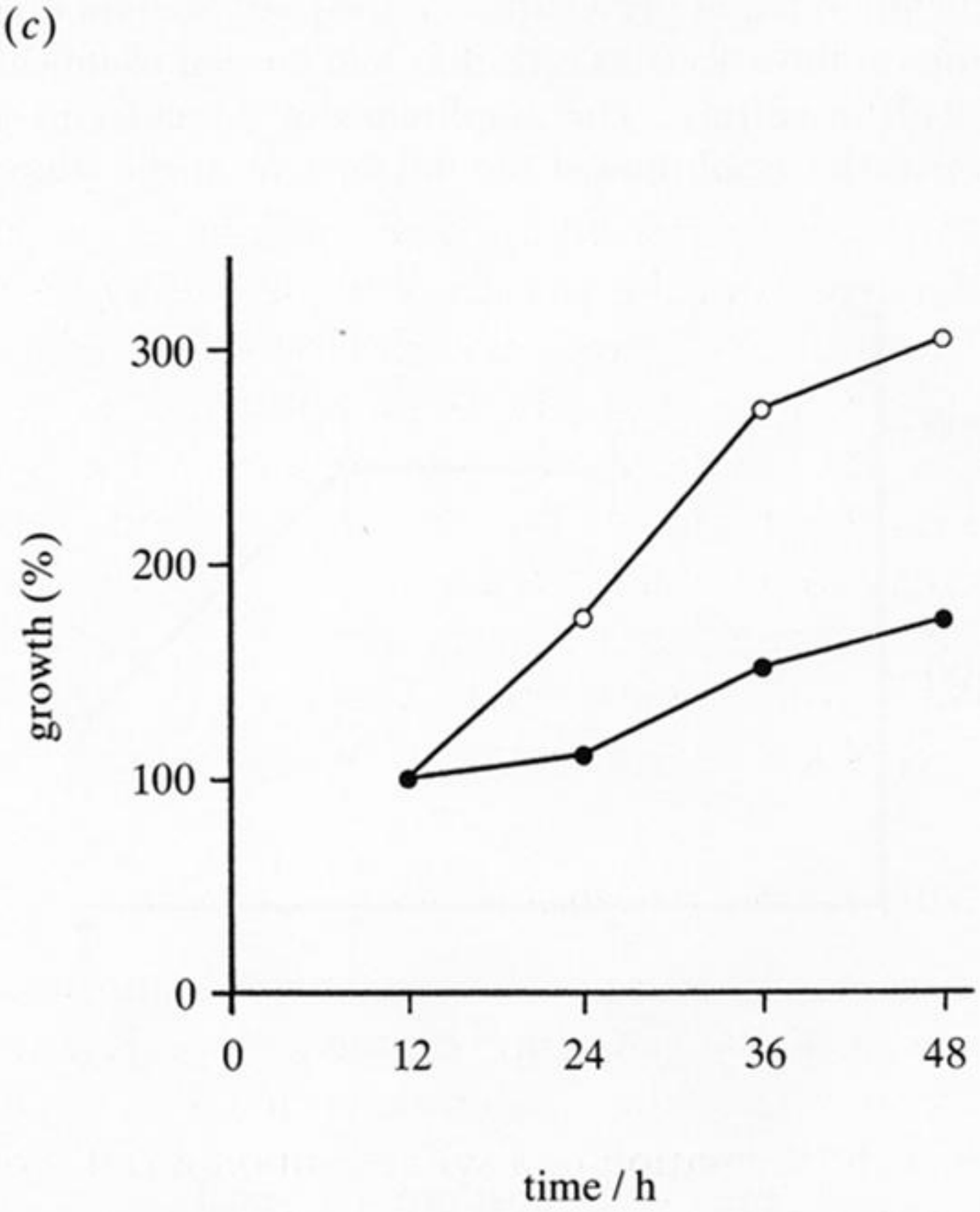

Figure 3. Decreased neurite outgrowth from postsynaptic compared with single cells plated on ECM-laminin substrate. (*a*) Outgrowth from a pair of cells and a single cell after 24 h. Although the patterns were similar, the total length of the processes was less in cell B (which was postsynaptic to cell A), than in the single cell C. Outgrowth of the presynaptic cell A occurs over the soma of B rather than on the substrate. (*b*) Mean values (±s.e.) of neurite outgrowth (in millimetres) of single cells (open circles) and postsynaptic cells (filled circles). All values after 12 h were significantly different ($p < 0.05$) (*c*) To compare rates of outgrowth, neurite length was normalized to values observed at 12 h. In postsynaptic cells, the rate as well as the amount of growth was reduced. Somata diameters are 70–80 µm.