Tyrosine phosphorylation during synapse formation between identified leech neurons

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1. We have examined whether tyrosine phosphorylation is required for synapse formation between identified neurons from the central nervous system of the leech in culture.

2. Within a few hours of contact with the cell body of the serotonergic Retzius neuron (R cell), the soma of the postsynaptic pressure-sensitive neuron (P cell), but not the R cell, could be labelled intracellularly with an antibody against phosphotyrosine residues. The labelling seemed specific for P cells contacted by R cells, as it was greatly reduced in pairs of either R or P cells and in single cells. Genistein (20 μM) and lavendustin A (10 μM), selective inhibitors of tyrosine kinases, blocked the labelling of contacted P cells, whereas their ineffective analogues (genistin and lavendustin B) had no effect on labelling.

3. R cell contact also induced the loss of an extrasynaptic, depolarizing response (due to modulation of cation channels) to serotonin (5-HT) in the P cell within a few days of juxtaposing cell bodies and within an hour of contact with growth cones. Treatment of the neurons with the tyrosine kinase inhibitors (but not the ineffective analogues) prevented the loss of the depolarizing response and of single cation channel modulation by 5-HT.

4. R cells formed inhibitory, Cl^--dependent synapses with P cells. Synapse formation was prevented by the tyrosine kinase inhibitors but not by their ineffective analogues. These compounds had no obvious effect on neurite outgrowth or cell adhesion. We conclude that tyrosine phosphorylation is a signal during the formation of this synapse.

Tyrosine phosphorylation is a key signal of normal and oncogenic cellular differentiation (Cantley *et al.* 1991; Greenwald & Rubin, 1992) and plays a variety of critical roles during development of the nervous system (Wagner, Mei & Huganir, 1991; Nairn & Shenolikar, 1992). Tyrosine kinases transduce growth factor signals (Schlessinger & Ulrich, 1992), influence growth cone dynamics and axon bundle formation (Elkins, Zinn, McAllister, Hoffmann & Goodman, 1990; Atashi, Klinz, Ingraham, Matten, Schachner & Maness, 1992; Bixby & Jhabvala, 1993; Wu & Goldberg, 1993), regulate synaptic function by phosphorylation of proteins associated with synaptic vesicles (Barnekow, Jahn & Schartl, 1990), and participate in long-term potentiation in the hippocampus (O'Dell, Kandel & Grant, 1991; Grant, O'Dell, Karl, Stein, Soriano & Kandel, 1992). Although tyrosine phosphorylation is important for neuronal differentiation, neurite outgrowth and synaptic function, its role in synapse formation in the nervous system is unclear.

The cellular and molecular mechanisms of synapse formation in the nervous system are difficult to study because of the limited accessibility and identification of the synaptic structures. These mechanisms have been characterized in considerable detail at the peripheral neuromuscular junction, where recent studies have suggested that tyrosine phosphorylation plays a crucial role in the clustering of acetylcholine receptors induced by cell contact (Baker & Peng, 1993; Peng, Baker & Dai, 1993) and by secreted factors such as agrin (Wallace, Qu & Huganir, 1991), and in the synthesis of receptors induced by the protein ARIA (acetylcholine receptor inducing activity; Falls, Rosen, Corfas, Lane & Fischbach, 1993). In order to investigate whether tyrosine phosphorylation is required for interneuronal synaptogenesis, we have examined the events leading to synapse formation between identified leech neurons. The central nervous system of the leech is simple in that it consists of repeating segmental ganglia, each containing only a few hundred neurons of large size. Many of these neurons have been identified, along with the synapses and circuits that they form (Muller, Nicholls & Stent, 1981). Moreover, isolated leech neurons in culture reform connections similar to those observed *in vivo* (Ready

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& Nicholls, 1979; Fuchs, Nicholls & Ready, 1981; Fuchs, Henderson & Nicholls, 1982; Chiquet & Nicholls, 1987), facilitating the study of neuronal recognition and synapse formation.

The best-studied synapse between cultured leech neurons is the inhibitory connection between the serotonergic Retzius neuron (R cell) and the pressure-sensitive neuron (P cell) (Fuchs *et al.* 1982; Dietzel, Drapeau & Nicholls, 1986; Kuffler, Nicholls & Drapeau, 1987; Drapeau & Sanchez-Armass, 1988). P cells have two different responses activated by serotonin (5-HT): a synaptically activated, Cl^--dependent conductance and an extrasynaptic, serotonergic cation conductance, each with a different pharmacology and dependent on distinct protein kinases (Sanchez-Armass, Merz & Drapeau, 1991). The depolarizing, extrasynaptic response may be involved in neuromodulation by 5-HT (Kristan & Nusbaum, 1982; Belardetti, Brunelli, Demontis & Sonetti, 1984). At sites of contact between R cells and P cells, the depolarizing response to 5-HT is reduced prior to formation of inhibitory synapses (Drapeau & Sanchez-Armass, 1988; Drapeau, Melinyshyn & Sanchez-Armass, 1989; Ching, Catarsi & Drapeau, 1993).

By using immunocytochemical and electrophysiological approaches together with selective inhibitors of tyrosine kinases, we have found evidence that tyrosine phosphorylation is required for synapse formation between these neurons.

METHODS

Cell cultures

R and P cells were isolated from the segmental ganglia (excluding reproductive ganglia) of the leech *Hirudo medicinalis* (Ricarimpex, Audenge, France) and cultured as described previously (Dietzel *et al.* 1986). Briefly, after exposing the desheathed ganglia to collagenase (Type XI; Sigma), the cell bodies of R and P cells were removed by aspiration into a micropipette. The cells were plated in the wells of either uncoated microtest culture dishes (Nunclon, Denmark) to permit neurite extension or in dishes coated with poly-lysine (Sigma), which prevents neurite extension, and were incubated in Leibovitz-15 medium (L-15; Gibco Canada, Burlington, Ontario, Canada) supplemented with 0·2 mg ml^{-1} gentamicin, 0·1 mg ml^{-1} ampicillin (Schering Canada Inc., Pointe Claire, Quebec, Canada) and 2% heat-inactivated fetal bovine serum (Gibco). In some experiments in which cell pairs were later separated (by repeated passage through the aspiration pipette), we used R cells mildly fixed for 30 min in 0·02% (w/v) paraformaldehyde and 0·03% (w/v) glutaraldehyde (Sigma) in phosphate-buffered saline (PBS); the fixed R cells were transferred at 10 min intervals into three serial droplets of L-15 medium in order to remove the aldehydes before being paired with untreated P cells (Drapeau *et al.* 1989).

Phosphotyrosine immunocytochemistry

The cells were plated either singly or, after being pushed together, as pairs on acid-etched coverslips (Fisher Scientific, Ontario, Canada) coated with poly-lysine and were cultured in L-15 medium; the medium was supplemented with serum only if the cultures were maintained for more than 2 days. No differences in the staining patterns were observed in the presence of serum. At different times (3 h to 4 days) the cells were fixed for 15–20 min in 4% paraformaldehyde PBS. After fixation, the coverslips were rinsed 3 times with PBS. The cells were permeabilized in 100% methanol for 20 min and then rinsed 3 times with PBS. Non-specific binding was blocked with 3% bovine serum albumin (Sigma) for 1 h. The cells were incubated in biotinylated anti-phosphotyrosine monoclonal antibody (clone 4G10; Upstate Biotechnology Industry, NY, USA) at dilutions ranging from 1:100 to 3:100 for 1 h. The coverslips were rinsed again in PBS and then incubated in streptavidin–Texas Red (Molecular Probes, Eugene, OR, USA) at concentrations of 1:250 to 1:450 for 30 min in the dark. Specimens were mounted in glycerol with 0·38% *n*-propyl gallate and 15% *p*-phenylenediamine (Sigma) and viewed with an epifluorescence microscope (Nikon). The experiments were evaluated blindly: cells were given a score for the presence or absence of staining but the drug treatment was unknown to the person examining the cells.

To obtain images of the cells, a confocal microscope equipped with a Kr–Ar laser was used (Leica). The specimens were excited at 514 nm and were scanned after using a 590 nm long-pass filter. Serial optical sections of ~1 μm in thickness were combined to reconstruct the entire staining pattern of the cells and copies were obtained with a Seikosha SG-3500 video printer.

Solutions

In order to isolate the cationic response to 5-HT, the Cl^- response was suppressed by recording in a Cl^--free solution of the following composition (mM): 130 Na_2SO_4, 4 K_2SO_4, 5 $MgSO_4$, 10 3,4-diaminopyridine, 10 glucose, 10 Hepes, brought to pH 7·4 with H_2SO_4 and to 330 mosmol. In some experiments, 100 μM $ZnCl_2$ (Sanchez-Armass *et al.* 1991) or 1 mM 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulphonic acid (SITS) (Drapeau & Sanchez-Armass, 1988) were added to block Cl^- channels, although in other experiments without addition of these blockers, the Cl^- response was undetectable. In order to isolate the Cl^- response, P cells were voltage clamped and currents were recorded in the following solution (Drapeau & Sanchez-Armass, 1988) (mM): 165 Tris(hydroxymethyl)aminomethane (TrisCl), 5 CsCl, 2 $MgCl_2$, 10 3,4-diaminopyridine, 10 glucose; pH 7·4. 5-HT (Sigma) at 100 μM was dissolved in the recording solution and applied to the P cell bodies by pressure ejection (Picospritzer; General Valve Corp., Fairfield, NJ, USA) with 100 ms pulses through a blunt pipette (15–20 μm diameter; Drapeau & Sanchez-Armass, 1988). 5-HT was applied focally (within 20 μm) onto the growth cones with a fine-tipped pipette (< 1 μm tip opening) containing a high 5-HT concentration (50 mM, pH 7·4) while recording from the cell body (Henderson, 1983; Ching *et al.* 1993).

For patch-clamp experiments the following filtered saline solution was used in the bath (mM): 155 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 Hepes, 10 glucose, brought to pH 7·4 and 330 mosmol; for filling of the recording pipettes, this solution was diluted by 10% in order to improve seal formation. 5-HT was applied by pressure ejection of 1 μl of a concentrated solution into the 10 μl wells to yield a final 5-HT concentration of 30 μM, which gave a maximal response (Drapeau, 1990).

In the experiments on synapse formation, the saline solution was modified by increasing $CaCl_2$ to 10 mM and by removing $MgCl_2$. Stock solutions in dimethyl sulphoxide (DMSO) of lavendustin A or B (LC Services, Woburn, MA, USA) were diluted 5000-fold to

yield a final concentration of 10 μM, and stock solutions of genistein (LC Services) or genistin (Extrasynthese, Orsay, France) were diluted 2000-fold to yield a final concentration of 20 μM; these low DMSO concentrations had no effect on the properties studied. In most of the experiments in which these compounds were used, P cells were left in droplets containing the compound for at least 2 h before plating them either alone or with R cells in the presence of the compound. In other experiments (Fig. 3*B*), no pretreatment was used.

Recordings

Intracellular voltage recordings were obtained with 70–100 MΩ microelectrodes for P cells and 30–50 MΩ pipettes for R cells filled with either 4 M potassium or caesium acetate. In the experiments carried out to detect synapses, 4 M potassium acetate or 3 M KCl electrodes were used to impale P cells. Action potentials in R cells were evoked by passing current using an electrometer with a bridge circuit (World Precision Instruments, New Haven, CT, USA) and, for synaptic potentials recorded with KCl electrodes,

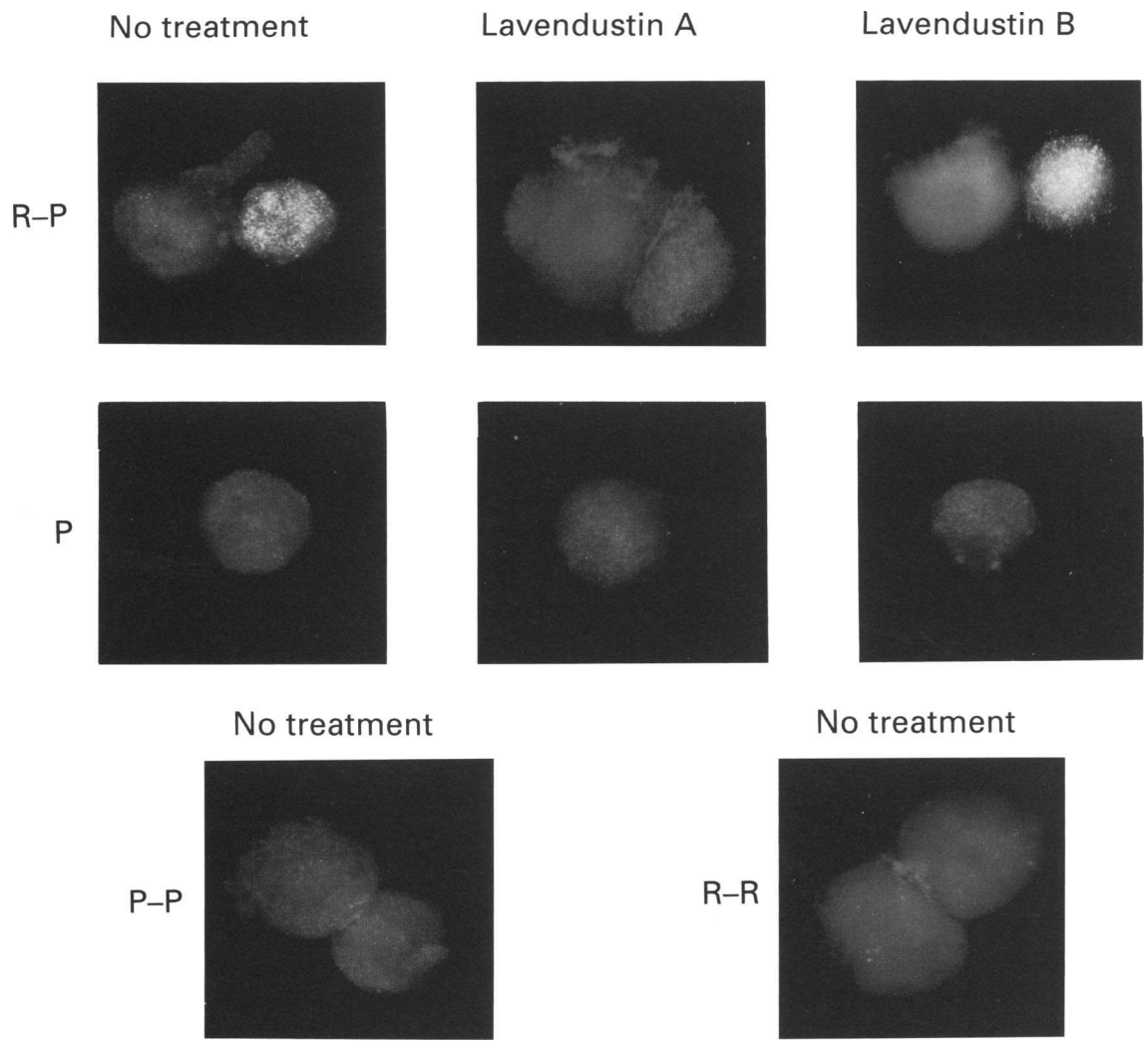

Figure 1. Phosphotyrosine labelling of R and P cells

Two days after plating either single cells or cell pairs, the cultured cells were fixed, permeabilized and stained with a monoclonal antibody to phosphotyrosine linked to the fluorophore Texas Red. The cells were then imaged in a confocal microscope, serial optical sections of the cells were superimposed and the composite images were printed. The top row shows R and P cell pairs (R cells always to the left), in the middle row are shown single P cells, and in the lower row are shown P cell pairs (left) and R cell pairs (right). The cells were plated without drug treatment (column on left and lower row) or after treatment with 10 μM of either lavendustin A (middle column) or lavendustin B (right column). Scale bar, 50 μm.

Table 1. Summary of the phosphotyrosine labelling of R and P cells

	R–P					
	R	P	P	R	P–P	R–R
Untreated	2/31	28/31*	2/24	3/26	4/20	0/16
Lavendustin B	4/39	33/39*	2/22	0/11	5/26	0/12
Lavendustin A	0/31	4/31	2/19	0/6	2/18	0/10

The results shown are for cells that were either not treated or treated with 10 μM of either lavendustin B or lavendustin A and stained as described in Fig. 1 by one person; a second, uninformed person rated the cells as being either stained or not (ratios are cells stained/total examined). The cells examined were R–P pairs (R cells in the first column, P cells in the second column), single P cells (third column) or R cells (fourth column), and pairs of P cells (fifth column) or R cells (sixth column). * Paired cells showing a highly significant difference (χ^2 test, $P < 0{\cdot}001$) in staining compared with the observations with unpaired cells; all other pairings were not significantly different from the observations with unpaired cells ($P > 0{\cdot}2$).

Cl^- was injected into the P cells by passing 1–2 nA of hyperpolarizing current for a few minutes.

Voltage-clamp current recordings were obtained using a single, 4 M caesium acetate electrode with a 15–20 MΩ resistance and using a sample-and-hold amplifier (Axoclamp-1A; Axon Instruments, Burlingame, CA, USA), as described previously (Drapeau & Sanchez-Armass, 1988). Cell-attached patches were formed on P cells, as described previously (Drapeau, 1990), using 5–10 MΩ electrodes that were coated to within 50 μm of the tip with dental wax to reduce the capacitance. Recordings were made using an Axopatch-1A patch clamp (Axon Instruments) and were low-pass filtered at 1 kHz (−3 dB) before digitization (Neuro Data Instruments Corp., New York, USA) and subsequent storage on videotape. For analysis of open probabilities, the analog signals were played back and digitized at 5 kHz and transferred to a hard disk for off-line analysis with a PC compatible computer using a TL-1 DMA Interface and the pCLAMP software package (Axon Instruments). Hard copies of the data were obtained using a QMS PS410 laser printer (QMS Inc., Mobile, AL, USA).

RESULTS

Selective tyrosine phosphorylation in P cells contacted by R cells

In order to determine whether cell contact induced tyrosine phosphorylation, we plated P cell bodies in contact with R cells on coverslips for a period of 3 h to 4 days. The cells were then fixed, permeabilized, and treated with a biotinylated anti-phosphotyrosine monoclonal antibody conjugated to avidin, followed by staining with streptavidin conjugated to Texas Red (see Methods). The contacted P cells showed a bright, punctate distribution of the fluorescence at the cell surface and, particularly after a few days, throughout the cell body in most (28 out of 31) of the cell pairs examined (Fig. 1 and Table 1). The punctate labelling of the P cells was not observed in the absence of the primary antibody or if the cells were not permeabilized, demonstrating specific intracellular labelling of the P cells. Staining was evident within 3 h after contact between the cells, the shortest period we could examine while retaining sufficient cell adhesion to the coverslips. In the majority of the experiments, cells were fixed and stained after 2 days in culture. After several days of contact, the intensity of the labelling decreased progressively, but this was not quantified.

In contrast to the P cell labelling, juxtaposed R cells were rarely stained (2 out of 31; Fig. 1 and Table 1). Similarly, we found that single cells as well as pairs of either P cells or R cells were labelled far less often (Fig. 1 and Table 1; χ^2 test, $P > 0{\cdot}2$ for paired compared with single cells), showing that only P cells contacted by R cells had elevated levels of tyrosine phosphorylation ($P < 0{\cdot}001$ compared with single P cells).

If the antibody labelling was indeed related to tyrosine phosphorylation in the P cell, inhibitors of tyrosine kinases should prevent this labelling. We therefore tested the effects of two specific inhibitors, lavendustin A and genistein, as well as their inactive forms, lavendustin B and genistin, respectively (Onoda *et al.* 1989; O'Dell *et al.* 1991; Hidaka & Kobayashi, 1992). The selective, high-affinity inhibitor lavendustin A, but not lavendustin B, greatly reduced the labelling of the P cells (Fig. 1 and Table 1), as did the lower-affinity inhibitor genistein (none of 8), but not genistin (7 out of 7; micrographs not shown). As only the inhibitory forms of these compounds affected the labelling, these results support the interpretation that tyrosine kinases participate in the increased phosphorylation observed in P cells contacted by R cells.

In order to determine if tyrosine phosphorylation has a functional role in synapse formation between the R and P cells, we examined the effects of the inhibitors of tyrosine kinases on two related events: the selection of 5-HT responses in the P cell and the subsequent formation of inhibitory synapses.

Selection of transmitter responses: induction by cell body contact

In previous work, we have shown that the extrasynaptic, cationic response to 5-HT in the P cell is lost upon contact with the R cell prior to synapse formation (Drapeau *et al.*

1989). In order to determine if this early physiological effect of cell contact depends on tyrosine phosphorylation, we recorded the depolarizing response under conditions which suppressed the Cl^--dependent response. At different times following the juxtaposition of R and P cell bodies, the P cells were recorded intracellularly in a Cl^--free solution (see Methods) while applying 100 μM 5-HT from a pressure pipette. At a holding potential of −50 mV (near the normal resting potential), the only detectable response to 5-HT is due to the activation of a monovalent cation conductance (Drapeau & Sanchez-Armass, 1988). Both single P cells plated for 3 days (Fig. 2*A*) and P cells recorded within a few hours of being paired with R cells (Fig. 2*B*) showed a depolarizing response of 3–4 mV when 5-HT was applied. While this response persisted in single P cells, in paired cells it decreased after 1 or 2 days of pairing and was abolished after 3 days (Fig. 2*A* and *B*).

To study whether the loss of the depolarizing response to 5-HT was reversible, the cells were separated after 3 days of contact. We failed to observe a significant reversal of the response for up to 5 days following separation (i.e. day 8 in Fig. 2*B*). Because it was previously shown that fixed R cells adhere to P cells and retain their ability to induce the loss of the cationic response (Drapeau *et al.* 1989), we also tried pairing the P cells with aldehyde-fixed R cells. In this case, the P cell depolarizing response gradually recovered up to 80% by 5 days following separation (Fig. 2*B*). Thus, the effect of contact with R cells was reversible for fixed R cells but not for unfixed R cells.

Selection of transmitter responses: block by tyrosine kinase inhibitors

In order to determine whether tyrosine phosphorylation is required during the selection of transmitter responses, we examined the effects of treating the cells with the tyrosine kinase inhibitors found to block phosphotyrosine labelling of the P cell. In the presence of lavendustin A, P cells showed a depolarizing response even after 3 or more days of contact with R cells (Figs 3*A* and 4); a similar result was obtained on incubation with genistein (3·0 ± 0·5 mV, mean ± S.E.M.; $n = 7$). On the contrary, contacted P cells exposed to inactive lavendustin B (Figs 3*A* and 4) lost the depolarizing response to 5-HT after 3 days of contact, as did cells exposed to genistin (0·06 ± 0·04 mV; $n = 8$), just as described above for untreated cells (Fig. 2). Neither lavendustin A nor B affected the responses in single P cells (Fig. 4).

With the aim of determining the time course for blocking the loss of the depolarizing response with inhibitors of tyrosine kinases, we examined the effects of treatments for various time periods in the presence of lavendustin A. As shown in Fig. 3*B*, we found that at least 24 h of treatment

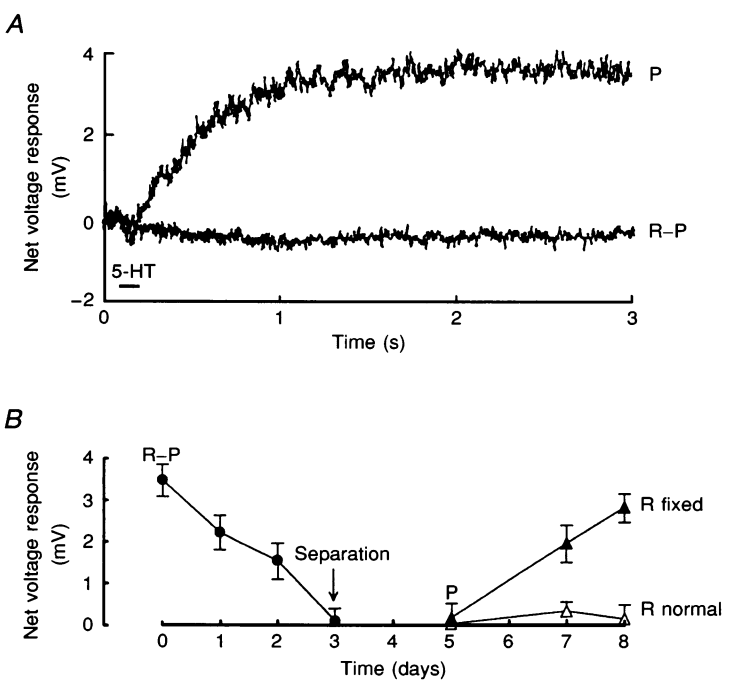

Figure 2. Time course of the depolarizing response to 5-HT and its loss

A, the net voltage response (relative to the resting potential of ~−50 mV) to a 100 μM 5-HT pulse (100 ms duration, as indicated by the bar) is shown for a single P cell (top trace) and for a P cell contacted by an R cell for 3 days in culture (bottom trace). *B*, the time course of the decline in the response to 5-HT of P cells after being placed into contact with an R cell is shown (●) for days 0–3. The recovery of the 5-HT response is shown after separating the P cells from either normal (△) or fixed R cells (▲) after 3 days of contact and testing 2–5 days later (i.e. on days 5–8). Data points are the means ± S.E.M. of 4–10 different experiments.

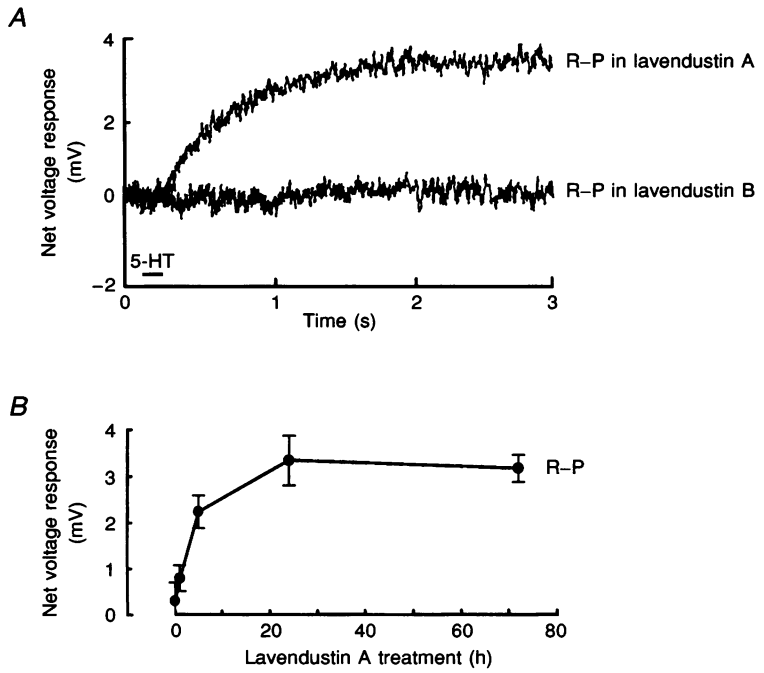

Figure 3. Time course of the lavendustin effect on the depolarizing response to 5-HT

A, the net responses to a pulse of 100 μM 5-HT (100 ms as indicated by the bar) in P cells contacted by R cells for 3 days in culture are shown for cells incubated in the presence of either 10 μM lavendustin A (top trace) or lavendustin B (bottom trace). *B*, the response to 5-HT is shown for P cells contacted by R cells for 3 days and treated with 10 μM lavendustin A for different periods of time after being paired. Data points are the means ± S.E.M. of 5–10 different experiments.

with the inhibitor, applied immediately after cell contact, was required in order to prevent the loss of the depolarizing response; shorter periods of exposure to lavendustin A resulted in partial loss of the depolarizing response to 5-HT. These results suggest that tyrosine kinase activity is required at least during the first day of contact between the cells. On the other hand, if lavendustin A was applied for 3 days after the cells had already been paired for 3 days

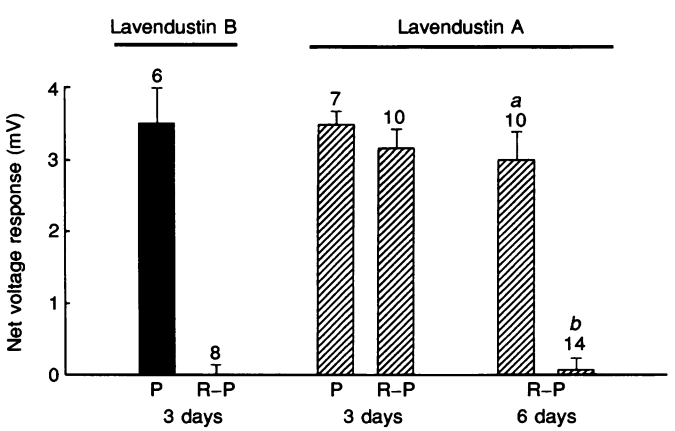

Figure 4. Effects of treatment with lavendustin A or B on the depolarizing response to 5-HT in single and contacted P cells

The filled columns on the left show the depolarizing response of P cells to pulses of 100 μM 5-HT recorded after 3 days of treating single P cells or R–P pairs with 10 μM lavendustin B. The pair of hatched columns on the left show the effect of treating single P cells or R–P cell pairs with 10 μM lavendustin A for 3 days. The pair of hatched columns at the right show the effect of treating R–P pairs with lavendustin A either starting the treatment immediately after pairing and recording after 6 days (*a*) or starting the treatment 3 days after pairing and recording 3 days later (*b*). Data points are the means ± S.E.M. for the number of experiments indicated above each column.

(i.e. for days 4–6), it was no longer effective compared with cells treated for the entire 6 day period (Fig. 4). This result shows that once the loss of 5-HT sensitivity was induced (after 3 days of contact), tyrosine kinase activity was not required to sustain it, suggesting a window of 1–3 days for the effect of cell contact.

We have shown previously that the cationic response is due to the activation of monovalent cation channels by 5-HT and protein kinase C. The cation channels can be recognized by their characteristic properties, including rare, brief inward openings with an amplitude of 3–4 pA at the resting potential, a conductance of 60 pS and a reversal potential of 10 mV (Drapeau, 1990; Catarsi & Drapeau, 1992; Ching *et al.* 1993; Catarsi & Drapeau, 1993). We observed that cation channels recorded in P cells contacted by R cells for 3 days lost their modulation by 5-HT in the presence of inactive lavendustin B ($P_{o,5\text{-HT}}$: $P_{o,control} = 1{\cdot}1 \pm 0{\cdot}1$, mean ± S.E.M.; $n = 14$; Fig. 5), as in untreated cells (Drapeau, 1990), but were still modulated by 5-HT when the cells were incubated with lavendustin A ($P_{o,5\text{-HT}}$: $P_{o,control} = 3{\cdot}7 \pm 0{\cdot}4$; $n = 9$; Fig. 5). These results suggest that the loss of sensitivity to 5-HT was due to a tyrosine kinase-dependent loss of modulation of the cation channels.

Selection of transmitter responses: induction by contact with growth cones

As shown previously, recordings from the P cell soma detected the cationic, depolarizing response to local application of 5-HT onto uncontacted growth cones but not onto growth cones contacted by R cell neurites (Ching *et al.* 1993). We were able to assess the timing of the loss of sensitivity to focally applied 5-HT by pushing an R cell body with a blunt micropipette until it contacted a P cell growth cone; after a period of contact, the R cell was moved so that 5-HT could be applied directly onto the same growth cone, which often remained adhered to the dish. One hour of contact was sufficient to reduce the cationic response to 15 ± 5% of the response observed with uncontacted growth cones (Fig. 6). Shorter periods of contact produced only a partial loss of the response but the precise time course was not determined. Application of lavendustin A during the 1 h period of contact with the R cell prevented the loss of responsiveness to 5-HT in contacted P cell growth cones (108 ± 6% of the response in uncontacted growth cones; mean ± S.E.M.; Fig. 6).

Block of synapse formation by tyrosine kinase inhibitors

We examined the effects of tyrosine kinase inhibitors on synapse formation by plating P and R neuronal cell bodies together for 6 days in their presence and recording synaptic interactions. In some experiments, the detection of synapses was facilitated by injecting Cl^- into the P cells in order to displace the Cl^- reversal potential from the resting potential. With lavendustin B treatment, twelve out of thirty-two pairs showed synaptic responses (Fig. 7), similar to the results obtained with untreated cells (9 out of 20 pairs in this study and about one-half of the pairs in our previous studies). In contrast, postsynaptic potentials were

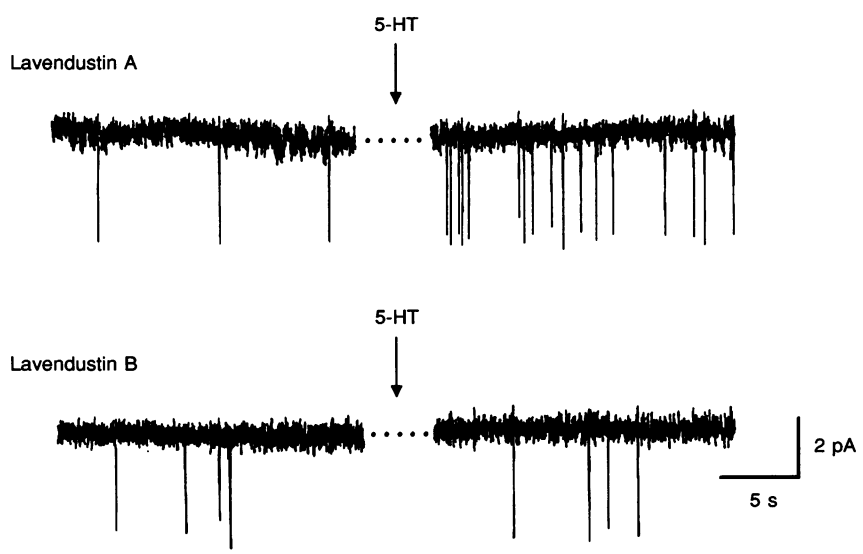

Figure 5. Effect of lavendustin on single cation channel modulation by 5-HT

Cation channel activity was recorded in cell-attached patches at the resting potential from a P cell contacted by an R cell for 3 days and treated either with 10 μM lavendustin A (top) or lavendustin B (bottom). The traces are 20 s samples from the recordings, which were interrupted for a few minutes during which 30 μM 5-HT was added, as indicated by the arrows. Several hundred openings were analysed (at high resolution, see Methods) in order to estimate the channel open probability before ($P_{o,control}$) and after ($P_{o,5\text{-HT}}$) addition of 5-HT.

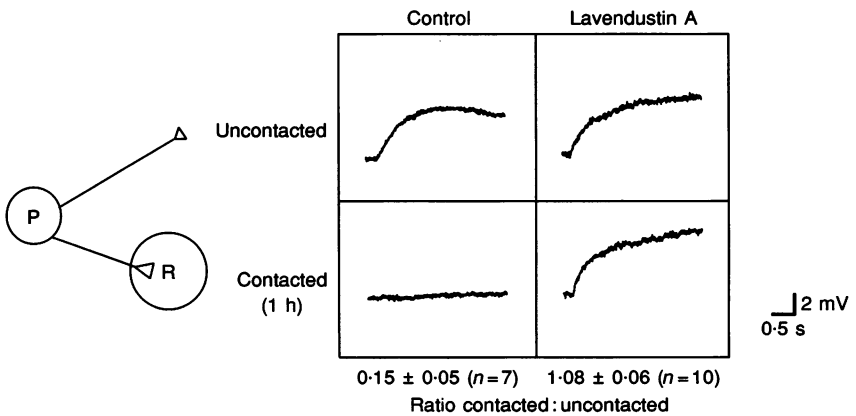

Figure 6. Effect of R cell contact and lavendustin A on the depolarizing response to 5-HT in P cell growth cones

Intracellular recordings were obtained from P cells for pulses of 5-HT (100 ms) applied locally from a fine-tipped pipette (< 1 μm opening) containing a high 5-HT concentration (50 mM, pH 7·4). The 5-HT was applied locally onto growth cones that were either not contacted (top traces) or contacted by an R cell that had been placed on them for 1 h and then removed for measuring the 5-HT response (lower traces). Measurements were made in the absence (left column) or presence (right column) of 10 μM lavendustin A added 1 h earlier. The ratios of the responses recorded in contacted relative to uncontacted growth cones (contacted : uncontacted) from the same P cells are shown at the bottom of the figure for experiments in the absence (left) or presence (right) of 10 μM lavendustin A (means ± S.E.M.).

observed far less frequently ($P < 0{\cdot}001$ by the χ^2 test) in the presence of either lavendustin A (2 out of 36 pairs; Fig. 7) or genistein (none of the 33 pairs tested; not shown).

The suppression of synaptic responses by these drugs could be due to the block of synapse formation. Alternatively, the drugs may have had indirect effects, such as block of cell adhesion or of the postsynaptic responsiveness to 5-HT. We tested for indirect effects of tyrosine kinase inhibitors as follows. In the presence of these drugs, the neurons remained well adhered to one another, as observed by the inability to separate the cells when they were displaced

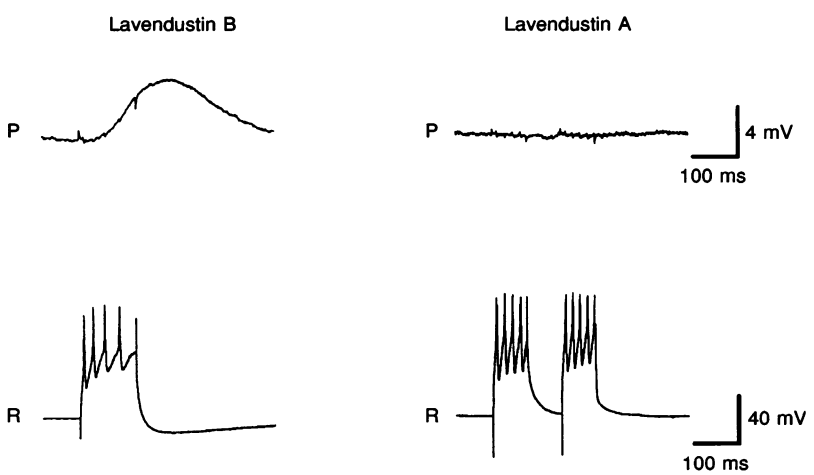

Figure 7. Effect of lavendustin on R–P cell synapse formation

The traces show the effects of intracellular stimulation of R cells (bottom) and voltage recordings from contacted P cells (top) after 6 days in the presence of either 10 μM lavendustin B (left) or lavendustin A (right). P cells were injected with Cl^- to reverse the Cl^- gradient; the extracellular solution contained 10 mM Ca^{2+} and 0 Mg^{2+}. For cells treated with lavendustin B (left), a train of 4 action potentials was elicited in the R cell, resulting in a summated synaptic response in the P cell. In contrast, for cells treated with lavendustin A (right), 2 trains of action potentials in the R cell failed to elicit a response in the P cell.

from the culture dish by moving the P cell with the recording microelectrode. Also, in separate experiments in which the cells were plated on uncoated dishes, neurite outgrowth was unaffected by exposure for 3 days (starting from cell plating) to lavendustin A or genistein. Thus, these drugs affected neither cell-to-cell adhesion nor neurite outgrowth.

To test for an effect of these drugs on the postsynaptic Cl^- conductance, we performed voltage-clamp recordings with P cells incubated in an impermeant cation solution (Drapeau & Sanchez-Armasss, 1988) and applied 5-HT from a pressure pipette, as described above for detecting the cationic response. The Cl^- conductance was retained after 6 days of incubation in the presence of lavendustin A, showing that the block of the synaptic response was not due to direct block of the Cl^- conductance by lavendustin A. We conclude that the tyrosine kinase inhibitors blocked synapse formation, although we did not carry out a morphological examination of the zone of contact.

DISCUSSION

Within a few hours of contact with R cells, P cells showed immunocytochemical labelling for tyrosine phosphorylation. Subsequently, the extrasynaptic response to transmitter (5-HT) was eliminated and inhibitory synapses were formed, as documented in our previous work (Drapeau *et al.* 1989). When P cell growth cones were contacted by R cells, the loss of the extrasynaptic response to 5-HT occurred far more rapidly (within 1 h) than it did in contacted cell bodies (after 3 days). All of these events were blocked by inhibitors of tyrosine kinases. Based on these results, we propose a model for the formation of synapses between R and P neurons and relate our observations to those made in other preparations.

Mechanism of R–P cell synapse formation

We propose the following model for the formation of synapses between R and P cells (Fig. 8): (1) at sites of contact specifically between R and P cell neurites or growth cones, a synaptogenic signal transduction pathway involving tyrosine phosphorylation is activated in the P cell; (2) a resultant cytoplasmic signal blocks the extrasynaptic response to 5-HT; (3) subsequent synaptic release of 5-HT results in selective activation of inhibitory Cl^- channels.

R cell-specific contact

Leech neurons in culture re-form synapses with their appropriate partners in the absence of other cells (Ready & Nicholls, 1979; Fuchs *et al.* 1981) or of an extracellular matrix (Kuffler *et al.* 1987), suggesting that they recognize one another by intrinsic cues. The specificity of neuronal recognition has been studied in the greatest detail for the formation of synapses between R and P cells. The R cell is the only serotonergic neuron known to form chemical synapses with the P cell (Acklin & Nicholls, 1990). In contrast to the R–P synapse observed in standard segmental ganglia, R cells from the reproductive ganglia do not innervate P cells either *in situ* or *in vitro* (Merz & Drapeau, 1994*a*), showing that the P cells are able to specifically recognize standard R cells.

Interestingly, the selective loss of the extrasynaptic, depolarizing response to 5-HT is also induced specifically by standard R cells and not by other neurons (Merz & Drapeau, 1992, 1994*a*), providing a measure of R–P cell interactions in the absence of a synaptically activated Cl^- conductance. We have shown that 5-HT is not the factor inducing this loss of response and that R cells fixed with aldehydes retain this interaction with P cells, suggesting that it is surface contact and not a diffusible factor that is responsible (Drapeau *et al.* 1989). In this report, we found that P cells recovered the cationic response only when separated from fixed (and not from living) R cells. One possibility is that suppression of the cationic response was mediated by the release of an unidentified factor by unfixed, but not fixed, R cells while they were paired with the P cells. An alternative and more likely hypothesis is

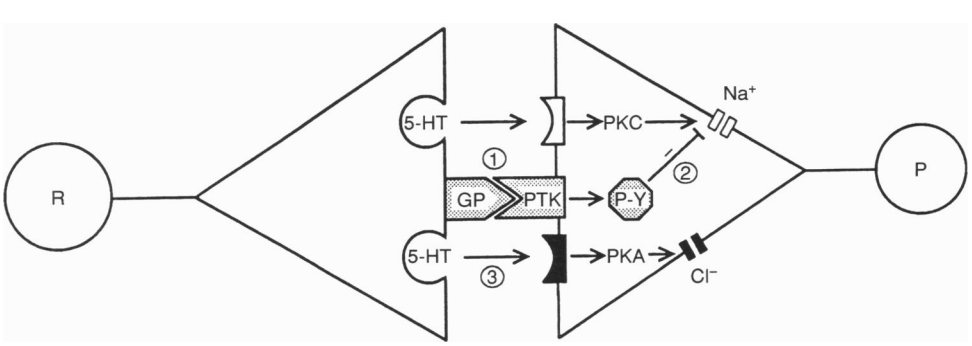

Figure 8. Model for synapse formation between R and P cells

(1) Contact by an R cell-specific surface glycoprotein (GP) activates a P cell protein tyrosine kinase (PTK). (2) A tyrosine phosphorylated (P-Y) cytoplasmic signal is generated and uncouples cation channel modulation by protein kinase C (PKC). (3) Subsequent synaptic release of 5-HT activates a distinct receptor (filled concave box) linked via protein kinase A (PKA) to inhibitory Cl^- channels.

that a small amount of membrane from living R cells remained attached to the P cells and maintained the suppression of the cationic response, but we have not investigated this at an ultrastructural level.

R cells treated with trypsin or the lectin wheat germ agglutinin (WGA) prior to fixation have no effect on the P cells' responses to 5-HT (Merz & Drapeau, 1994*b*). These results demonstrate that R cell contact, presumably mediated by surface glycoproteins, induces early physiological changes in the P cell during synaptogenesis. Since WGA (Merz & Drapeau, 1994*b*) and, as shown in this study, tyrosine kinase inhibitors block both the selection of 5-HT responses and synapse formation, the early selection of responses is likely to be triggered during the recognition process and is thus intrinsic to synaptogenesis.

P cell tyrosine phosphorylation

Phosphotyrosine labelling was detected in P cells contacted by R cells but not in P–P or R–R cell pairs, consistent with the cell-specific functional interactions during synapse formation described above. Since R cell pairs formed synapses (Fuchs *et al.* 1981) but were not tyrosine phosphorylated, our results suggest that this is a restricted rather than a general signal during synapse formation. It will be of interest to determine whether tyrosine phosphorylation occurs during the formation of synapses between other neurons, with other partners of the P cell, or is specific for the R–P synapse.

Selective inhibitors of tyrosine kinases blocked P cell labelling, the selection of 5-HT responses, and synapse formation, demonstrating a critical role for tyrosine phosphorylation in the P cell during innervation by the R cell. The simplest interpretation of the effect of R cell contact is that it activates a P cell tyrosine kinase. Additional events such as the suppression of a tyrosine phosphatase or increased accessibility of a substrate may also occur. The lack of effect of the tyrosine kinase inhibitors after the loss of the cationic response was established suggests a transient activation of this signalling pathway.

We observed intracellular labelling of the P cell surface within a few hours of contact with R cells and, particularly after a few days, throughout the cell body. A variety of proteins are likely to be tyrosine phosphorylated since some differentiation signals mediated by cell contact and by growth factors during development of the nervous system are dependent on receptor-mediated tyrosine phosphorylation and activation of cytosolic signals (Wagner *et al.* 1991; Nairn & Shenolikar, 1992; Keegan & Halegoua, 1993). The P cell body labelling was observed within the shortest period we could test (a few hours after plating) and was maintained for a few days before it appeared to decline. Similarly, at the frog neuromuscular junction, motoneuron contact induces a punctate tyrosine phosphorylation of the muscle cell that lasts for at least 2 days (Baker & Peng, 1993).

The loss of the extrasynaptic response to 5-HT in P cell growth cones, which are more akin to the sites where synapses are formed *in vivo* in the leech, occurred far more rapidly (within 1 h of contact) than in the cell bodies. The faster time course in the smaller growth cones is consistent with the generation of a diffusible factor, as suggested by the intracellular distribution of phosphotyrosine labelling and as detected by previous experiments with membrane patches inserted into the cytoplasm of contacted P cell bodies (Catarsi & Drapeau, 1993).

Inhibitory synapses and extrasynaptic modulation

Although the modulation of extrasynaptic channels was lost as rapidly as an hour after contact with P cell growth cones, chemical synapses between cultured leech neurons were not detected until after several hours or even a few days (Liu & Nicholls, 1989). Interestingly, a similar delay between cell contact and synapse formation may occur during embryonic development in the leech as spontaneous synaptic potentials are observed within several hours to a day following the growth of neuropilar processes (Kuwada & Kramer, 1983). The early loss of the extrasynaptic response to 5-HT in the P cell may be required so that 5-HT release by the R cell at a later stage activates only the appropriate, inhibitory Cl^- response. Although the Cl^- current is activated by 5-HT application even after application of tyrosine kinase inhibitors, the lack of Cl^--dependent synaptic responses under this condition suggests that the assembly of a functional (inhibitory) synapse is similarly dependent on tyrosine kinase activity.

The contact-dependent selection of 5-HT responses would limit the inhibitory, synaptic actions of 5-HT to sites of contact in the neuropil; on cell bodies, chronic exposure to 5-HT desensitizes the Cl^--dependent response and thus activates only the depolarizing response (Henderson *et al.* 1983; Drapeau & Sanchez-Armass, 1988).

Tyrosine phosphorylation as a synaptogenic signal

During formation of the neuromuscular junction, tyrosine phosphorylation is a regulatory signal in muscle. It increases the rate of receptor desensitization by acetylcholine (Huganir, 1991) and is associated with the clustering of acetylcholine receptors that is induced by motoneuron contact (Baker & Peng, 1993; Peng *et al.* 1993) and by agrin (Wallace *et al.* 1991) and with the induction of receptor synthesis by ARIA (Falls *et al.* 1993). Likewise, tyrosine phosphorylation appears to signal the contact-

mediated regulation of postsynaptic transmitter sensitivity during synapse formation between R and P cells. These observations suggest an important role for tyrosine phosphorylation as a synaptogenic signal at sites of contact between some synaptic partners.

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