FOREIGN CONNECTIONS ARE FORMED *IN VITRO* BY *APLYSIA CALIFORNICA* INTERNEURONE L10 AND ITS *IN VIVO* FOLLOWERS AND NON-FOLLOWERS

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Summary

Aplysia californica interneurone L10 forms a set of presynaptic connections with many postsynaptic 'follower' cells in the abdominal ganglion. These followers do not connect back to L10. The present study tests whether the direction and sign of these connections are obligatory and are reconstructed when neuronal processes regenerate *in vitro*. L10 was co-cultured with one of six different followers and two non-followers.

1. *In vitro* connections that preserve the sign of those formed *in vivo* were made by L10 onto neurones L11, L12 and L13. The connections consisted of inhibitory postsynaptic potentials (IPSPs) with characteristic fast and slow components.

2. *In vitro* connections that did not preserve the sign of connections found *in vivo* were made by L10 onto R15, R16 and L7. Neurones R15 and R16 receive excitatory inputs from L10 *in vivo* and L7 receives a dual-action input *in vivo*, with inhibition followed by excitation. A purely inhibitory connection from L10 was formed *in vitro* onto all these cells.

3. Connections that have never been observed *in vivo* in terms of both direction and sign were formed *in vitro*. Followers L7, L11, L12, L13 and R16 and non-follower L14A formed novel connections onto L10. All these connections were inhibitory and some were strong. For example, IPSPs with a magnitude of 20 mV were observed in L10 following a single action potential in L13.

Our results show that identified *Aplysia* neurones can form stereotyped specific connections *in vitro*. The specificity is different from that in the intact ganglion. The ubiquity of novel connections suggests that restrictions imposed on synaptogenesis in the animal are distinct from those regulating synapse formation in culture.

Introduction

A prominent feature of invertebrate nervous systems is the invariance of their neuronal connectivity. Different animals of the same species display stereotyped synaptic connections between identified cells. This anatomical simplicity has prompted the use of invertebrates in the search for functionally defined neuronal circuits. Indeed, much of our current understanding of patterned motor output derives from studies of identified sets of invertebrate neurones that are activated during breathing, locomotion or ingestive behaviour (for a recent review see Getting, 1989). Functional circuits associated with behaviour such as learning and memory are also accessible in the invertebrate nervous system (for a recent review see Byrne, 1987).

The invariance of neuronal connections in invertebrates suggests that the synaptic specificity may be obligatory. The present work addresses this hypothesis by constructing small circuits *in vitro* with identified neurones from *Aplysia californica*. This approach follows the work of Ready and Nicholls (1979), who showed that identified neurones from the leech can establish chemical connections *in vitro*. The techniques of invertebrate cell culture have since been extended to other preparations, notably *Aplysia californica* (Kaczmarek *et al.* 1979; Dagan and Levitan, 1981; Schacher and Proshansky, 1983), the pond snail *Helisoma trivolvis* (Wong *et al.* 1981), the pond snail *Lymnaea stagnalis* (Kostenko *et al.* 1983), the squid *Loligo pealei* (Parsons and Chow, 1989) and to crustaceans (Cooke *et al.* 1989). Co-cultures of identified neurones have been used to study the development of electrotonic connections (Hadley *et al.* 1983; Bodmer *et al.* 1984), the development and physiology of chemical synapses (Fuchs *et al.* 1981; Fuchs *et al.* 1982; Camardo *et al.* 1983; Haydon, 1988) and mechanisms for short-term and long-term synaptic modification (Rayport and Schacher, 1986; Montarolo *et al.* 1986; Dale *et al.* 1988). Recently, co-cultured invertebrate neurones have been used to examine emergent properties of neuronal circuits (Kleinfeld *et al.* 1990; Parsons *et al.* 1989a; Syed *et al.* 1989).

Previous studies have shown that connections not found *in vivo* can occur *in vitro*. Nicholls and coworkers used co-cultured identified leech cells and found that many cells formed connections *in vitro* that largely resembled those in the ganglion, while others formed novel, albeit weak, synapses in culture (Fuchs *et al.* 1981; Fuchs *et al.* 1982; Arechiga *et al.* 1986; Vyklicky and Nicholls, 1988). In co-cultures of *Aplysia* neurones, Schacher *et al.* (1985) found that giant neurone R2 formed novel connections *in vitro*, including a connection from R2 to interneurone L10. Novel connections among co-cultured left upper quadrant (LUQ) neurones (Parsons *et al.* 1989) and other identified neurones (Kleinfeld *et al.* 1990) have also been observed. In contrast, Schacher and coworkers found that the synaptic specificity of L10 for three groups of cells, i.e. LUQ cells, right upper quadrant (RUQ) cells and R14, was maintained *in vitro* (Camardo *et al.* 1983; Schacher, 1988). The results for *Aplysia* suggest that L10 may be unusual in its ability to maintain specificity in culture.

Here we examine whether the preservation of synaptic specificity of L10 is

common or is exceptional in co-cultures with other *Aplysia* neurones. The systematic study of this question is facilitated by the results of recent studies of the cellular and synaptic properties of L10 *in situ* (Frazier *et al.* 1967; Kandel *et al.* 1967; Koester and Kandel, 1977; Koester and Alevizos, 1989). In particular: (i) L10 makes connections onto a large number of cells *in vivo*; many of these are followers and do not make connections back onto L10; (ii) the sign of the postsynaptic response is different for different followers; (iii) many followers are relatively large and thus amenable to isolation and study in culture.

We assayed the connections formed by co-cultured pairs of neurones, consisting of L10 and a follower or non-follower. Two additional co-cultures that did not involve L10 were examined in conjunction with these studies. Our focus was on describing the patterns of synaptic specificity *in vitro*. Biophysical aspects of the novel connections are not emphasized in the present work.

Preliminary reports of this work have been presented (Kleinfeld *et al.* 1988; Obaid *et al.* 1989; Parsons *et al.* 1989*b*).

Materials and methods

Culture techniques

Juvenile *Aplysia californica* (1–10 g; stage 12), raised in mariculture, were either a gift of the Howard Hughes Medical Institute (Woods Hole, MA) or purchased from Sea Life Supply (Sand City, CA). Identified neurones in the abdominal ganglion were located by their relative position and size and by the morphology of their axonal branching patterns (Frazier *et al.* 1967; Kandel *et al.* 1967). Neurones were isolated and maintained in cell culture as described earlier (Dagan and Levitan, 1981; Schacher and Proshansky, 1983; Kleinfeld *et al.* 1990). Pairs of neurones were plated on the same day with their axons crossed approximately 200 μm below the soma. This procedure ensured that the neurones remained in close proximity prior to their attachment to the culture dish. The morphology and relative position of the cells was sketched at the time of each plating. Extensive outgrowth was present 2–3 days after plating, as shown for co-cultured L10 and R15 in Fig. 1. Co-cultures in which either cell lost its axonal segment were rejected for further study; such cells may be incapable of forming chemical connections (Schacher *et al.* 1985; Kleinfeld *et al.* 1990).

A potential systematic error in our experiments arose from incorrect identification of neurones. This error was judged to be negligible for particularly distinctive cells such as L7 and R15. For other neurones this error was estimated to be no more than 10%.

Electrophysiology

Standard current-clamp methods were used. Neurones were penetrated with single-barrel microelectrodes filled with 4 mol l^{-1} potassium acetate at their tips and backfilled with 3 mol l^{-1} KCl ($R_E = 15$–20 MΩ). Electrotonic coupling between neurones was measured in response to small, hyperpolarizing currents as
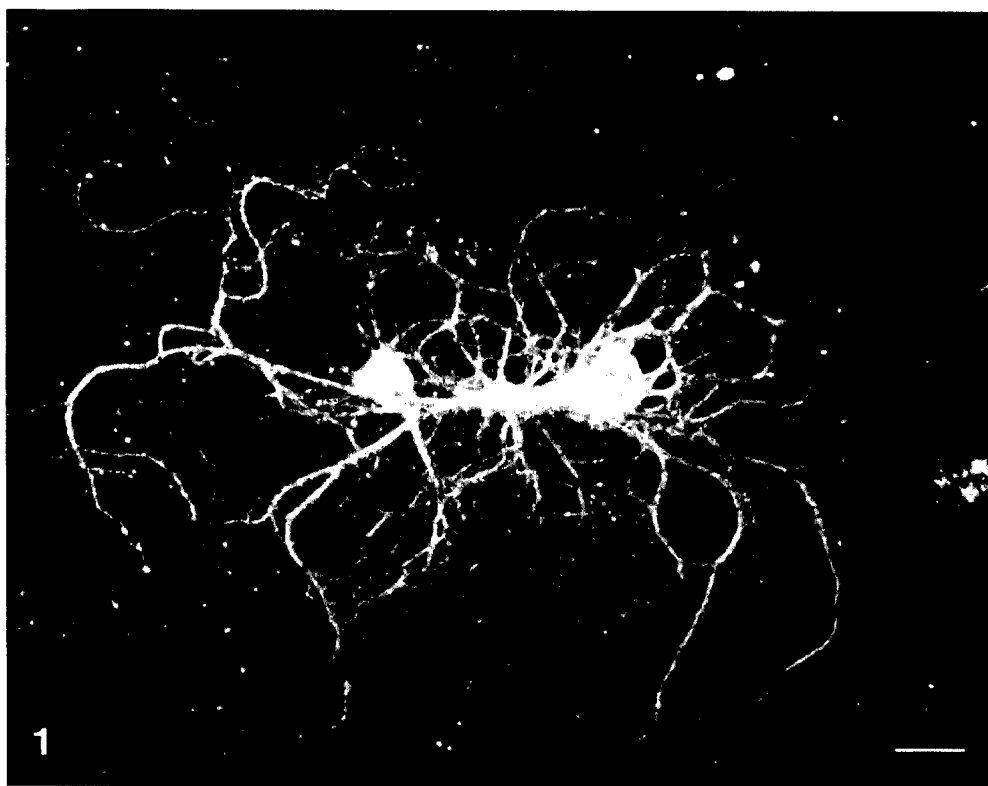
Fig. 1. A co-culture of L10 (right) and R15 (left) after 3 days *in vitro*. The extensive array of fine neuronal processes corresponds to regenerated outgrowth. The co-culture was photographed at the time of intracellular recording using darkfield illumination. Scale bar, 100 μm.

described previously (Bennett, 1977). Postsynaptic potentials were measured in response to action potentials elicited in the presynaptic cell by the injection of brief, suprathreshold current pulses. For some measurements the postsynaptic cell was penetrated with separate current-passing and voltage-sensing electrodes. A synapse was judged to be present if we observed a postsynaptic response with a magnitude of at least 5 mV after 1–10 spikes in the presynaptic neurone; significant responses less than 5 mV in amplitude following multiple spikes in the presynaptic cell were never observed. A synapse was judged to be absent if a significant postsynaptic response was not observed after firing the presynaptic cell at the high rate of 10–20 spikes s^{-1} for 5 s; this criterion was modified for L10/R15 co-cultures (see Results).

All recordings were made in an artificial sea water (ASW) that consisted of 460 mmol l^{-1} NaCl, 10 mmol l^{-1} KCl, 10 mmol l^{-1} Hepes, 10 mmol l^{-1} glucose, 55 mmol l^{-1} MgCl$_2$ and 10 mmol l^{-1} CaCl$_2$, pH 7.6, at room temperature. Co-cultures were examined 3–5 days after plating.

Results

We examined co-cultures of L10 with six of its followers in the ganglion (L7, L11, L12, L13, R15 and R16) and two non-followers (a second L10 and L14A). *In situ*, L10 forms purely inhibitory inputs to L11, L12 and L13, purely excitatory inputs to R15 and R16, and a dual-action input to L7, with inhibition followed by excitation (Kandel *et al.* 1967) (Fig. 2). None of these followers or non-followers is reported to form connections back to L10.

The firing properties of the neurones, either alone or in co-culture, are discussed first. We then present results on the novel reciprocal connections and unidirectional connections that are established *in vitro*. A large number of co-cultures were prepared so that the probability of forming a given type of synapse could be established (Table 1).

Neuronal firing properties in vitro

The firing properties of many neurones in the intact ganglion have been characterized. Interneurone L10 produces an aperiodic bursting output *in vivo* (Frazier *et al.* 1967; Koester *et al.* 1974). Follower R15 produces a stereotypic ('parabolic') bursting output consisting of trains of action potentials separated by periods of hyperpolarization (Strumwasser, 1965; Alving, 1968). In contrast, followers L7, L11, L12, L13 and R16 and the non-follower L14A produce non-bursting output (Frazier *et al.* 1967). We first examined the firing properties of these cells in culture.

Interneurone L10 was studied in two different culture environments, i.e. isolated L10 neurones ($N=10$) or co-cultures of L10 with one of its followers ($N=77$) (Fig. 1). Intracellular recordings showed that about half the L10s fired
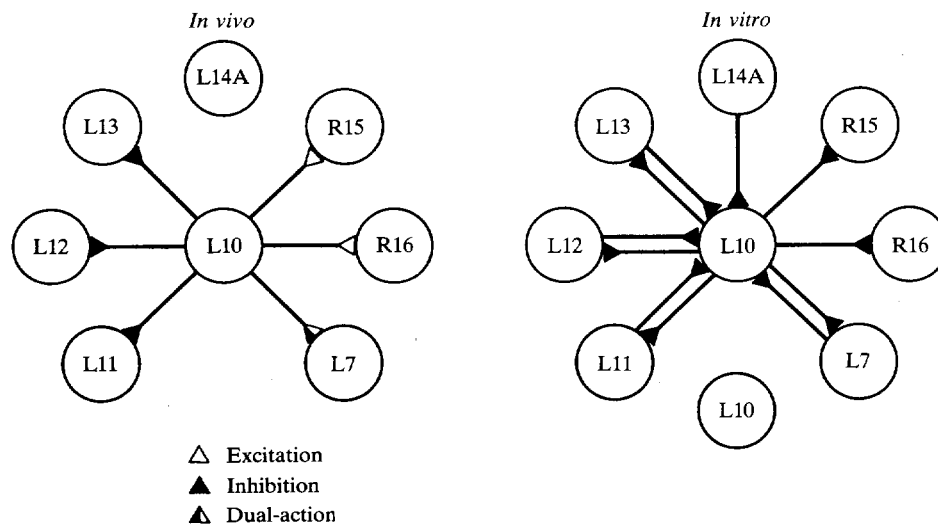

Fig. 2. Schematic drawing of the connections formed by L10 to selected followers and non-followers *in vivo* and *in vitro* (Kandel *et al.* 1967; Wachtel and Kandel, 1971).

Table 1. *Inhibitory connections in co-cultures with L10*

Co-cultured neurone	Connection from L10[a] (%)	Connection to L10[a] (%)	Reciprocal connections (%)	N
LUQ (L2–L6)	55	35	25	87[b]
L7	45	45[c]	35	24[d]
L10	5[c]	0	0	19
L11	70	**30**	20	19[f]
L12	80	**80**	80	9
L13	45	**90**	45	13
L14A	10[e]	**55**	10	9
R15	**60**	0	0	5[g]
R16	**85**	0	0	7

[a] Entries indicate the percentage of co-cultures in which a connection was observed. Those in bold type correspond to connections that have not been observed *in vivo* (see Fig. 2).

[b] Data from Kleinfeld *et al.* (1990). Inhibitory connections from L10 to LUQ, but not from LUQ to L10, were reported by Camardo *et al.* (1983) and Schacher *et al.* (1985).

[c] In one additional instance we observed an excitatory connection.

[d] Schacher (1988) observed a dual-action synapse, with excitation preceding inhibition, from L10 to L7.

[e] Only a single instance with this connection was observed.

[f] Inhibitory connections from L10 to L11, but not from L11 to L10, were reported by Schacher *et al.* (1985).

[g] Schacher (1988) reports an excitatory connection from L10 to R15.

with a rhythmic pattern of output activity, regardless of the culture environment. A typical pattern consisted of well-defined bursts of action potentials, lasting for 25–50 s, separated by longer periods of silence (Fig. 3A,B). The rhythmic, bursting pattern continued unabated for up to 10 h. On rare occasions ($N=3$) we observed a rhythmic output pattern with a relatively short period between bursts (Fig. 3C). The rhythmic bursting patterns observed *in vitro* are distinctly periodic compared with those observed *in vivo*.

Two features of an individual burst were seen with every L10. (i) The amplitude of the action potentials within a burst increased with time until it reached a plateau value 5–10 s after the onset of the burst (Fig. 3B,C). (ii) The burst terminated with a subthreshold depolarization (Fig. 3B,C).

L10 neurones that did not burst could be induced to fire tonically by the constant injection of small depolarizing currents. A current of about 0.1–0.5 nA was needed to drive a cell just above its threshold for firing, at which it fired at a rate of 2–3 spikes s^{-1}. The firing properties of cultured L10s were uncorrelated with the input resistance of the cell ($R_{In}=35\pm15$ MΩ; s.d.) and the basis for the heterogeneity among L10 cells from different animals is unclear.

Follower R15 neurones, cultured in isolation, produced an output pattern consisting of bursts of action potentials with a pronounced hyperpolarization between bursts (Fig. 4A). These features closely approximate to the behaviour
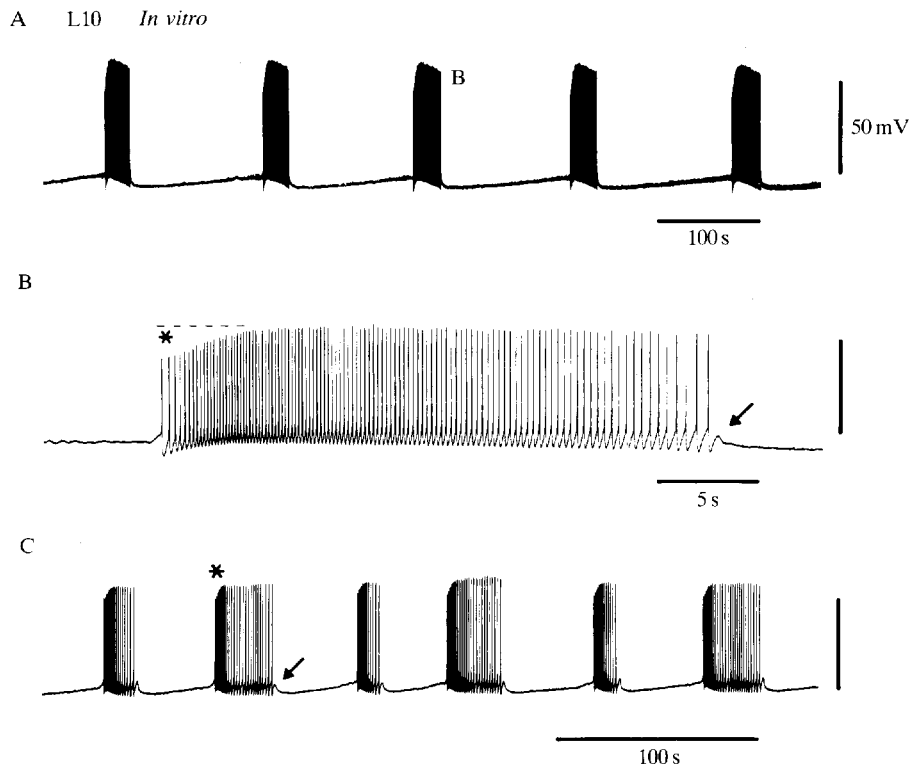
A   L10   *In vitro*

B

C

Fig. 3. Rhythmic bursting by isolated L10 cells in culture. (A) Output from an L10 cell maintained for 5 days *in vitro*. The duration and period of these bursts were typical of those observed in the majority of L10 cells. (B) Expanded record of the burst labelled B in part A. The stereotypic features of the firing pattern are highlighted. These include an increase in the height of the spikes and the period between spikes at the onset of a burst (*) and a subthreshold depolarization at the termination of a burst (arrow). (C) Output from a different L10, maintained for 4 days *in vitro*. The duration and period of the bursts for this cell were not typical of that observed in the majority of L10 cells. However, the stereotypic features observed at the onset and the termination of each burst were present (cf. B and C).

reported for R15 *in vivo* (Strumwasser, 1965; Meech, 1979). Similar results were found with other cultured R15 cells ($N=6$). A typical burst pattern of R15 co-cultured with L10 is shown in Fig. 4B. The bursts occurred periodically, but the durations of the bursts were shorter than those reported for R15 *in vivo*. Bursting by R15 *in vitro* has also been reported by Kramer *et al.* (1988).

None of the other neurones we examined (L7, L11, L12, L13, L14A and R16) exhibited rhythmic bursting *in vitro*, in agreement with their behaviour *in vivo*. Each of these cells could be induced to fire tonically by the constant injection of small, depolarizing currents. The behaviour of L14A was notable in that its rate of firing tended to accelerate, for a period of about 30 s, following the onset of activity. A similar increase in firing rate occurs *in vivo* (Carew and Kandel, 1977).
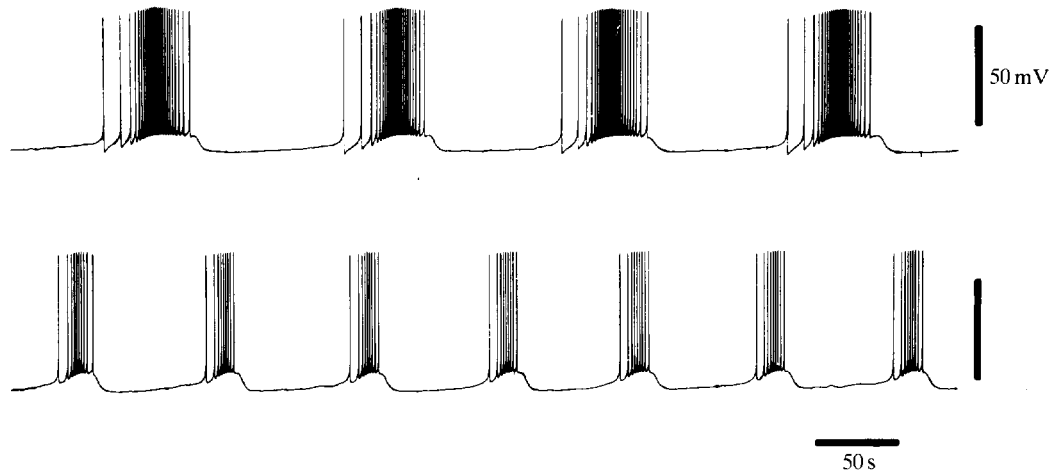
A   R15   *In vitro*

B

50 s

Fig. 4. Rhythmic bursting by R15 cells in culture. (A) The output from an isolated R15 cell maintained for 3 days *in vitro*. The duration, period and shape of the bursts were very similar to the observed behaviour of R15 *in vivo* (see text). (B) The output from an R15 cell co-cultured with an L10 cell and maintained for 4 days *in vitro*. The output from this cell is typical of those used to study the interaction between L10 and R15.

Reciprocal connections in vitro

Co-cultures of L10 paired with followers L7, L11, L12 or L13 formed reciprocal inhibitory connections. The connections from L10 to each of L11, L12 and L13 were reminiscent of those *in vivo* (Kandel *et al.* 1967). The connection from L10 to L7 differed; a dual-action synapse, with inhibition followed by excitation, occurs *in vivo*. Novel connections were also observed: each of these followers formed a connection to L10. These results are summarized in Fig. 2 and Table 1.

Neurone L13 formed a particularly strong novel connection to L10. Typically, inhibitory postsynaptic potentials (IPSPs) with a magnitude of 10–20 mV followed a single action potential in L13 (Fig. 5A–C). This IPSP did not facilitate but it did fatigue to about half its maximum value after repeated stimulation of L13. A connection from L13 to L10 occurred in all but one co-culture (12 out of 13 co-cultures). In addition, the type of connection found *in vivo* from L10 to L13 was present in about half the cultures (Fig. 5D). *In vitro*, the IPSP in L13 was observable following a brief train of action potentials in L10 but not following a single action potential, in contrast to the connection from L13 to L10.

Neurone L7 also formed strong, novel synaptic connections to L10 (12 out of 24 co-cultures). We observed IPSPs with a magnitude of 2–5 mV following a single action potential in L7 (Fig. 6A–C). Further, we typically observed spontaneous, brief hyperpolarizing events about 50 s after the initial IPSP (Fig. 6C).

A solely inhibitory input from L10 to L7 was also present in about half the cultures (Fig. 6D). There was a significant probability, at the level of two standard
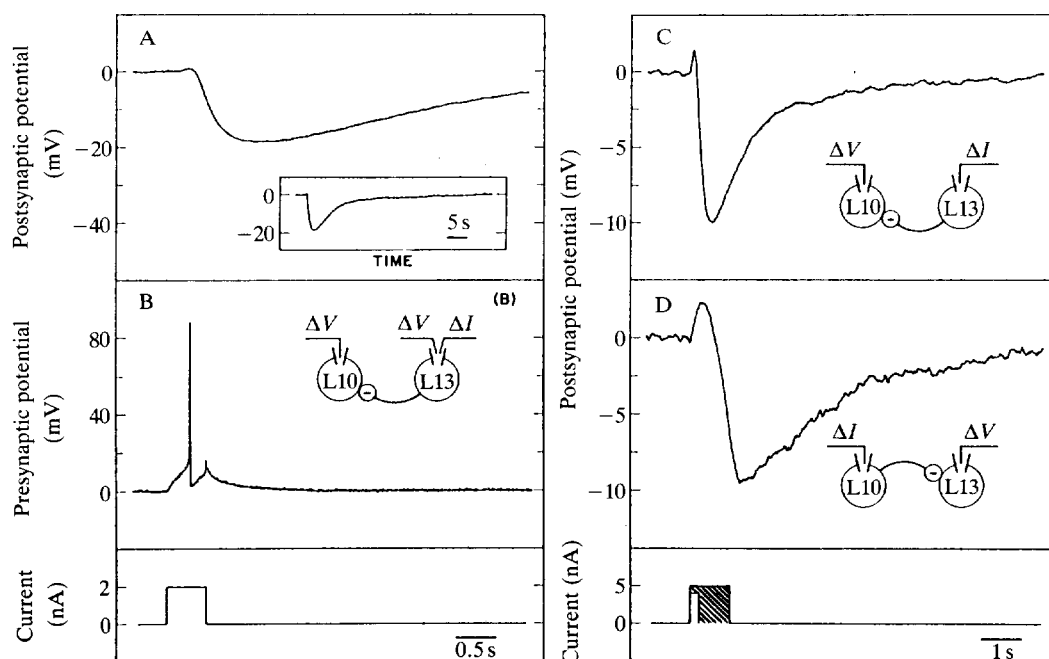
Fig. 5. The synaptic interactions between co-cultured L10 and L13 neurones. These cells were examined after 5 days *in vitro*. (A,B) The postsynaptic potential (PSP) observed in L10 following a single action potential in L13. The potential of the postsynaptic neurone was initially -52 mV. Note the relatively large amplitude of the IPSP; the inset shows that the cell potential recovered to its initial value. (C,D) Reciprocal inhibitory connections observed in a different co-culture. The response in L10 was elicited by a single action potential in L13. The response in L13 was caused by a train of about 10 action potentials in L10; no response was seen following a single action potential. Note that the initial increase in potential was caused by electrical coupling between the neurons ($\kappa = \Delta V_{Post}/\Delta V_{Pre} \approx 0.2$). The unshaded current pulse corresponds to C and the shaded pulse corresponds to D.

deviations, that co-cultured L7 and L10 cells that expressed one connection would express the reciprocal connection. It was interesting that there were no instances in which an excitatory response or a dual-action response was observed in L7. This is in contrast to the response observed *in vivo* (Wachtel and Kandel, 1971) and previously reported to occur *in vitro* (Schacher, 1988).

It was surprising that L10, a cholinergic neurone (Giller and Schwartz, 1971), did not elicit a dual-action response in L7 *in vitro*. In particular, the dual-action response characterized *in vivo* can be mimicked by the application of acetylcholine to L7 (Frazier *et al.* 1967). One possible explanation for this loss is that L7 may lose the capacity for dual-action responses to cholinergic stimulation *in vitro*. To test this we analyzed co-cultures of L7 with R2 ($N=3$); R2, like L10, is cholinergic (Giller and Schwartz, 1971), although it may contain additional transmitters (Schacher *et al.* 1985). We found that single action potentials in R2 produced an
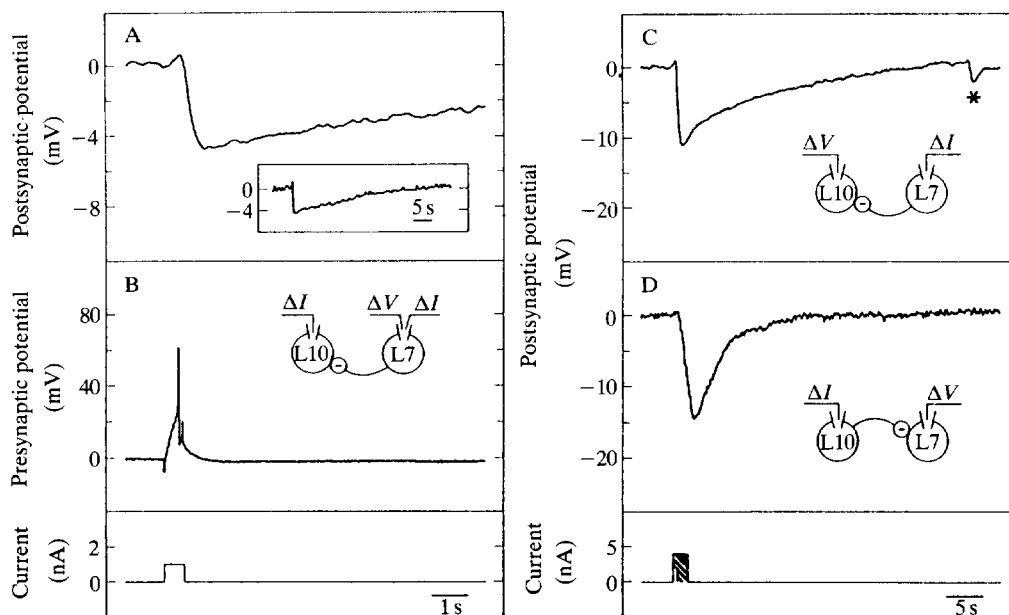
Fig. 6. The synaptic interactions between co-cultured L10 and L7 neurones. These cells were examined after 3 days *in vitro*. (A,B) The IPSP observed in L10 following a single action potential in L7. The potential of the postsynaptic neurone was initially −50 mV. The inset shows that the cell potential recovered to its initial value. (C,D) Reciprocal inhibitory connections observed in the same co-culture. The response in L10 was elicited by multiple action potentials in L7. Typically, we observed that a spontaneous PSP occurred after such presynaptic stimulation (*). The response in L7 was caused by a train of action potentials in L10; no response was seen following a single action potential.

excitatory postsynaptic potential (EPSP) in L7, while rapid firing unmasked an IPSP. The dual-action response, with inhibition followed by excitation, is clearly seen following a burst of action potentials in R2 (Fig. 7). The *in vitro* response in L7 following activation of R2 was similar to that observed *in vivo* following excitation of L10 (Wachtel and Kandel, 1971). The synapse from R2 to L7 is novel and demonstrates that L7 is capable of forming a dual-action synapse *in vitro*.

The connection from L10 to followers L11 and L12 observed *in vivo* (Kandel *et al.* 1967) was also present *in vitro* (Fig. 8B,D). However, L11 and L12 formed novel inhibitory connections to L10 (Fig. 8A,C). Unlike the situation with L13 or L7, a single action potential in L11 elicited a relatively weak ($\Delta V < 1$ mV) response in L10. However, a strong summed response was observed following a brief train of action potentials in L11 (Fig. 8A). Connections of similar strength were observed for the connection from L12 to L10 (Fig. 8C). These novel connections were found in about three-quarters of the co-cultures studied (13 out of 19 for L11/L10 and 7 out of 9 for L12/L10).

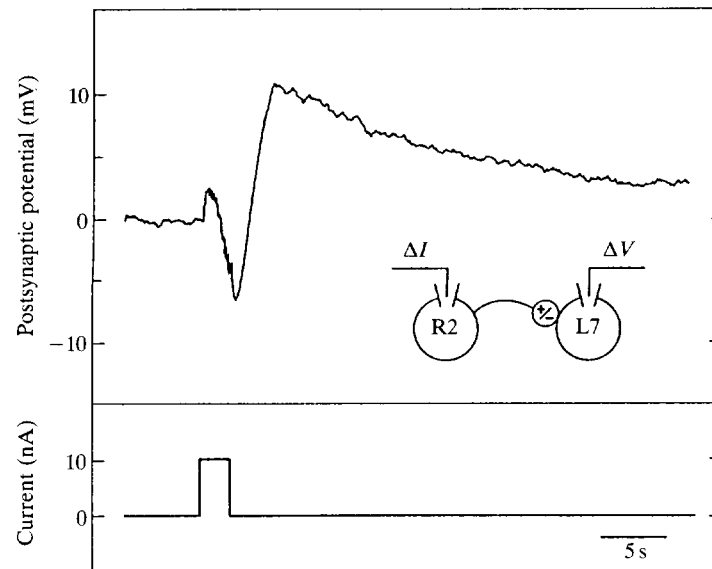In the light of the partial preservation of the synaptic function observed *in vivo*

Fig. 7. A novel dual-action synapse formed between co-cultured R2 and L7 neurones. These cells were examined after 4 days *in vitro*. An initial inhibitory response in R2 is followed by an excitatory response. The resting potential of L7 was −54 mV. The presynaptic cell R2 was activated by a relatively strong current pulse to demonstrate clearly the initial IPSP. Note that the initial increase in potential was caused by electrical coupling between the neurones ($\kappa \approx 0.2$).

for the L10 to L7 connection, it was of interest to examine the biophysical properties of L10 connections to followers L11, L12 and L13 (Figs 5D, 8B,D). We considered these properties in the light of the novel input from L10 to L7 (Fig. 6D). The postsynaptic response was examined in selected cells using a two-electrode current-clamp. The results for L11 co-cultured with L10 are shown in Fig. 9A. With L11 poised at −45 mV, just below its threshold potential, action potentials in L10 elicited both fast IPSPs and a slow IPSP. The fast IPSP inverted at a potential of about −60 mV and showed some evidence of facilitation (Fig. 9A). It could be reversibly blocked by the addition of 100 μmol l^{-1} tubocurarine to the ASW (data not shown). This response was similar to the chloride-mediated IPSP characterized *in situ* (Kehoe, 1972*a*). The slow IPSP inverted at a potential of about −80 mV (Fig. 9A). It was not affected by tubocurarine but was blocked by the addition of 500 μmol l^{-1} tetraethylammonium to the ASW (data not shown). This response was similar to the potassium-mediated IPSP that occurs *in vivo* (Kehoe, 1972*b*). These and related data for co-cultures with L12 ($N=4$) show that L10 was capable of forming inhibitory connections in culture that functioned similarly to those in the ganglion. Furthermore, the strength of these synapses was maintained with repeated bursting output from L10. This was demonstrated in a co-culture of L10 with L11, where L11 had been activated by the
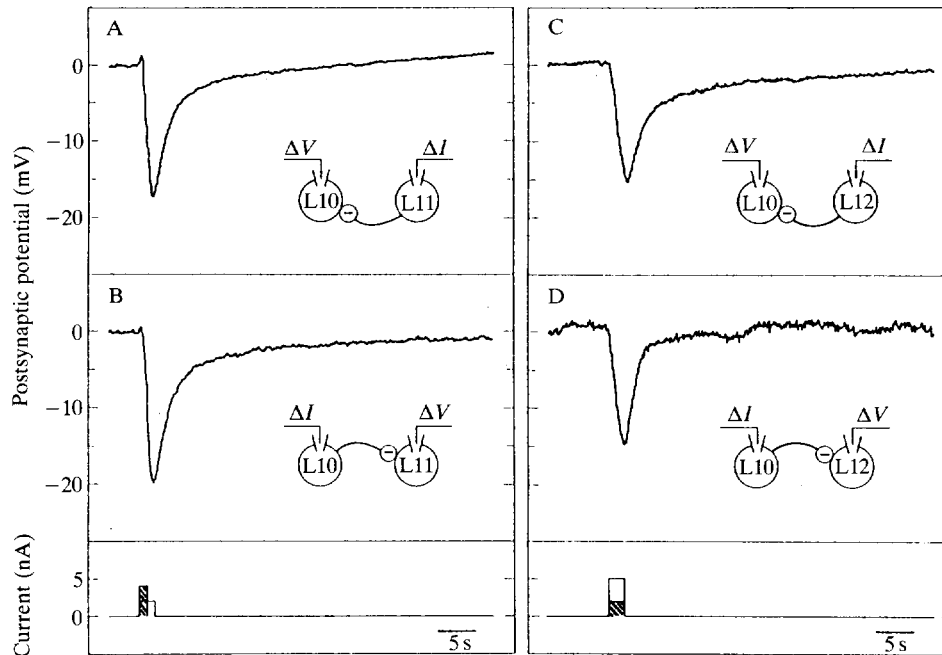
Fig. 8. Reciprocal synaptic interactions in co-cultures of L10 with followers L11 and L12. (A,B) Reciprocal inhibitory connections observed between co-cultured L10 and L11 neurones after 3 days *in vitro*. Both responses were elicited by multiple action potentials in the presynaptic neurone. (C,D) Reciprocal inhibitory connections observed between co-cultured L10 and L12 neurones after 3 days *in vitro*. Both responses were elicited by multiple action potentials in the presynaptic neurone. Note the relatively rapid decay of the PSP in L12.

injection of a small, constant current (Fig. 9B). The bursts in L10 cause the functional inhibition of L11 over repeated periods of about 10^3 s.

Unidirectional connections in vitro

Neurones R15 and R16 receive excitatory input *in vivo* (Kandel *et al.* 1967). *In vitro*, L10 also made connections onto these cells, but the input was inhibitory rather than excitatory. Reciprocal connections of these followers back onto L10 were not observed *in vitro*. In addition, L14A, a non-follower of L10, formed a novel inhibitory connection to L10.

The inhibition of R15 by L10 was initially deduced from the effects of L10 on the pattern of rhythmic bursting in R15. Activation of L10 resulted in a marked increase in the period between bursts in R15 (Fig. 10A). Further, action potentials in L10 could terminate a burst in R15 prematurely. This functional assay implies that L10 inhibits R15, although we did not observe IPSPs in R15 during the period between bursts (Fig. 10A). However, IPSPs were observed in R15 in response to stimulation of L10 when R15 was depolarized to a potential of at least −50 mV (Fig. 10B). For these measurements we waited until R15 stopped firing at each
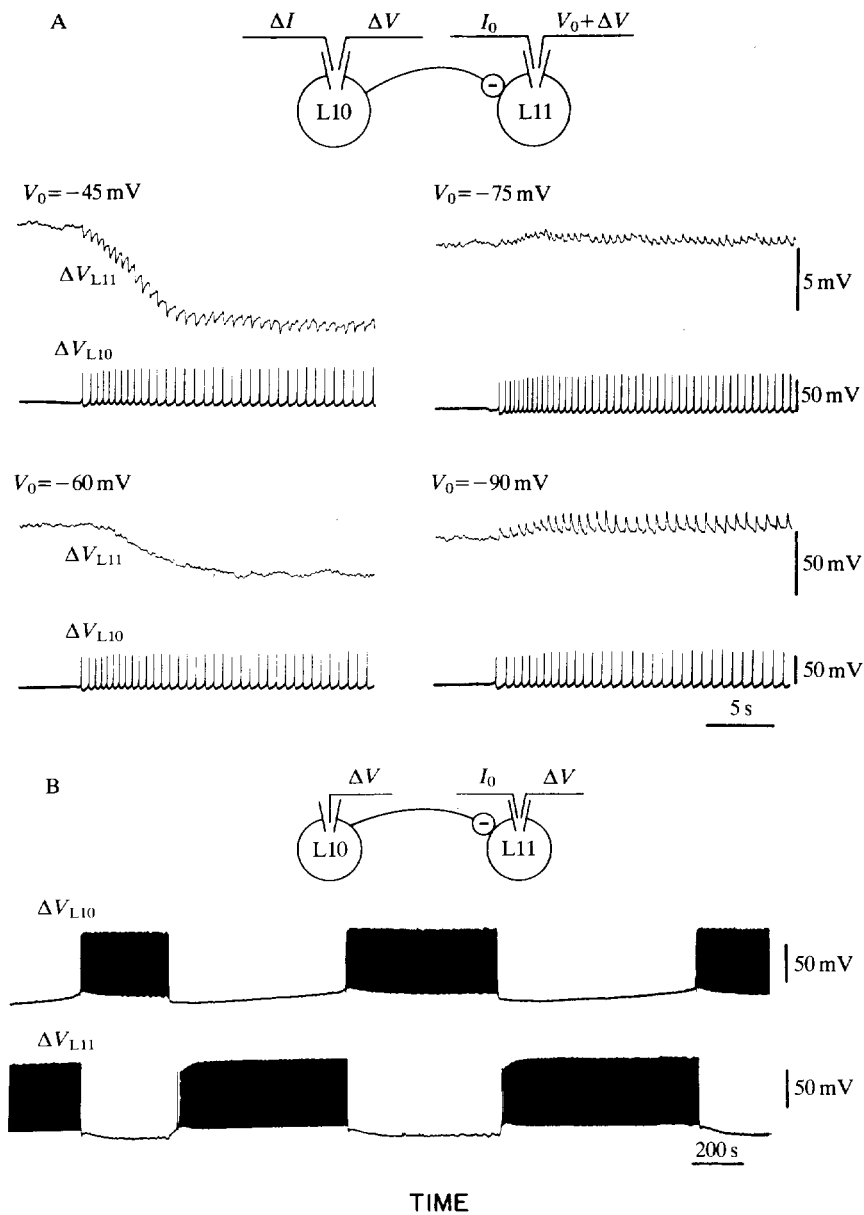
Fig. 9. Biophysical properties of the connection from L10 to L11. (A) Demonstration of both fast and slow components in the inhibitory response. The postsynaptic cell was probed with a two-electrode current-clamp. A fast IPSP that inverted at $V_0 \approx -60$ mV and a slow IPSP that inverted weakly at $V_0 \approx -80$ mV are seen. L10 was activated by the intracellular injection of 0.5 nA of current. The postsynaptic response in this co-culture was similar to that of *Aplysia* cells *in vivo*. (B) Oscillating neuronal output from a circuit of co-cultured L10 and L11 cells. L10 produced a rhythmic, bursting output with a period that varied between 500 and 1000 s. L11 was induced to fire by the intracellular injection of 0.2 nA of current in the absence of synaptic input from L10. The oscillating output resulted from the persistent inhibition of L11 by L10 when L10 was active.
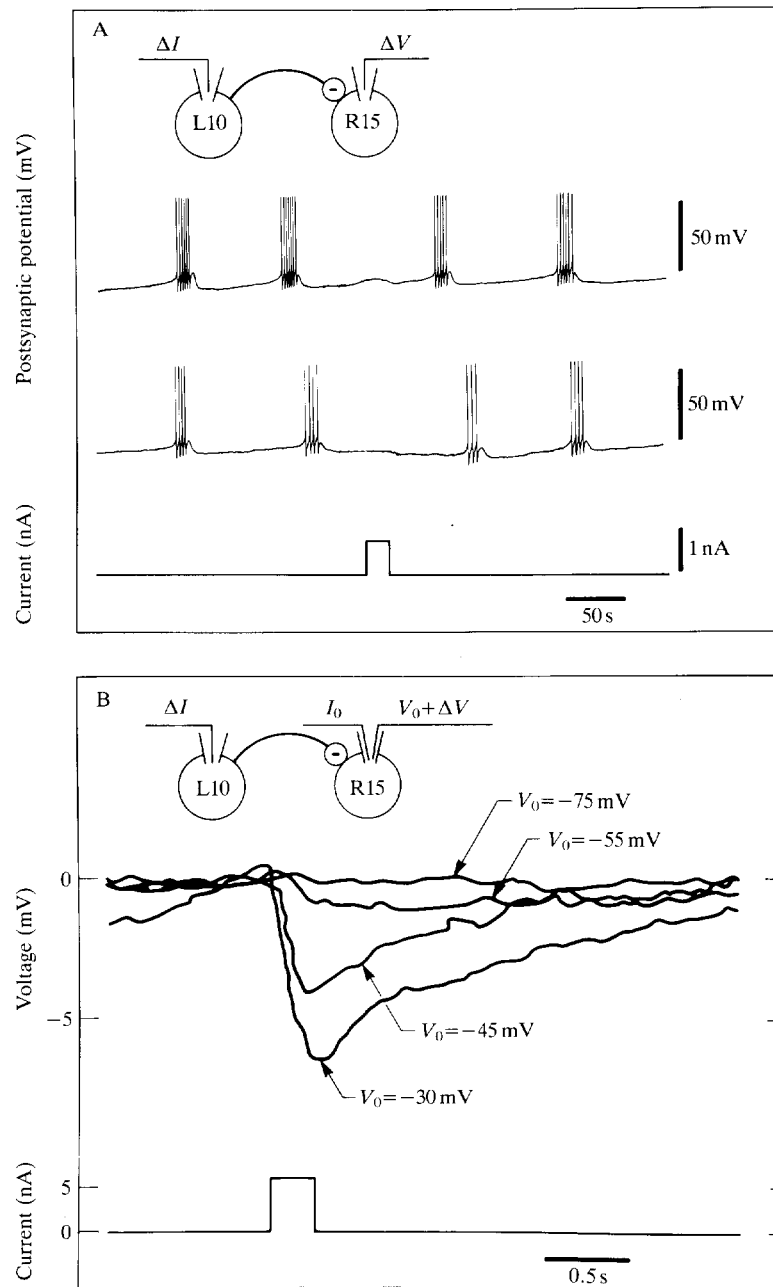
Fig. 10. Unidirectional synaptic interactions observed in co-cultures of L10 with follower R15 after 3 days *in vitro*. (A) Brief activation of L10 during rhythmic, bursting output by R15 causes the period between bursts to increase; compare A with Fig. 4B. (B) The IPSP induced in R15 by a train of about five action potentials induced in L10 by a current pulse. Note that R15 had to be depolarized to a potential considerably above its interburst potential for the IPSP to be apparent.
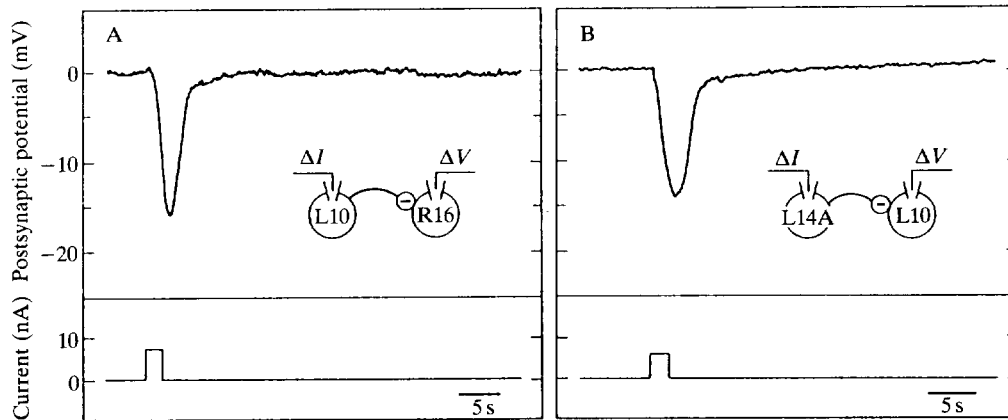
Fig. 11. Unidirectional synaptic interactions in co-cultures of L10 with follower R16 and non-follower L14A. (A) The inhibitory connection observed in a co-culture of L10 and R16 after 3 days *in vitro*. The initial potential of the postsynaptic cell was about −55 mV and the PSP was elicited by multiple action potentials in the presynaptic neurone. (B) The inhibitory connection observed in a co-culture of L10 and L14A after 3 days *in vitro*. The initial potential of the postsynaptic cell was about −50 mV. The PSP was elicited by multiple action potentials in the presynaptic neurone.

level of depolarization. There were no instances in which an excitatory response in R15 was observed.

Inhibitory connections were also formed from L10 to R16 (6 out of 7 instances) (Fig. 11A). IPSPs with magnitudes of 1–2 mV were observed following single action potentials in L10. The recovery time of the IPSP was relatively fast (about 1 s), like that observed with R15. However, the IPSP in R16 was clearly discernible at its resting potential, −55 mV, in contrast to the IPSP in R15 (cf. Figs 10B, 11A). There were no instances in which an excitatory response in R16 was observed. Further, we never observed a connection from R16 to L10.

A novel inhibitory connection to L10 was formed by L14A (Fig. 11B). The IPSPs had a magnitude of 1–2 mV following single action potentials in L10. There was only one instance of a connection from L10 to L14A (*N*=12). A single occurrence is an unreliable indicator (see Materials and methods) and we conclude that L10 does not innervate L14A.

Connections among co-cultured L10 cells

The results above show that L10 is capable of forming atypical inhibitory connections with a variety of other neurones from the abdominal ganglion. We therefore investigated whether one L10 could innervate other L10 cells *in vitro*. Only a single instance, out of 38 possible instances, was observed. This small occurrence is an unreliable indicator (see Materials and methods). We thus conclude that L10 does not innervate other L10 cells *in vitro*.

Discussion

A well-defined specificity of connections was found in co-cultures of *Aplysia californica* interneurone L10 with *in situ* followers and non-followers (Fig. 2 and Table 1). This specificity partially coincided with that observed *in vivo* (Kandel *et al.* 1967). *In vivo* connections whose sign was preserved *in vitro* were made by L10 onto neurones L11, L12 and L13 (Figs 5D, 8B,D). Connections *in vitro* that did not preserve the sign of corresponding synapses *in vivo* were made by L10 onto R15, R16 and L7 (Figs 6D, 10A, 11A). Connections that have never been observed to occur *in vivo*, in terms of either direction or sign, were formed *in vitro* between L10 and followers L7, L11, L12, L13 and R16 and non-follower L14A (Figs 5D, 6D, 8B,D, 11A,B). All these novel connections were inhibitory and some were strong (Fig. 5A,C).

The synaptic response observed in two of our co-cultures differs from that reported by Schacher (1988). In those studies the connections between L10 and followers L7 and R15 preserved their *in vivo* specificity. The basis for the differences with these particular synapses is unclear. However, novel inhibitory connections in addition to those reported here have been observed *in vitro*. A novel connection from R2 to L10 has been reported by Schacher *et al.* (1985) and connections involving L10 and identified cerebral neurones are discussed by Chiel *et al.* (1986).

It is striking that all the novel connections to L10 *in vitro* were inhibitory (Table 1). Similarly, the known connections to L10 *in vivo* from other interneurones are also exclusively inhibitory (Kandel *et al.* 1967; Koester and Alevizos, 1989). This suggests that the specificity of these connections may be regulated by the postsynaptic cell, L10. Postsynaptic regulation of the synaptic morphology of *Aplysia* sensory neurones for motor neurone L7 has been demonstrated by Glanzman *et al.* (1989). A precedent for postsynaptic regulation of synapse formation has also been described in mammalian nervous systems (Sur *et al.* 1988). The morphology of presynaptic, but foreign, inputs is adjusted and resembles the morphology of the native input (Metin and Frost, 1989).

The followers that formed novel connections to L10 *in vitro* do not innervate L10 *in vivo*. One limiting factor that could account for the absence of these novel connections *in vivo* is physical proximity. However, many of these followers receive input from L10 in the ganglion (Frazier *et al.* 1967) and their processes are in intimate contact. Our results suggest that it is unlikely that physical separation prevents the formation of such connections *in vivo*. It should be noted that, *in vivo*, L10 is innervated by other interneurones (Kandel *et al.* 1967; Segal and Koester, 1980). These presynaptic neurones may effectively compete for L10 territory and thus prevent the followers from forming reciprocal connections onto L10. A role for synaptic competition is suggested by the reduced frequency of novel connections for specific followers when other followers are present *in vitro* (D. Kleinfeld, T. D. Parsons and F. Raccuia-Behling, unpublished results). Thus, the use of co-cultures of L10 with single followers may optimize the conditions for the formation of novel connections. A precedent for this interpretation comes

from the observed elimination of foreign inputs onto salamander muscle fibres by native inputs (Dennis and Yip, 1978).

References

ALVING, B. O. (1968). Spontaneous activity in isolated somata of *Aplysia* pacemaker neurons. *J. gen. Physiol.* **51**, 29–45.

ARECHIGA, H., CHIQUET, M., KUFFLER, D. P. AND NICHOLLS, J. G. (1986). Formation of specific connections in culture by identified leech neurones containing serotonin, acetylcholine and peptide transmitters. *J. exp. Biol.* **126**, 15–31.

BENNETT, M. V. L. (1977). Electrical transmission: A functional analysis and comparison to chemical transmission. In *Handbook of Physiology*, section 1, *The Nervous System*, vol. 1 (ed. E. R. Kandel), pp. 357–416. Bethesda: American Physiology Society.

BODMER, R., DAGAN, D. AND LEVITAN, I. B. (1984). Chemical and electrotonic connections between *Aplysia* neurons in primary culture. *J. Neurosci.* **4**, 228–233.

BYRNE, J. H. (1987). Cellular analysis of associative learning. *Physiol. Rev.* **67**, 329–439.

CAMARDO, J., PROSHANSKY, E. AND SCHACHER, S. (1983). Identified *Aplysia* neurons form specific chemical synapses in culture. *J. Neurosci.* **3**, 2814–2820.

CAREW, T. J. AND KANDEL, E. R. (1977). Inking in *Aplysia*. I. Neural circuit of an all-or-none behavioral response. *J. Neurophysiol.* **40**, 692–707.

CHIEL, H. J., TANK, D. W. AND CONNOR, J. A. (1986). Presynaptic inhibition reconstructed *in vitro*. *Soc. Neurosci. Abstr.* **12**, 1338.

COOKE, I., GRAFT, R., GRAU, S., HAYLETT, B., MEYER, D. AND RUBEN, P. (1989). Crustacean peptidergic neurons in cultures show immediate outgrowth in simple media. *Proc. natn. Acad. Sci. U.S.A.* **86**, 402–406.

DAGAN, D. AND LEVITAN, I. B. (1981). Isolated identified *Aplysia* neurons in cell culture. *J. Neurosci.* **1**, 736–740.

DALE, N., SCHACHER, S. AND KANDEL, E. R. (1988). Long-term facilitation in *Aplysia* involves increase in transmitter release. *Science* **239**, 282–285.

DENNIS, M. J. AND YIP, J. W. (1978). Formation and elimination of foreign synapses on adult salamander muscle. *J. Physiol., Lond.* **274**, 299–310.

FRAZIER, W. T., KANDEL, E. R., KUPFERMANN, I., WAZIRI, R. AND COGGESHALL, R. E. (1967). Morphology and functional properties of identified neurons in the abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **30**, 1288–1351.

FUCHS, P. A., NICHOLLS, J. G. AND READY, D. F. (1981). Membrane properties and selective connexions of identified leech neurones in culture. *J. Physiol., Lond.* **316**, 203–223.

FUCHS, P. A., HENDERSON, L. P. AND NICHOLLS, J. G. (1982). Chemical transmission between individual Retzius and sensory neurons of leech in culture. *J. Physiol., Lond.* **323**, 195–209.

GETTING, P. A. (1989). Emerging principles governing the operation of neural networks. *A. Rev. Neurosci.* **11**, 185–204.

GILLER, E., JR. AND SCHWARTZ, J. H. (1971). Choline acetyltransferase in identified neurons of abdominal ganglion of *Aplysia californica*. *J. Neurophysiol.* **34**, 93–107.

GLANZMAN, D. L., KANDEL, E. R. AND SCHACHER, S. (1989). Identified target motor neuron regulates neurite outgrowth and synapse formation of *Aplysia* sensory neurons *in vitro*. *Neuron* **3**, 441–450.

HADLEY, R. D., KATER, S. B. AND COHAN, C. S. (1983). Electrical synapse formation depends on interaction of mutually growing neurites. *Science* **221**, 466–468.

Haydon, P. G. (1988). The formation of chemical synapses between cell-cultured neuronal somata. *J. Neurosci.* **8**, 1032–1038.

Kaczmarek, L., Finbow, M., Revel, J. and Strumwasser, F. (1979). The morphology and coupling of *Aplysia* bag cells within the abdominal ganglion and in cell culture. *J. Neurobiol.* **10**, 535–550.

Kandel, E. R., Frazier, W. T., Waziri, R. and Coggeshall, R. E. (1967). Direct and common connection among identified neurons in *Aplysia. J. Neurophysiol.* **30**, 1352–1376.

Kehoe, J. (1972*a*). Ionic mechanisms of a two-component cholinergic inhibition in *Aplysia* neurones. *J. Physiol. Lond.* **225**, 85–114.

Kehoe, J. (1972*b*). The physiological role of three acetylcholine receptors in synaptic transmission in *Aplysia. J. Physiol., Lond.* **225**, 147–172.

Kleinfeld, D., Raccuia, G. F. and Chiel, H. J. (1988). Reciprocal inhibition between identified *Aplysia* neurons maintained in culture. *Biophys. J.* (Abstr.) **53**, 427a.

Kleinfeld, D., Raccuia-Behling, F. and Chiel, H. J. (1990). Circuits constructed from identified *Aplysia* neurons exhibit multiple patterns of persistent activity. *Biophy. J.* **57**, 697–715.

Koester, J. and Alevizos, A. (1989). Innervation of the kidney of *Aplysia* by L10, the LUQ cells, and an identified peripheral motoneuron. *J. Neurosci.* **9**, 4078–4088.

Koester, J. and Kandel, E. R. (1977). Further identification of neurons in the abdominal ganglion of *Aplysia* using behavioral criteria. *Brain Res.* **121**, 1–20.

Koester, J., Mayeri, E., Liebeswar, G. and Kandel, E. R. (1974). Neural control of circulation in *Apysia.* II. Interneurons. *J. Neurophysiol.* **37**, 476–496.

Kostenko, M. A., Musienko, V. S. and Smolikhina, T. I. (1983). Ca^{2+} and pH affect the neurite formation in cultured mollusc isolated neurons. *Brain Res.* **276**, 43–50.

Kramer, R. H., Levitan, E. S., Carrow, G. M. and Levitan, I. B. (1988). Modulation of a subthreshold-calcium current by the neuropeptide FMRFamide in *Aplysia* neuron R15. *J. Neurophysiol.* **60**, 1728–1738.

Meech, R. W. (1979). Membrane potential oscillations in molluscan 'burster' neurones. *J. exp. Biol.* **81**, 93–112.

Metin, C. and Frost, D. O. (1989). Visual experiences of neurons in somatosensory cortex of hamsters with experimentally induced retinal projections to somatosensory thalmus. *Proc. natn. Acad. Sci. U.S.A.* **86**, 357–361.

Montarolo, P. G., Goelet, P., Castellucci, V. F., Morgan, J., Kandel, E. R. and Schacher, S. (1986). A critical time window for macromolecular synthesis in long-term heterosynaptic facilitation in *Aplysia. Science* **234**, 1249–1254.

Obaid, A. L., Kleinfeld, D., Parsons, T. D., Raccuia, G. F. and Salzberg, B. M. (1989). R15 and other rhythmically active *Aplysia* neurons exhibit endogenous bursting in culture: Possible role as actuators of *in vitro* neuronal ensembles. *Biophys. J.* (Abstr.) **55**, 175a.

Parsons, T. D. and Chow, R. (1989). Neurite outgrowth in primary cultures of neurons from the squid, *Loligo pealei. Neurosci. Lett.* **97**, 23–28.

Parsons, T. D., Kleinfeld, D., Raccuia-Behling, F. and Salzberg, B. M. (1989). Optical recording of the electrical activity of synaptically interacting *Aplysia* neurons in culture using potentiometric probes. *Biophys. J.* **56**, 213–221.

Parsons, T. D., Salzberg, B. M., Obaid, A. L., Raccuia-Behling, F. and Kleinfeld, D. (1989*a*). Synaptic interactions in assemblies of cultured *Aplysia* neurons. II. Dynamic patterns of effective connectivity. *Soc. Neurosci. Abstr.* **15**, 1140.

Parsons, T. D., Raccuia, G. F., Salzberg, B. M. and Kleinfeld, D. (1989*b*). Novel synaptic connections are formed among identified *Aplysia* neurons in culture. *Biophys. J.* (Abstr.) **55**, 175a.

Rayport, S. G. and Schacher, S. (1986). Synaptic plasticity *in vitro*: Cell culture of identified *Aplysia* neurons mediating short-term habituation and sensitization. *J. Neurosci.* **6**, 759–763.

Ready, D. F. and Nicholls, J. G. (1979). Identified neurons isolated from leech CNS make selective connections in culture. *Nature* **182**, 67–69.

Schacher, S. (1988). Identified *Aplysia* neurons maintained in dissociated cell culture: Regeneration, synapse formation and synaptic plasticity. In *Cell Culture Approaches to Invertebrate Neuroscience* (ed. D. J. Beadle, G. Lees and S. B. Kater), pp. 53–84. New York: Academic Press.

SCHACHER, S. AND PROSHANSKY, E. (1983). Neurite regeneration by *Aplysia* neurons in dissociated cell culture: Modulation by *Aplysia* hemolymph and the presence of the initial axonal segment. *J. Neurosci.* **3**, 2403–2413.

SCHACHER, S., RAYPORT, S. G. AND AMBRON, R. T. (1985). Giant *Aplysia* neuron R2 reliably forms strong chemical connections *in vitro*. *J. Neurosci.* **5**, 2851–2856.

SEGAL, M. M. AND KOESTER, J. (1980). Different cholinergic synapses converging onto neurons in *Aplysia* produce the same synaptic action. *Brain Res.* **199**, 459–465.

STRUMWASSER, F. (1965). The demonstration and manipulation of a circadian rhythm in a single neuron. In *Circadian Clocks* (ed. J. Ashoff), pp. 442–462. New York: North-Holland Publishing Company, Oxford University Press.

SUR, M., GARRAGHTY, P. E. AND ROE, A. W. (1988). Experimentally induced visual projections into auditory thalmus and cortex. *Science* **242**, 1437–1441.

SYED, N. I., LUKOWIAK, K. AND BULLOCK, A. G. M. (1989). *In vitro* reconstruction of the central pattern generator (CPG) underlying respiratory behavior in *Lymnaea*. *Soc. Neurosci. Abstr.* **15**, 1047.

VYKLICKY, L. AND NICHOLLS, J. G. (1988). Specificity of connections formed by nociceptive cells of the leech in tissue culture. *J. exp. Biol.* **134**, 17–26.

WACHTEL, H. AND KANDEL, E. R. (1971). Conversion of synaptic excitation to inhibition at a dual chemical synapse. *J. Neurophysiol.* **34**, 56–68.

WONG, R. G., HADLEY, R. D., KATER, S. B. AND HAUSER, G. C. (1981). Neural outgrowth in molluscan organ and cell cultures: The role of conditioning factor(s). *J. Neurosci.* **1**, 1008–1021.