Journal of Physiology (1993), **468**, *pp.* 425–439
With 5 figures
Printed in Great Britain

SELECTION OF TRANSMITTER RESPONSES AT SITES OF NEURITE CONTACT DURING SYNAPSE FORMATION BETWEEN IDENTIFIED LEECH NEURONS

BY SHIM CHING, STEFANO CATARSI AND PIERRE DRAPEAU*

From the Centre for Research in Neuroscience and Departments of Biology and Neurology, McGill University and The Montreal General Hospital Research Institute, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4

(*Received 3 September 1992*)

SUMMARY

1. Pressure sensitive (P) neurons of the leech *Hirudo medicinalis* show both an inhibitory, Cl^--dependent response and a depolarizing, cationic response to pipette application of serotonin (5-HT). Serotonergic Retzius (R) neurons in culture reform inhibitory, Cl^--dependent synapses with P neurons but fail to elicit the extrasynaptic, depolarizing response to 5-HT. We have examined the localization of the selection of 5-HT responses by testing the sensitivity of P cell growth cones and neurites to 5-HT application.

2. As measured by intracellular recording at the P cell soma, synaptic release of 5-HT from R cell processes activated only the Cl^--dependent response in P cell neurites. Focal application of 5-HT from a micropipette depolarized uncontacted P cell growth cones and neurites. In contrast, processes from the same P cells that were contacted by R cells were rarely depolarized by 5-HT application unless the application pipette was moved along the neurites away from the sites of contact.

3. The channels underlying the depolarizing response to 5-HT were identified in patch clamp recordings from P cell growth cones. These cation channels showed rare, brief openings in the absence of 5-HT. Application of 5-HT in the bath (outside the patch pipette) increased channel activity in uncontacted P cell growth cones but not in growth cones of the same P cells contacted by R cells.

4. We conclude that the selection of transmitter responses during synapse formation was localized to discrete sites of contact between the synaptic partners.

INTRODUCTION

The ability of a neuron to function as part of a complex network, receiving and communicating information at a multitude of synapses, requires the formation of appropriate connections during development. A variety of factors influence the

* To whom correspondence should be addressed at Department of Neurology, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4.

MS 1748

formation and maintenance of synaptic connections (Easter, Purves, Rakic & Spitzer, 1985). The early establishment of appropriate responses at sites of contact between presynaptic growth cones and neurites of synaptic targets may be crucial in the normal ontogeny of neural systems since competition for synaptic space is high during development (Purves & Lichtman, 1985).

Studies dealing with the specificity of synapse formation must eventually look to the growth cone, the neuronal structure that senses the environment while navigating towards and ultimately contacting its postsynaptic targets. The difficulties in observing the initial synaptogenic events in the developing nervous system are many: growth cones and their sites of contact may be inaccessible for experimental manipulation, the influence of the synaptic elements upon each other may be impossible to separate from other determinants and the cellular identity of growth cones difficult to elucidate in a tissue as complex as the nervous system.

At the accessible neuromuscular junction, the initial events during synapse formation have been established. Growth cones of cholinergic neurons in culture are able to release acetylcholine (Hume, Role & Fischbach, 1983; Young & Poo, 1983) and their efficiency in releasing transmitter increases upon muscle cell contact (Chow, 1989). The presynaptic motoneuron induces acetylcholine receptor (AChR) clustering in muscle cells at sites of contact (Anderson & Cohen, 1977; Bloch & Pumplin, 1988; Berg, Boyd, Halvorsen, Higgins, Jacob & Margiotta, 1989). Concomitantly, the functional properties of AChRs are altered (Katz & Miledi, 1972; Neher & Sakmann, 1976) as a result of the switching of a receptor subunit (Mishina *et al.* 1986). Post-translational changes also occur, as protein kinases regulate desensitization (Huganir & Greengard, 1990) and possibly aggregation of AChRs (Wallace, Qu & Huganir, 1991).

While mature neuromuscular transmission is mediated by a single type of AChR, studies of a variety of preparations show that neurons can display a multitude of responses to one transmitter that are selectively activated by different presynaptic inputs. As first described for *Aplysia* neurons, several different responses are elicited in individual neurons by 5-HT (Gerschenfeld, 1973), only some of which are mediated by any single presynaptic neuron (Gerschenfeld & Paupardin-Tritsch, 1974). Mammalian neurons also exhibit a variety of receptor subtypes whose selective activation depends on the pattern of presynaptic inputs (Nicoll, 1988; Nicoll, Malenka & Kauer, 1990). At a molecular level, neuronal receptors are often composed of several subunits in a variety of combinations responsible for the diversity of receptor subtypes and responses to transmitter in the nervous system (Olsen & Tobin, 1990; Seeburg *et al.* 1990). How appropriate synaptic responses are selected during synaptogenesis remains unknown.

In the simple nervous system of the leech, pressure sensitive (P) neurons *in vivo* and *in vitro* exhibit both inhibitory and excitatory responses to application of 5-HT (Henderson, 1983). Presynaptic input from the serotonergic Retzius (R) neuron activates only the Cl^--dependent hyperpolarizing response in the neuropil (Fuchs, Henderson & Nicholls, 1982). Neurohormonal secretion of 5-HT into the blood sinus bathing the ganglia, which modulates behaviour such as swimming (Kristan & Nusbaum, 1982), may selectively activate extrasynaptic, depolarizing responses in the cell bodies without reaching the synapses buried in the neuropil.

Cultured leech neurons reform specific synapses and are a useful preparation for studying the mechanism of neuronal synaptogenesis (Nicholls, 1987). When R and P somata are juxtaposed in culture where they reform inhibitory synapses, the depolarizing response is absent at synaptic sites and is markedly reduced in the extrasynaptic P cell body (Drapeau & Sanchez-Armass, 1988). Other results indicate that contact with the R cell membrane does not affect Cl^- responses and reduces the cationic response prior to synapse formation (Drapeau, Melinyshyn & Sanchez-Armass, 1989) and that the selection of transmitter responses is restricted to contact specifically with the R cell (Merz & Drapeau, 1992). These results suggest that the loss of the extrasynaptic response occurs upon contact between growth cones and neurites as synapses are formed in the developing neuropil of the ganglion (Drapeau & Sanchez-Armass, 1989).

As a test of this hypothesis, we have used the techniques of intracellular and single channel recording to investigate the selection of 5-HT responses at sites of contact between neurites and growth cones of R and P cells in culture. Our results demonstrate that the selection of 5-HT responses occurs at discrete sites of contact specifically between the synaptic partners.

METHODS

Cultures

R and P cells from the segmental ganglia of the leech *Hirudo medicinalis* (obtained from Ricarimpex, Audenge, France) were isolated and cultured as described previously (Dietzel, Drapeau & Nicholls, 1986). Briefly, ganglia were desheathed with fine forceps and treated with collagenase (Type XI, Sigma Chemical Co. St Louis, MO, USA) and the conspicuous somata of the R and P neurons were removed by aspiration into a micropipette. P cells were cultured singly or were co-cultured with R cells placed a few cell diameters apart on uncoated wells of microtest culture dishes (Nunclon, Denmark) in Leibovitz-15 culture medium (Gibco Canada, Burlington, ON, Canada) supplemented with gentamicin (0·2 mg/ml; Schering Canada Inc. Pointe Claire, Quebec). The cells survived for a few days and most extended neurites tipped with growth cones within a day in culture. To keep the cells alive for longer periods, the medium was supplemented with 2% heat-inactivated fetal bovine serum (Gibco Canada) two days following plating. Neurites continued to grow for at least a week. The neurons could be distinguished easily by their characteristic sizes (40–50 μm diameter for P cells and 60–80 μm for R cells) and the darker appearance of the R cells under phase contrast optics. Experiments were typically conducted 2–7 days following plating.

Solutions

Culture medium was replaced with a normal physiological solution composed of (mM): 155 NaCl, 5 KCl, 1 $MgCl_2$, 1 $CaCl_2$, 10 glucose and 10 Hepes (pH 7·4). For focal application of transmitter, we used pressure ejection (100 ms pulses at 10 psi: Picospritzer, General Valve Corp. Fairfield, NJ, USA) of 50 mM 5-HT (Sigma Chemical Co. USA) dissolved in normal solution (pH 7·4) from a pipette of 70–100 MΩ in resistance (< 1 μm tip). For patch clamp experiments, 5-HT was applied either by superfusion with 30 μM 5-HT in normal solution or by pressure ejection from a capillary tube of 1 μl of a concentrated solution of 5-HT into the 10 μl wells of culture dishes to yield a final 5-HT concentration of 30 μM.

Recordings

Intracellular recordings were made using 20–25 MΩ electrodes pulled from thin wall capillary tubing (1·0 mm o.d.; Frederick Haer Co. Brunswick, ME, USA) filled with 4 M KCl or caesium acetate. Action potentials in R cells were evoked by passing current using a bridge circuit and synaptic potentials in P cells were recorded using an Axoclamp-2a amplifier (Axon Instruments,

Burlingame, CA, USA). Cl^- were injected into the P cells via the KCl recording electrode by passing hyperpolarizing current.

Patches were formed on P cell growth cones using 10–20 MΩ electrodes coated to within 50 μm of their tips with dental wax. Electrodes were filled with filtered normal solution diluted by 10% to improve seal formation. Seal resistances were in the range of 2–10 GΩ. Patch clamp recordings were performed with an Axopatch-1a patch clamp (Axon Instruments). Records were low pass filtered at 1 or 2·5 kHz (−3 dB), as indicated, before digitization (Neuro Data Instruments Corp. New York, NY) and subsequent storage on video tape. For analysis (Sakmann & Neher, 1983), the analog signals were digitized at five times the −3 dB frequency, transferred to a hard disk and analysed off-line either with an LSI-11/73 computer (Digital Equipment Corp. Maynard, MA, USA) and an Indec Systems Interface (Sunnyvale, CA, USA) or an IBM PC compatible with a TTL-1 DMA Interface and the pCLAMP software package (Axon Instruments). Dwell time histograms were analysed in accordance with Sigworth & Sine (1987) in order to facilitate curve fitting. A Colorpro plotter (Hewlett Packard Canada Ltd. Montreal, Quebec) and a QMS-PS410 laser printer (QMS Inc. Mobile AL, USA) were used to obtain hardcopies of the data.

RESULTS

Synapse formation between growth cones and neurites in culture

Isolated leech neuronal somata form selective synapses in the absence of other cell types when the cell bodies are juxtaposed in culture (Nicholls, 1987). We investigated if synapses could also form between neurites of cultured R and P cells. Most of the neurons plated on tissue culture plastic extended fine neurites of a few micrometres in width, tipped with roughly hemispherical growth cones of 5–15 μm in diameter (see Fig. 1). Neurites extended within hours following plating of the cell bodies and continued growing for several days. Similarities in morphology with processes and growth cones extended on an extracellular matrix extract or on the lectin concanavalin A (ConA) as substrate (Chiquet & Nicholls, 1987) were apparent. When the neurites of co-cultured R and P cells contacted one another (Fig. 1), Cl^--dependent inhibitory synapses were observed within a few days in ~ 1/4 of the cell pairs. A train of action potentials elicited by current injection in the presynaptic R cell usually failed to evoke a noticeable response in the P cell recorded at its resting potential of −50 mV (Fig. 1*A*), which is near the Cl^- equilibrium potential (Drapeau & Sanchez-Armass, 1988). While recording with electrodes filled with 4 M KCl, the injection of Cl^- by hyperpolarizing pulses reversed the Cl^- gradient without changing the resting potential and could reveal long-lasting, depolarizing synaptic responses to single action potentials (Fig. 1*B*). The Cl^--dependent synapses formed between R and P cell neurites were similar to those previously characterized for the juxtaposition of somata *in vitro* (Drapeau & Sanchez-Armass, 1988) and in the neuropil *in vivo* (Fuchs *et al.* 1982).

Extrasynaptic response to 5-HT

To measure the extrasynaptic response of P cells to 5-HT, we applied focally a solution of 50 mM 5-HT by pressure ejection from a fine tipped micropipette. The spread of the response to the cell soma was recorded with an intracellular electrode (Henderson, 1983; Pellegrino & Simonneau, 1984). We used caesium acetate electrodes in order to increase the input resistance and to maintain the Cl^- gradient (near the resting potential), thereby permitting the selective detection of the depolarizing, extrasynaptic response to 5-HT. Depolarizing responses were elicited

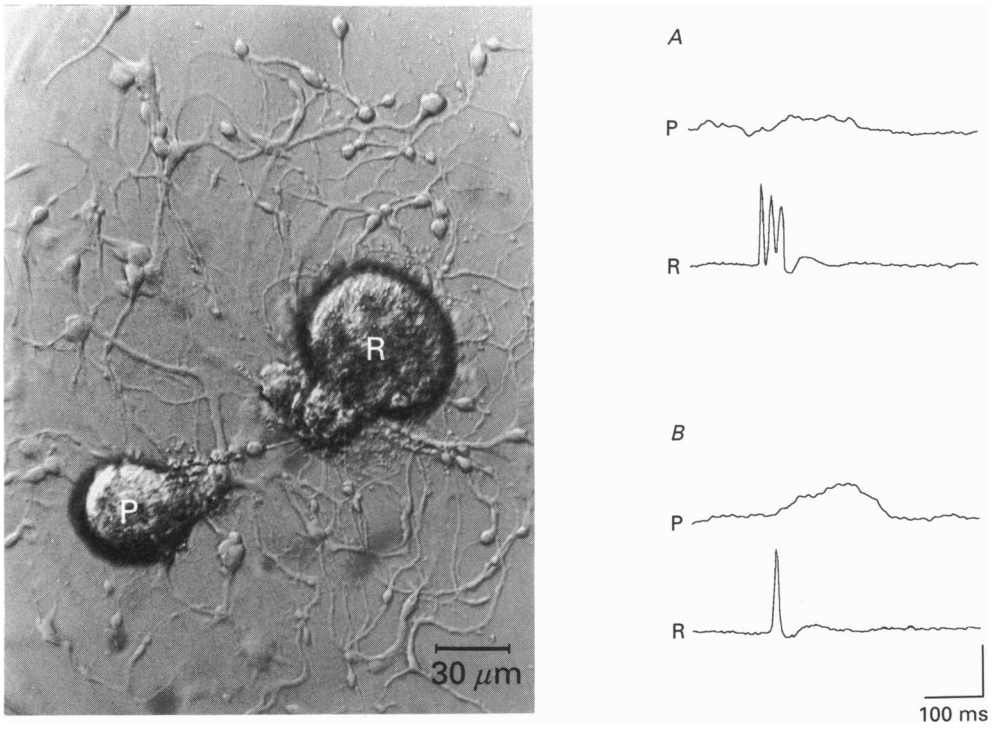

Fig. 1. Cl^--dependent synapses between processes of cultured R and P neurons. The photomicrograph shows representative R and P cells after 7 days in culture and visualized with Hoffman modulation optics. *A*, a train of action potentials elicited in the presynaptic R cell failed to elicit a significant synaptic response in the postsynaptic P cell held at −50 mV. *B*, injection of Cl^- by applying hyperpolarizing current via the 4 M KCl filled recording electrode was able to reverse the Cl^- gradient so that a long-lasting, depolarizing synaptic response to a presynaptic spike was seen. The vertical calibration bar corresponds to 0·5 mV in the P cell recordings and 25 mV in the R cell traces. Three trials were averaged in each recording.

by application of 5-HT to P cell bodies and processes regardless of their length as long as they were not contacted by R cells (Fig. 2). The largest responses were observed in the somata, which often brought the membrane potential to the threshold for an action potential (Fig. 2*A*). The application of 5-HT was highly localized as moving the application pipette more than 20 μm away (e.g. above the cell body or process) resulted in the loss of responsiveness.

Effect of R cell contact on the extrasynaptic response to 5-HT

The size of the extrasynaptic responses varied with the site of 5-HT application, as expected for a differential distribution of receptors for 5-HT on the P cell neurites (Pellegrino & Simonneau, 1984). However, the effect of R cell neurite and growth cone contact on similar structures of the P cell was unambiguous. For the five cell pairs examined, each of the twenty uncontacted P cell processes tested showed a

depolarizing response to 5-HT application of several millivolts in amplitude (Fig. 2*B*). In contrast, nearly all (14 out of 16) contacted processes tested were *not* depolarized by 5-HT application at the site of contact (Fig. 2*C* and *E*).

Contact with growth cones and neurites of another (non-synaptic) P cell did not affect the depolarizing response to 5-HT, consistent with the specific effect of R cell

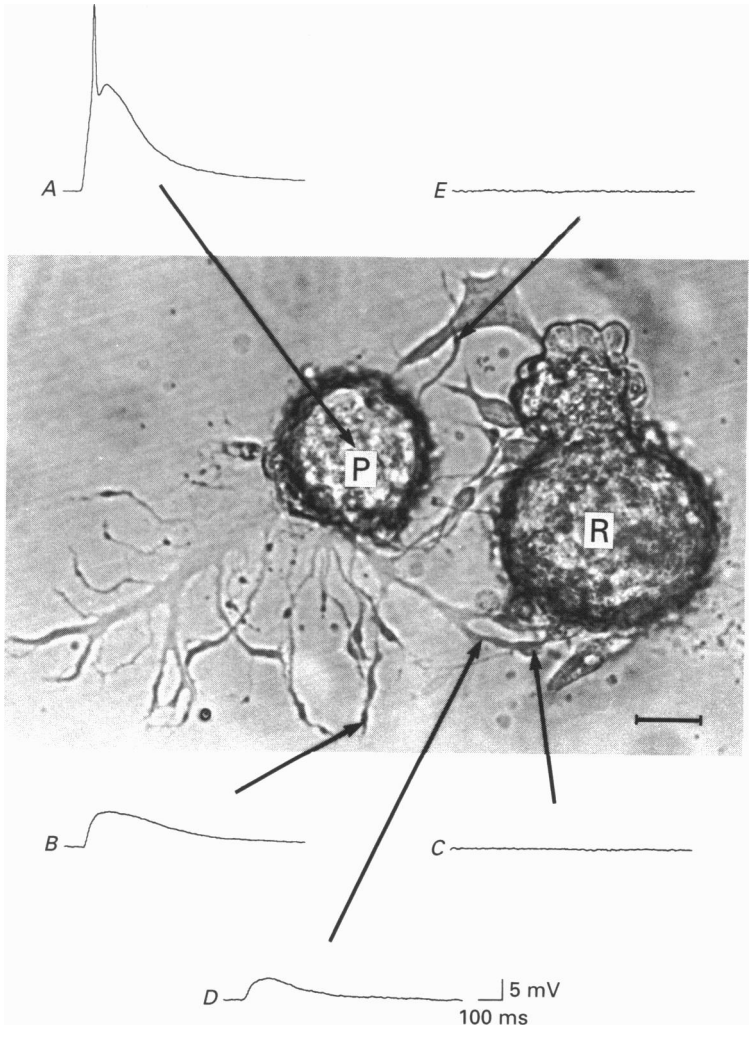

Fig. 2. Extrasynaptic response to 5-HT in P cell neurites and growth cones following R cell contact. The phase-contrast photomicrograph shows an R and P cell pair after 2 days in culture. Intracellular recording with a 4 M caesium acetate-filled electrode measured the responses to focal pressure ejection of 50 mM 5-HT from a micropipette. *A*, application of 5-HT to the P cell soma evoked an action potential. *B*, application of 5-HT to P cell growth cones not contacted by the R cell resulted in a large depolarization. In P cell growth cones contacted by an R cell neurite (*C*) or growth cone (*E*), the depolarizing response to 5-HT was absent. *D*, movement of the application pipette 20 μm proximal to the point of contact of the neurite shown in *C* elicited a depolarizing response.

contact (Merz & Drapeau, 1992) and discounting the effect of contact as merely a diffusional barrier to 5-HT. Application of 5-HT at least 20 μm proximal to the site of contact elicited a depolarizing response (Fig. 2*D*). Because of the limited spatial resolution of 5-HT application, this observation suggests that the selective suppression of the extrasynaptic response to 5-HT was restricted to the discrete sites of contact between the cells.

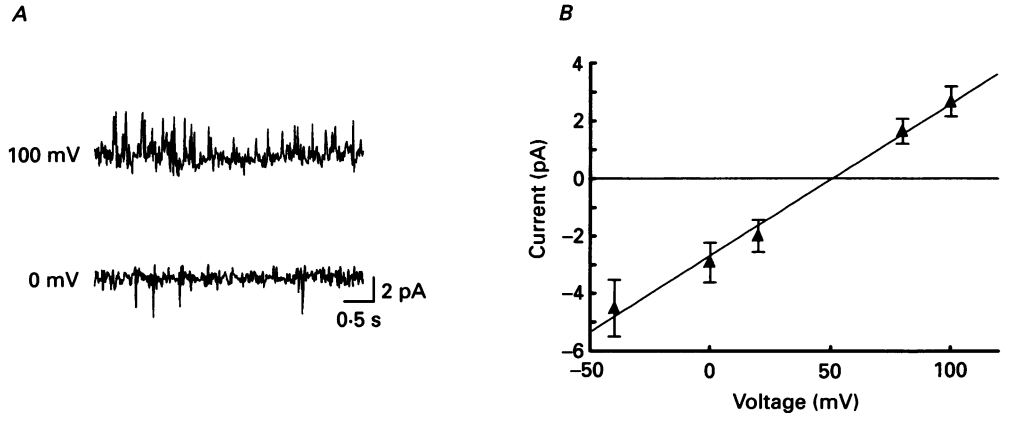

Fig. 3. Current–voltage relationship of the growth cone cation channel. P cell-attached patches were recorded in growth cones of uncontacted P cells. *A*, recordings at the resting potential (0 mV pipette potential) detected inward currents of ~ -3 pA in amplitude (lower trace). The effect of a large depolarization (-100 mV pipette potential) was both to increase the probability of opening and reverse the polarity of the opening events (upper trace). *B*, current–voltage relationship for the same patch held at various potentials. The slope conductance for the line fit by linear regression is 50 pS and the reversal potential is 50 mV from rest. Depolarization represents hyperpolarization of the pipette and downward current transitions correspond to inward currents relative to the membrane. Records were low-pass filtered at 1 kHz (-3 dB) before digitization.

Cation channels activated by 5-HT in P cell growth cones

We sought to determine if R cell contact eliminated 5-HT modulation of the depolarizing channels described in P cell body-attached (Drapeau, 1990) and excised patch clamp studies (Catarsi & Drapeau, 1992). Cell-attached patch clamp recordings (Hamill, Marty, Neher, Sakmann & Sigworth, 1981) of P cell growth cones detected the cation channels underlying the extrasynaptic response to 5-HT (Fig. 3*A*). The channels had properties similar to those characterized in the P cell soma (Drapeau, 1990). The channels had a brief (< 1 ms) mean open time and opened approximately twice per second, with a probability of opening (P_{open}) at rest of $\sim 5 \times 10^{-4}$. Channel activity at the resting potential increased several-fold in the presence of 30 μM 5-HT applied in the bath outside the patch pipettes. This is consistent with 5-HT activation of the second messenger protein kinase C (PKC) as established previously for macroscopic (Sanchez-Armass, Merz & Drapeau, 1991) and single channel currents (Drapeau, 1990; Catarsi & Drapeau, 1992).

We determined the conductance of the growth cone cation channels by holding the patches at varying potentials. Depolarization of the patches reversed the current

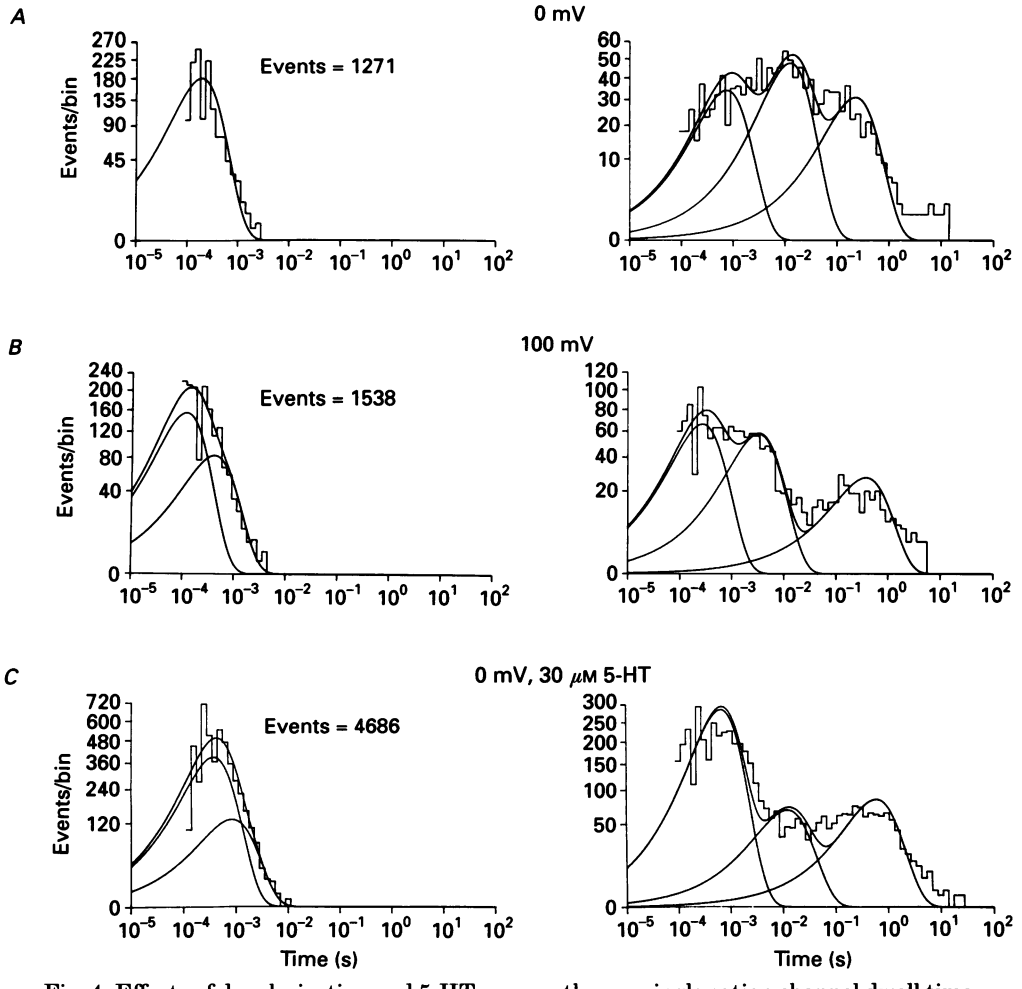

Fig. 4. Effects of depolarization and 5-HT on growth cone single cation channel dwell time distributions. Records were low-pass filtered at 2·5 kHz (−3 dB) before digitization. This patch appeared to contain only one channel, as estimated from the approximation:

$$\text{Probability of 2 channels} = 1/[1+(P_{\text{open}}/2)]^{\text{events}} = 2{\cdot}6 \times 10^{-4},$$

so that the probability of a single channel was approximately 99·97%. The durations of the events (openings on the left, closures on the right) were binned logarithmically (10 bins per decade). The number of events in each bin is plotted on a square root scale against the logged bin sizes (Sigworth & Sine, 1987). The smooth curves are the individual exponential fits used to generate the probability density functions, which are displayed as the enveloping curves. *A* shows recordings under control conditions at the resting potential, *B* represents events recorded at 100 mV depolarized from rest, and results in *C* were obtained following bath application of 30 μM 5-HT at the resting potential. The time constants and fractions of the events used for least square fitting of each component in the histograms were: *A*, open time, 0·19 ms and 1·0; *A*, closed time 0·75 ms and 0·30, 12·6 ms and 0·42, 224 ms and 0·28; *B*, open time 0·11 ms and 0·65, 0·40 ms and 0·35; *B*, closed time 0·28 ms and 0·44, 3·4 ms and 0·38, 376 ms and 0·18; *C*, open time 0·38 ms and 0·75, 0·84 ms and 0·25; *C*, closed time 0·67 ms and 0·65, 12·6 ms and 0·16, 631 ms and 0·19. The total number of events is indicated for each pair of histograms.

polarity and increased channel activity (Fig. 3*A*, upper trace). The current–voltage relationship for the growth cone cation channel is shown in Fig. 3*B*. The mean current amplitude estimated from Gaussian fits to opening events at various holding potentials indicated a reversal potential of ~ 50 mV depolarized from rest and an ohmic conductance of ~ 50 pS. These results are consistent with both the reversal

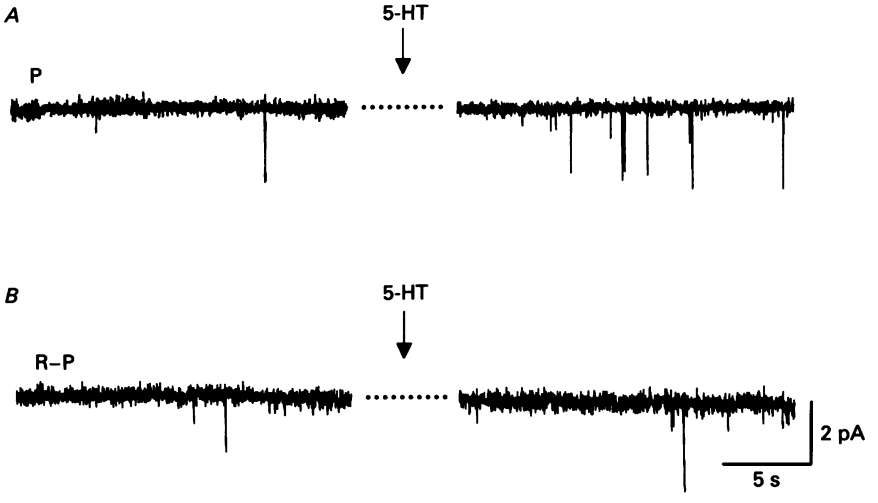

Fig. 5. Modulatory effects of 5-HT on cation channel activity in uncontacted and contacted growth cones. In a P cell growth cone not contacted by an R cell (*A*), addition (outside the patch) of 30 μM 5-HT increased the probability of cation channel opening, as represented in the upper traces. In contrast, as shown in *B*, the activity of cation channels in a P cell growth cone of the same cell contacted by an R cell (R–P) was unaffected by the addition of 5-HT.

potential of the macroscopic cation current activated by 5-HT in P cells (Drapeau & Sanchez-Armass, 1988) and the conductance of the soma cation channel (Drapeau, 1990; Catarsi & Drapeau, 1992). However, channels in several patches, particularly from small growth cones, had conductances as low as half that seen in Fig. 3. Assuming the same specific membrane resistivity for growth cones as observed for somata, we estimate that the resistance of small growth cones may be as high as that of the open channels and could thereby have impeded current flow in some recordings, resulting in an underestimate of channel conductance. The reversal potential for single channel currents and the voltage responses to 5-HT application described above are consistent with a normal resting potential in the growth cones.

Further evidence for the identity of growth cone cation channels was found in the dwell time distributions for a recording from an uncontacted P cell growth cone patch containing only one channel. At rest (in the absence of 5-HT), the probability density function for the open time distribution could be fitted with a single exponential with a time constant of 0·19 ms, while the closed time distribution required a minimum of three exponentials (Fig. 4*A*). Depolarizing holding potentials or bath application of 30 μM 5-HT revealed a second class of longer duration openings and reduced the value of the time constants of the closed time distribution exponentials (Fig. 4*B* and

C). Similar dwell time distributions and sensitivity to depolarization and 5-HT were reported for P cell body-attached patch clamp recordings (Drapeau, 1990). Combined with the results described above, these observations reliably identified the cation channels in growth cone patches.

Effect of R cell contact on cation channel modulation

By applying 5-HT in the bath while recording from P cell-attached growth cone patches, we were able to study the effect of R cell contact on 5-HT modulation of cation channel activity. Typical records obtained from an uncontacted growth cone in the presence and absence of 5-HT are shown in Fig. 5*A*. Application of 5-HT increased P_{open} 5·5 ± 1·5-fold (mean ± S.E.M. $n = 9$) over control. In a contacted growth cone of the same P cell (Fig. 5*B*), 5-HT failed to increase P_{open}. On average, channel activity in contacted growth cones was not significantly affected by 5-HT, displaying a mean 1·1 ± 0·1-fold (mean ± S.E.M. $n = 6$) increase from control. For the pooled data, the difference in 5-HT activation between uncontacted and contacted growth cones was statistically significant ($P < 0·001$, two-tailed Student's t test). This localization of the loss of response to individual, contacted growth cones was seen in all experiments. Additionally, the fraction of patches containing channels suggested no reduction in the number of channels in uncontacted (15/38 patches or 40%) compared with contacted growth cones (13/28 patches or 46%). Thus, contact with the R cell reduced channel activation by 5-HT but not channel density.

DISCUSSION

R and P cells formed synapses between their processes that were analogous to those reported for cell body pairing *in vitro* and between similar processes in the neuropil *in vivo* (Fuchs *et al.* 1982). Voltage clamp studies of the more accessible synapses formed between juxtaposed somata have shown that the synaptic responses are due to activation of a long-lasting Cl^- current (Drapeau & Sanchez-Armass, 1988) which is mediated by protein kinase A (Sanchez-Armass *et al.* 1991). Synapses formed within a few days following contact between outgrowing neurites, consistent with the delay observed for synapse formation between juxtaposed cell bodies. Connections have been reported to form within hours of contact between the axonal stumps (Liu & Nicholls, 1989). However, we observed a lower efficiency of synapse formation between neurites as compared to juxtaposed somata. Some synapses may not have been detected due to the electrotonic decay of the responses recorded in the remote cell bodies.

Our results show that the selection of transmitter responses between R and P cells occurred at sites of contact between growth cones and neurites, presumably the sites at which synapses are later formed. By the localized application of 5-HT, we resolved a response that was eliminated solely at regions of contact between the synaptic pair, thus leaving extrasynaptic regions in the same P cells unaffected. Furthermore, single channel recordings demonstrated that cation channels were present in both contacted and uncontacted P cell growth cones but that 5-HT no longer modulated channel activity in patches from contacted growth cones. P cell body-attached

(Drapeau, 1990) and excised membrane patch clamp studies (Catarsi & Drapeau, 1992) have shown that the loss of the extrasynaptic response to 5-HT is due to the failure of PKC to activate the channels following neuronal contact.

In analogy with the detailed studies of synaptogenesis at the neuromuscular junction, where subunit switching alters AChR activity during development (Mishina *et al.* 1986), our findings suggest that a modification of transmitter-activated channel properties also occurs during neuronal synapse formation. However, as discussed below, several important differences were observed in the synaptogenic interactions between cultured leech neurons.

The preferential insertion of AChR subunits in muscle cells at sites of motoneuron contact occurs by the targeting of transcriptional products from subsynaptic nuclei (Witzemann, Brenner & Sakmann, 1991). The finding that Cl^--dependent synapses between R and P cells develop in culture within hours of contact between cell bodies or axon stumps (Liu & Nicholls, 1987) suggests a more rapid modification than the changing of subunit composition. Since protein kinases regulate desensitization (Huganir & Greengard, 1990) and possibly clustering (Wallace *et al.* 1991) of AChRs, covalent modification of the extrasynaptic channels in P cells may be similarly induced by neuronal contact.

At the neuromuscular junction, factors putatively secreted from nerve endings, such as agrin (Nitkin *et al.* 1987), ARIA (Falls, Harris, Johnson, Morgan, Gorfas & Fischbach, 1990) and calcitonin gene related peptide (New & Mudge, 1986; Fontaine, Klarsfeld, Hokfelt & Changeux, 1986), alter AChR subunit expression and aggregation. Contact with the R cell but not other neurons, including *non-synaptic* serotonergic cells, induces the loss of cation current modulation in the P cell (Merz & Drapeau, 1992). Furthermore, aldehyde-fixed R cells can still reduce the extrasynaptic response to 5-HT (Drapeau *et al.* 1989) and treatment with trypsin or wheat germ agglutinin prevents the effect of the R cell (Merz & Drapeau, 1991). These results suggest that an R cell-specific surface molecule induces the loss of extrasynaptic channel modulation in the P cell. Interestingly, surface molecules have been implicated in inducing the choice of cell fates during development (Greenwald & Rubin, 1992). Our results suggest that neuronal contact may play a similar role in inducing functional changes during synaptogenesis.

In contrast to the innervation of polynucleated muscle cells by a single nerve fibre, the events underlying neuronal synaptogenesis are likely to differ significantly. The formation of synapses at extensive distances away from the cell body requires locally acting cues between appropriate partners. The localization of transmitter receptors and ion channels at distinct cellular sites is a critical aspect of neuronal development that generates the requisite subcellular machinery underlying proper physiological function (Hille, 1992). For example, the distribution of glycine receptors in rat spinal cord is confined to postsynaptic complexes (Triller, Cluzeaud, Pfeiffer, Betz & Korn, 1985). Similarly, $GABA_A$ receptor subunit distribution in developing hippocampal neurons in culture becomes localized from a widespread cellular distribution to only the somata and dendrites (Killisch, Dotti, Laurie, Luddens & Seeburg, 1991). Distinct from the clustering of receptor/channel complexes, our observations indicate that transmitter responses can also be localized by the selective loss of channel modulation at sites of neuronal contact. This mechanism may be particularly

important for localizing the responses of modulatory transmitters such as 5-HT which act through second messengers (Peroutka, 1988).

In addition to modifying postsynaptic responses, neuronal contact also alters presynaptic properties. For example, an increase in acetylcholine release from growth cones of cultured *Xenopus* motoneurons is observed following contact with muscle cells (Chow, 1989). Furthermore, appropriate *Helisoma* neurons in culture show an increase in synaptic efficacy resulting from postsynaptic contact, an effect which also seems to be localized to only contacted growth cones (Haydon & Zoran, 1989).

While the presence of ion channels in growth cones has been previously documented in several preparations (O'Lague, Huttner, Vandenberg, Morrison-Graham & Horn, 1985; Belardetti, Schacher & Siegelbaum, 1986; Lipscombe, Madison, Poenie, Reuter, Tsien & Tsien, 1988), the changes that these channels undergo during their interaction with the extracellular environment are not well understood. Calcium channels in growth cones of *Helisoma* neurons differ between growing and non-growing states (Haydon, Cohan, McCobb, Miller & Kater, 1985). Exposure to 5-HT raises intracellular calcium levels and inhibits neurite outgrowth by causing growth cone collapse in neuron B19 of *Helisoma* (Haydon, McCobb & Kater, 1984). Calcium channel density in sprouting PC12 cells is high in the growth cone membrane while low in the neurite shaft (Streit & Lux, 1989). The distribution of calcium channels in processes of cultured leech neurons appears to be influenced by growth upon different substrates (Ross, Arechiga & Nicholls, 1987). Our findings present a modification of channel properties triggered by specific neuronal contact that results in the altering of growth cone physiology during synaptogenesis.

Synaptogenesis results from a complex series of events that begins with process outgrowth and ends with the formation and refinement of connections between neurons. The rearrangement of synaptic connections through competition is an important step during development of both vertebrate (Hubel, Wiesel & LeVay, 1977) and invertebrate (Gao & Macagno, 1987*a*,*b*) nervous systems. By investigating the changes that occur at the level of potential synaptic sites during the formation of specific synapses between two identified neurons, we have established that neuronal contact triggers early functional events during synapse formation. Neurite outgrowth and subsequent contact in the neuropil would lead to the loss of the excitatory, extrasynaptic response at sites of contact so that newly formed synapses would only activate the inhibitory, synaptic response to 5-HT. At such a time, when synaptic competition is high (Purves & Lichtman, 1985), the selection of transmitter responses could be advantageous for the formation of appropriate connections following neuronal contact. While coincident pre- and postsynaptic activity is thought to be critical for the stabilization of excitatory synapses (Shatz, 1990), the sorting of inhibitory connections may depend on activity-independent interactions such as neuronal contact.

This work was supported by a postdoctoral award from the CNR of Italy (S. Catarsi) and an award from the FRSQ and grants from the MRC and FCAR of Canada (P. Drapeau).

REFERENCES

ANDERSON, M. J. & COHEN, M. W. (1977). Nerve induced and spontaneous redistribution of acetylcholine receptors on cultured muscle cells. *Journal of Physiology* **268**, 757–773.

BELARDETTI, F., SCHACHER, S. & SIEGELBAUM, S. A. (1986). Action potentials, macroscopic and single channel currents recorded from growth cones of *Aplysia* neurones in culture. *Journal of Physiology* **374**, 289–313.

BERG, D. K., BOYD, R. T., HALVORSEN, S. W., HIGGINS, L. S., JACOB, M. H. & MARGIOTTA, J. F. (1989). Regulating the number and function of neuronal acetylcholine receptors. *Trends in Neurosciences* **12**, 16–21.

BLOCH, R. J. & PUMPLIN, D. W. (1988). Molecular events in synaptogenesis: nerve–muscle adhesion and postsynaptic differentiation. *American Journal of Physiology* **254**, C345–364.

CATARSI, S. & DRAPEAU, P. (1992). Loss of extrasynaptic channel modulation by protein kinase C underlies the selection of serotonin responses in an identified leech neuron. *Neuron* **8**, 275–281.

CHIQUET, M. & NICHOLLS, J. G. (1987). Neurite outgrowth and synapse formation by identified leech neurones in culture. *Journal of Experimental Biology* **132**, 191–206.

CHOW, I. (1989). Cell–cell interaction during synaptogenesis. *Journal de Physiologie* **84**, 121–127.

DIETZEL, I. D., DRAPEAU, P. & NICHOLLS, J. G. (1986). Voltage dependence of 5-hydroxytryptamine release between identified leech neurons in culture. *Journal of Physiology* **372**, 191–205.

DRAPEAU, P. (1990). Loss of channel modulation by transmitter and protein kinase C during innervation of an identified leech neuron. *Neuron* **4**, 875–882.

DRAPEAU, P., MELINYSHYN, E. & SANCHEZ-ARMASS, S. (1989). Contact-mediated loss of the non-synaptic response to transmitter reinnervation of an identified leech neuron in culture. *Journal of Neuroscience* **9**, 2502–2508.

DRAPEAU, P. & SANCHEZ-ARMASS, S. (1988). Selection of postsynaptic serotonin receptors during reinnervation of an identified leech neuron in culture. *Journal of Neuroscience* **8**, 4718–4727.

DRAPEAU, P. & SANCHEZ-ARMASS, S. (1989). Parallel processing and selection of the responses to serotonin during reinnervation of an identified leech neuron in culture. *Journal of Neurobiology* **20**, 312–325.

EASTER, S. S. JR, PURVES, D., RAKIC, P. & SPITZER, N. C. (1985). The changing view of neural specificity. *Science* **230**, 507–511.

FALLS, D. L., HARRIS, D. A., JOHNSON, F. A., MORGAN, M. M., GORFAS, G. & FISCHBACH, G. (1990). M_r 42,000 ARIA: a protein that may regulate the accumulation of acetylcholine receptors at developing chick neuromuscular junctions. *Cold Spring Harbor Symposia in Quantitative Biology* **55**, 397–406.

FONTAINE, B., KLARSFELD, A., HOKFELT, T. & CHANGEUX, J. P. (1986). Calcitonin gene-related peptide, a peptide present in spinal cord motoneurons, increases the number of acetylcholine receptors in primary cultures of chick embryo myotubes. *Neuroscience Letters* **71**, 59–64.

FUCHS, P. A., HENDERSON, L. P. & NICHOLLS, J. G. (1982). Chemical transmission between individual Retzius and sensory neurones of the leech in culture. *Journal of Physiology* **323**, 195–210.

GAO, W.-Q. & MACAGNO, E. R. (1987*a*). Extension and retraction of axonal projections by some developing neurons in the leech depend upon the existence of neighbouring homologues. I. The HA cells. *Journal of Neurobiology* **18**, 43–59.

GAO, W.-Q. & MACAGNO, E. R. (1987*b*). Extension and retraction of axonal projections by some developing neurons in the leech depend upon the existence of neighbouring homologues. II. The AP and AE neurons. *Journal of Neurobiology* **18**, 295–313.

GERSCHENFELD, H. M. (1973). Chemical transmission in invertebrate central nervous systems and neuromuscular junctions. *Physiological Reviews* **53**, 1–119.

GERSCHENFELD, H. M. & PAUPARDIN-TRITSCH, D. (1974). On the transmitter function of 5-hydroxytryptamine at excitatory and inhibitory monosynaptic junctions. *Journal of Physiology* **243**, 457–481.

GREENWALD, I. & RUBIN, G. M. (1992). Making a difference: the role of cell–cell interactions in establishing separate identities for equivalent cells. *Cell* **68**, 271–281.

HAMILL, O. P., MARTY, A., NEHER, E., SAKMANN, B. & SIGWORTH, F. J. (1981). Improved patch-

clamp techniques for high-resolution current recording from cells and cell-free membrane patches. *Pflügers Archiv* **391**, 85–100.

HAYDON, P. G., COHAN, C. S., MCCOBB, D. P., MILLER, H. R. & KATER, S. B. (1985). Neuron-specific growth cone properties as seen in identified neurons of *Helisoma*. *Journal of Neuroscience Research* **13**, 135–147.

HAYDON, P. G., MCCOBB, D. P. & KATER, S. B. (1984). Serotonin selectively inhibits growth cone motility and synaptogenesis of specific identified neurons. *Science* **226**, 561–564.

HAYDON, P. G. & ZORAN, M. J. (1989). Formation and modulation of chemical connections: evoked acetylcholine release from growth cones and neurites of specific identified neurons. *Neuron* **2**, 1483–1490.

HENDERSON, L. P. (1983). The role of 5-hydroxytryptamine as a transmitter between identified leech neurones in culture. *Journal of Physiology* **339**, 309–324.

HILLE, B. (1992). *Ionic Channels of Excitable Membranes*. Sinauer Associates, Inc. Sunderland, MA, USA.

HUBEL, D. H., WIESEL, T. N. & LEVAY, S. (1977). Plasticity of ocular dominance columns in the monkey striate cortex. *Philosophical Transactions of the Royal Society* B **278**, 377–409.

HUGANIR, R. L. & GREENGARD, P. (1990). Regulation of neurotransmitter receptor desensitization by protein phosphorylation. *Neuron* **5**, 555–567.

HUME, R. I., ROLE, L. W. & FISCHBACH, G. D. (1983). Acetylcholine release from growth cones detected with patches of acetylcholine receptor-rich membranes. *Nature* **305**, 632–634.

KATZ, B. & MILEDI, R. (1972). The statistical nature of the acetylcholine potential and its molecular components. *Journal of Physiology* **224**, 665–699.

KILLISCH, I., DOTTI, C. G., LAURIE, D. J., LUDDENS, H. & SEEBURG, P. H. (1991). Expression patterns of $GABA_A$ receptor subtypes in developing hippocampal neurons. *Neuron* **7**, 927–936.

KRISTAN, W. B. & NUSBAUM, M. P. (1982). The dual role of serotonin in leech swimming. *Journal de Physiologie* **78**, 743–747.

LIPSCOMBE, D., MADISON, D. V., POENIE, M., REUTER, H., TSIEN, R. Y. & TSEIN, R. W. (1988). Spatial distribution of calcium channels and cytosolic calcium transients in growth cones and cell bodies of sympathetic neurons. *Proceedings of the National Academy of Sciences of the USA* **85**, 2398–2402.

LIU, Y. & NICHOLLS, J. G. (1989). Steps in the development of electrical and chemical synapses by pairs of identified leech neurons in culture. *Proceedings of the Royal Society* B **236**, 253–268.

MERZ, D. C. & DRAPEAU, P. (1991). Cell-specific surface component selects the response to transmitter during synapse formation between identified leech neurons. *Neuroscience Abstracts* **17**, 736.

MERZ, D. C. & DRAPEAU, P. (1992). Cell-specific contact selects responses to transmitter in an identified leech neuron. *Proceedings of the Royal Society* B **248**, 129–133.

MISHINA, M., TAKAI, T., IMOTO, K., NODA, M., TAKAHASHI, T., NUMA, S., METHFESSEL, C. & SAKMANN, B. (1986). Molecular distinction between fetal and adult forms of muscle acetylcholine receptor. *Nature* **321**, 406–411.

NEHER, E. & SAKMANN, B. (1976). Noise analysis of drug induced voltage clamp currents in denervated frog muscle fibres. *Journal of Physiology* **258**, 705–729.

NEW, H. V. & MUDGE, A. W. (1986). Calcitonin gene-related peptide regulates muscle acetylcholine receptor synthesis. *Nature* **323**, 809–811.

NICHOLLS, J. G. (1987). The search for connections: studies of regeneration in the nervous system of the leech. In *Magnes Lecture Series*, vol. **2**, pp. 1–86. Sinauer Associates Inc., Sunderland, MA, USA.

NICOLL, R. A. (1988). The coupling of neurotransmitter receptors to ion channels in the brain. *Science* **241**, 545–551.

NICOLL, R. A., MALENKA, R. C. & KAUER, J. A. (1990). Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiological Reviews* **70**, 513–565.

NITKIN, R. M., SMITH, M. A., MAGILL, C., FALLON, F. R., YAO, Y. M. M., WALLACE, B. G. & MCMAHAN, U. J. (1987). Identification of agrin, a synaptic organizing protein from *Torpedo* electric organ. *Journal of Cell Biology* **105**, 2471–2478.

O'LAGUE, P. H., HUTTNER, S. L., VANDENBERG, C. A., MORRISON-GRAHAM, K. & HORN, R. (1985). Morphological properties and membrane channels of growth cones induced in PC12 cells by nerve growth factor. *Journal of Neuroscience Research* **13**, 301–321.

OLSEN, R. W. & TOBIN, A. J. (1990). Molecular biology of $GABA_A$ receptors. *FASEB Journal* **4**, 1469–1480.

PELLEGRINO, M. & SIMONNEAU, M. (1984). Distribution of receptors for acetylcholine and 5-hydroxytryptamine on identified leech neurones growing in culture. *Journal of Physiology* **352**, 669–684.

PEROUTKA, S. J. (1988). 5-Hydroxytryptamine receptor subtypes. *Annual Review of Neuroscience* **11**, 45–60.

PURVES, D. & LICHTMAN, J. W. (1985). *Principles of Neural Development*. Sinauer Associates Inc. Sunderland, MA, USA.

ROSS, W. N., ARECHIGA, H. & NICHOLLS, J. G. (1987). Influence of substrate on distribution of calcium channels in identified leech neurons in culture. *Proceedings of the National Academy of Sciences of the USA* **85**, 4075–4078.

SAKMANN, B. & NEHER, E. (1983). *Single Channel Recording*. Plenum, New York, USA.

SANCHEZ-ARMASS, S., MERZ, D. C. & DRAPEAU, P. (1991). Distinct receptors, second messengers and conductances underlying the dual responses to serotonin in an identified leech neuron. *Journal of Experimental Biology* **155**, 531–547.

SEEBURG, P. H., WISDEN, W., VERDOORN, T. A., PRITCHETT, D. B., WERNER, P., HERB, A., LÜDDENS, H., SPRENGEL, R. & SAKMANN, B. (1990). The $GABA_A$ receptor family: molecular and functional diversity. *Cold Spring Harbor Symposia in Quantitative Biology* **55**, 29–40.

SHATZ, C. J. (1990). Impulse activity and the patterning of connections during CNS development. *Neuron* **5**, 745–756.

SIGWORTH, F. J. & SINE, S. M. (1987). Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophysical Journal* **52**, 1047–1054.

STREIT, J. & LUX, H. D. (1989). Distribution of calcium currents in sprouting PC12 cells. *Journal of Neuroscience* **9**, 4190–4199.

TRILLER, A., CLUZEAUD, F., PFEIFFER, F., BETZ, H. & KORN, H. (1985). Distribution of glycine receptors at central synapses: an immunoelectron microscopy study. *Journal of Cell Biology* **101**, 683–688.

WALLACE, B. G., QU, Z. & HUGANIR, R. L. (1991). Agrin induces phosphorylation of the nicotinic acetylcholine receptor. *Neuron* **6**, 869–878.

WITZEMANN, V., BRENNER, H.-R. & SAKMANN, B. (1991). Neural factors regulate AChR subunit mRNAs at rat neuromuscular synapses. *Journal of Cell Biology* **114**, 125–141.

YOUNG, S. H. & POO, M.-M. (1987). Spontaneous release of neurotransmitter from growth cones of embryonic neurons. *Nature* **305**, 634–637.