Synapse Formation Between Isolated Axons Requires Presynaptic Soma and Redistribution of Postsynaptic AChRs

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Submitted 7 October 2002; accepted in final form 9 January 2003

INTRODUCTION

To establish the precise synaptic connectivity that is the basis of neural network organization and function in the adult brain, developing neurons must extend their axonal and dendritic processes (i.e., growth cones) toward their potential target cells. Following target cell recognition, neurite outgrowth is terminated and synapses begin to develop. It is generally accepted that, prior to contacting their synaptic partners, both presynaptic elements are ready for synaptic transmission (Haydon and Drapeau 1995). For instance, recent studies have shown that various pre- (Ahmari et al. 2000) and postsynaptic (Levi et al. 1999; O'Brien et al. 1997; Rao et al. 1998) components of the synaptic machinery may be preassembled in “packets” prior to target cell contact. On contact, these “ready made” synaptic components can be dispatched immediately to designated synaptic sites, thus allowing a “fast-track” synaptogenetic program to proceed in the absence of gene transcription and new protein synthesis. These studies thus suggest that various proteins required for synaptic programs are most likely present in the extrasomal compartments (i.e., axons and dendrites) and that the synaptogenesis may proceed in the absence of somata-based signaling.

Recent studies on isolated axons from cultured Aplysia neurons demonstrate the requirement of de novo protein synthesis in the formation and modulation of newly formed synaptic connections, though the precise site (i.e., pre- vs. postsynaptic) for this protein synthesis and the underlying mechanisms remain unresolved. For instance, Trudeau and Castellucci (1995) and Martin et al. (1997) have shown that long-term synaptic potentiation (which requires new synapses) at the sensorimotor synapse does not involve new protein synthesis in the postsynaptic cell (motor neuron), whereas Sherff and Carew (1999) have shown that blocking protein synthetic machinery in postsynaptic neurons prevents long-term facilitation. Coulson and Klein (1997) on the other hand showed that neither prenor postsynaptic protein synthesis is required for synapse formation and synaptic plasticity at soma–soma synapses between cultured Aplysia neurons. In contrast, Feng et al. (1997), have shown that synaptogenesis between paired Lymnaea somata is contingent on de novo protein synthesis. More recently, Schacher and Wu (2002) have shown that, although protein synthesis in both pre- and postsynaptic axons is required for continued synapse formation, these steps do not, however, involve the soma of either cell.

To decipher the precise contributions of pre- and postsynaptic somata and to determine the involvement of extrinsic trophic factors in synapse formation, we have attempted to reconstruct synapses between the isolated axons of identified Lymnaea neurons. Axons severed immediately after neuronal isolation were juxtaposed in cell culture and synapses were tested electrophysiologically. We provide evidence that the presynaptic, but not the postsynaptic cell body is required for new synapses between soma–axon pairs. The formation of the cholinergic synapse between presynaptic soma and postsynaptic axon pairs requires gene transcription and protein synthesis specifically in the presynaptic neuron. Moreover, this synap-
togenesis is contingent on extrinsic, brain-derived trophic factors and is mediated through receptor tyrosine kinases. Neither protein translation nor gene transcription is required postsynthetically for synapse formation. However, cell–cell contact and extrinsic trophic factors function in concert to enhance the postsynaptic responsiveness to exogenously applied acetylcholine at synaptic versus nonsynaptic sites.

This study has been published previously, in part, in the form of abstracts (Meems et al. 2001; van Minnen et al. 2000).

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Animals

Lymnaea stagnalis were maintained at room temperature in a well-aerated aquarium containing filtered pond water. For experiments involving cell isolation, snails approximately 1–2 month old (shell length 18–20 mm) were used, while conditioned medium (CM) was prepared from 2–3 month old animals (shell length 25–30 mm).

Cell culture

Neurons were isolated from the central ring ganglia and maintained in cell culture as described previously (Ridgway et al. 1991; Syed et al. 1990, 1999). Briefly, snails were anesthetized with 10% Listerine solution (ethanol, 21.9%; methanol, 0.042%) in normal Lymnaea saline containing (in mM) 51.3 NaCl, 1.7 KCl, 4.0 CaCl₂, and 1.5 MgCl₂ buffered to pH 7.9 with HEPES. The central ring ganglia were then washed several times (3 washes, 15 min each) with normal saline containing antibiotic (gentamycin, 50 μg/ml). The central ring ganglia were then treated with enzyme (trypsin) followed by enzyme inhibitor (trypsin inhibitor) and pinned down at the bottom of a dissection dish. All procedures were performed under sterile culture conditions.

CM was prepared by incubating gentamycin (20 μg/ml)-treated ganglia in Sigmacote-treated glass petri dishes, containing defined medium (DM, L-15; Life Technologies, Gaithersburg, MD; Special Order). DM consisted of serum-free, 50% L-15 medium with added inorganic salts (in mM): 40 NaCl, 1.7 KCl, 4.1 CaCl₂, 1.5 MgCl₂, and 10 HEPES, pH 7.9, and 20 μM gentamycin. The ganglia were incubated in a humidifier for 3–4 days (Syed et al. 1991) and the resulting CM was frozen (−20°C) until used.

The identified neurons (somata and initial axon segment, Fig. 1A) were isolated by applying gentle suction through a fire-polished, Sigmacote (Sigma, St. Louis, MO)-treated pipette. The isolated neurons were then plated on poly-L-lysine–pretreated glass coverslips (Ridgway et al. 1991) in either DM or CM. Axons were isolated by first plating the cell body along with its intact axon segment in cell culture and allowing to adhere to the poly-L-lysine–coated dish. The axon was then immediately severed from the cell body by using a sharp glass pipette, and the severed cell body was subsequently removed from the culture dish (Fig. 1B). Soma–axon synapses were prepared by juxtaposing the soma to the isolated axon (Fig. 1C). Axon–axon synapses were prepared by juxtaposing the axon segments of the identified neurons, followed by removal of both somata.

In some experiments, isolated cells were initially plated on hemolymph-pretreated culture dishes (to prevent adhesion) containing CM. After 12–18 h, the cells were transferred to normal poly-L-lysine–coated dishes and paired in CM.

For experiments involving anisomycin pretreatment, LPeD1 axon was cultured on poly-L-lysine–coated dishes containing CM alone or CM + anisomycin. After 12–18 h, the CM containing anisomycin was replaced with fresh CM and VD4 was paired with the axon. The VD4 was first maintained in hemolymph-pretreated dishes containing either CM alone or CM + anisomycin. After 12–18 h, VD4 was removed from hemolymph-pretreated dishes and paired with LPeD1 axon on normal poly-L-lysine dishes containing CM.

Electrophysiology

Neuronal activity was monitored using conventional intracellular recording techniques, as described previously (Syed and Winlow 1991). Glass microelectrodes (1.5-μm ID, World Precision Instruments, Sarasota, FL) were filled with a saturated solution of K₂SO₄ (resistance, 20–40 MΩ). An inverted microscope (Axiovert 135, Zeiss, Thornwood, NY) was used to view the neurons, which were impaled by Narashige (Tokyo) micromanipulators (MM202 and MM 204). Amplified electrical signals (Neuro Data Instrument Corp.) were displayed on a digital storage oscilloscope (PM 3394, Philips, Eindhoven, The Netherlands) and recorded on a chart recorder (TA 240S, Gould, Cleveland, OH).

Lucifer yellow injection and Syto 16 staining

Lucifer yellow (3–5%) was injected ionophoretically and the cells were processed as described previously (Hamakawa et al. 1999). To
stain the VD4 nucleus, cells were fixed in paraform aldehyde (1% in water) and incubated in Syto 16 (1:500 in PBS) for 30 min. The labeled cells were viewed under a fluorescent microscope (Zeiss-Axioscope) and photographed as described previously (Hamakawa et al. 1999).

**Transcription, translation, and receptor tyrosine kinase experiments**

To test whether synapse formation was mediated through receptor tyrosine kinases (RTK), a nonspecific RTK blocker, Lavendustin A (LavA, 10 μM), and its inactive analog, Lavendustin B (LavB, 10 μM), were used. Gene transcription and protein synthesis were perturbed by actinomycin D (1 μg/ml) and anisomycin (12.5 μg/ml), respectively.

Acetylcholine chloride was obtained from RBI. Mecamylamine, anisomycin, actinomycin D, LavA, and LavB were obtained from Sigma. Lucifer yellow and Syto 16 were obtained from Molecular Probes. *Lymnaea*-epidermal growth factor (L-EGF) was extracted and purified from *Lymnaea* albumen glands by Gregg T. Nagle, Ph.D. (University of Texas Medical Branch, Marine Biomedical Institute, Medical Research Building, Galveston, TX).

**RESULTS**

Specific synapses between VD4 and LPeD1 axons are maintained in the absence of their somata

To provide direct evidence (i.e., in the absence of other neurons and glia) that synaptic transmission between axons severed from their respective soma remains functional for days, synapses were examined in cell culture. Specifically, identified presynaptic (VD4) and postsynaptic (LPeD1) neurons (Fig. 1A) were juxtaposed such that their respective axons overlaid each other. Induced action potentials in VD4 generated 1:1 excitatory postsynaptic potentials (EPSPs) that were similar to those recorded previously (Woodin et al. 2002). Following the demonstration of an excitatory synapse, either VD4 or LPeD1 axon or both axons were severed and their cell bodies were removed from the culture dish. B: 12–24 h after soma removal (inset), the excitatory synapse remained intact and action potentials in VD4 continued to elicit 1:1 EPSPs in LPeD1 (n = 6). C: summary data showing the efficacy of synaptic transmission between somata and severed axons of VD4 and LPeD1. Synapses were tested electrophysiologically on Day 1 and either one or both somata were removed and synapses retested on either Day 2 or Day 3. For VD4 axon/LPeD1 soma pairs the mean EPSP amplitude was 9.3 ± 2.1 mV on day 1, 9.1 ± 1.4 mV on day 2, and 5.9 ± 0.9 mV on day 3. For VD4 soma/LPeD1 axon pairs the mean EPSP amplitude was 10.4 ± 2.7 mV on day 1, 8.0 ± 1.3 mV on day 2 and 5.9 ± 0.7 mV on day 3. For the axon-axon pairs the mean EPSP amplitude was 10.0 ± 3.8 mV on day 1, 8.6 ± 1.5 mV on day 2, and 5.9 ± 0.2 mV on day 3. Although the efficacy of synaptic strength on day 2 was similar to that recorded on day 1 (with the exception of the VD4 soma/LPeD1 axon pair), a significant reduction was observed on day 3 (P < 0.01 for all pairs).
synaptic transmission was independent of whether VD4 \((n = 5)\) or LPeD1 soma \((n = 6)\) or both \((n = 6)\) were removed (Fig. 2C). Although the efficacy of synaptic potentials recorded from severed axons did not change significantly on day 2 (mean EPSP amplitude day 1 \(= 10.0 \pm 3.8\) and day 2 \(= 8.5 \pm 1.5\) mV), a significant reduction in the EPSPs amplitude was observed on day 3 \((5.9 \pm 0.2\) mV, \(P < 0.01\)). These data thus demonstrate that the isolated axons can indeed maintain synapses in culture for several days.

Presynaptic but not postsynaptic soma is required for excitatory synapse formation between VD4 and LPeD1

To determine the involvement of both somata in new synapse formation, we tested whether severed axons were capable of establishing new synapses in the absence of their somata. Axons were paired either in a soma–axon or axon–axon configuration. Specifically, either VD4 soma or its severed axon was juxtaposed against LPeD1 soma or its severed axon. Synapses were tested electrophysiologically after 12–24 h. We discovered that pairing VD4 and LPeD1 axons did not result in synapse formation between the paired axons, whereas normal synapses formed when pre- and postsynaptic axons were attached to their respective soma (data not shown). That is, induced action potentials in the presynaptic axon (Fig. 3A) failed to generate electrophysiologically detectable responses in the postsynaptic axon \((n = 13)\). Similarly, pairing the severed axon from VD4 with LPeD1 soma also did not result in synapse formation \((n = 10)\) (Fig. 3B). However, when VD4 soma was paired with the LPeD1 axon, induced action potentials in VD4 soma generated 1:1 EPSPs in the LPeD1 axon (Fig. 3C). The VD4-induced EPSPs in LPeD1 were blocked completely and reversibly (Fig. 3D) by the ACh antagonist mecamylamine \((1 \mu M)\) \((n = 6)\), suggesting that, as observed in vivo and also in a soma–soma configuration (Woodin et al., 2002), the synaptic transmission between VD4 soma and its LPeD1 axon is cholinergic. These data demonstrate that 1) severed axons from postsynaptic neurons are capable of synaptogenesis and 2) the presynaptic but not postsynaptic soma is required for synapse formation. Our results thus suggest the importance of presynaptic genetic and protein synthetic machinery during synaptogenesis.

Excitatory synapse formation between VD4 soma and LPeD1 axon pairs requires trophic factors, gene transcription, and de novo protein synthesis and is mediated by receptor tyrosine kinases

We have previously demonstrated that specific excitatory synapse formation between soma–soma paired \(Lymnaea\) neurons requires trophic factor–mediated activation of receptor tyrosine kinases, which in turn activates synapse specific gene transcription and de novo protein synthesis (Hamakawa et al., 1999). To test whether soma–soma synaptogenesis also requires extrinsic trophic factor–mediated gene transcription and protein synthesis, VD4 somata were paired with LPeD1 axons either in CM (contains trophic factors) or DM (no trophic factors). Intracellular recordings revealed that, when paired in CM, 100% of VD4 soma and LPeD1 axon pairs developed excitatory synapses \((n = 50)\). In DM, however, only 12% of the soma–axon pairs developed synapses \((n = 25)\) (Fig. 4).

These experiments thus demonstrate the requirement of CM-derived trophic factors in synapse formation between soma–axon pairs.

Next, to determine whether the CM-induced excitatory synapse formation involved gene transcription and de novo protein synthesis, the VD4 soma and LPeD1 axon were paired in CM containing either a transcription inhibitor (actinomycin D, 1 \(\mu g/ml\)) or a protein synthesis blocker (anisomycin, 12.5 \(\mu g/ml\)). Both actinomycin D \((n = 6)\), and anisomycin \((n = 6)\) completely blocked synapse formation between VD4 soma and LPeD1 axon, suggesting that gene transcription and de novo protein synthesis are required for synapse formation between soma–axon pairs (Fig. 4). Because trophic factor–induced excitatory synapse formation is known to involve RTK activity, we next tested whether synapse formation between soma–axon pairs also requires CM-mediated gene transcription and protein synthesis via RTK. VD4 soma and LPeD1 axon were paired in CM containing either LavA (RTK inhibitor 10 \(\mu M\)) or its inactive isofrom LavB (10 \(\mu M\)). LavA \((n = 8)\), but not LavB \((n = 8)\) completely blocked excitatory synapse formation be-
CM + anisomycin and synaptic connections were tested under normal recording conditions, no synapses were detected between the paired cells (n = 6) (Fig. 5). Next, either VD4 soma or LPeD1 axon were independently pretreated with anisomycin overnight (see METHODS) and subsequently paired in normal CM for 5 h before intracellular recordings. We found that blocking presynaptic (n = 7), but not postsynaptic (n = 6) protein synthesis prior to pairing perturbed synapse formation (Fig. 5). Taken together, these data show that the postsynaptic axon does not require de novo protein synthesis for synapse formation and that the protein synthesis–dependent step underlying synaptogenesis occurs only in presynaptic somata.

CM does not alter the postsynaptic responsiveness to exogenous ACh

As shown above, both pre- and postsynaptic partners require CM for excitatory synapse formation. If the postsynaptic axon does not involve a trophic factor–induced, protein synthesis–dependent step, then how does CM affect LPeD1 axon during synapse formation? One possibility could be that CM alters the responsiveness of LPeD1 axon to VD4’s transmitter (i.e., ACh). To test this possibility, individually isolated LPeD1 axons were plated either in CM or DM and ACh was applied exogenously (10⁻⁶ M, 400 ms) under a fast perfusion system (Feng et al. 2002). Regardless of their culture conditions [i.e., CM (n = 17) or DM (n = 27)], pressure-applied ACh induced an excitatory response in all LPeD1 axons (data not shown).

Because CM does not change the responsiveness of postsynaptic axon to ACh, we next probed the possibility that cell–cell contact in CM may alter the responsiveness of LPeD1 axon to ACh such that its receptors selectively redistributed to the site of contact between the cells.

Does the protein synthesis–dependent step underlying excitatory synapse formation involve presynaptic soma or the postsynaptic axon?

To test whether the CM-induced protein synthesis–dependent step underlying synapse formation occurred in the presynaptic soma or the postsynaptic axon, both were pretreated separately with anisomycin (12.5 μg/ml) for 12–24 h in normal CM. When both the soma and the axon were directly paired in...
Trophic factors and the presynaptic cell contact alter the responsiveness of LPeD1 axon to exogenously applied ACh.

To test whether VD4 alters the responsiveness of LPeD1 axon in CM to exogenously applied ACh, this transmitter was first pressure applied to a single LPeD1 axon at both ends (see Fig. 1B). Pressure pulses of ACh either at the distal or proximal site produced almost identical excitatory responses in the isolated axon \( (n = 13) \) (Fig. 6A).

We next sought to determine whether postsynaptic axons paired with presynaptic soma displayed differential responses to ACh at the synaptic compared with the extrasynaptic site. Simultaneous intracellular recordings were first made to demonstrate synapses between VD4 soma and the LPeD1 axon \( (n = 7) \) (Fig. 6B1, inset). ACh was then tested (as above) for its effects at both the synaptic and the extrasynaptic site (away from the contact). In seven out of seven preparations, a single pulse of ACh applied directly at the contact site between VD4 and LPeD1 axon produced a strong excitatory response in the axon, which in most instances generated several action potentials (Fig. 6B1). Identical pressure application of ACh to the same axon, albeit at the extrasynaptic site (see Fig. 1C), produced only small (approximately 10 mV) subthreshold, depolarizing responses in the paired axon (Fig. 6B2). These data demonstrate that, while both single and paired LPeD1 axons in CM respond to exogenously applied ACh, these responses, however, differ qualitatively in the paired axon at the synaptic versus the extrasynaptic site. These results show that VD4 contact in the presence of CM induces ACh receptors to arrange such that they selectively localize at the synaptic site. It is important to note that, in all instances, axons were held at the same membrane potential \( (\sim 58 \text{ mV}) \).

To test whether VD4 contact with LPeD1 axon in DM alone was also sufficient to alter the responsiveness of LPeD1 axons to ACh, either single or paired axons were examined in DM. We found that both proximal and distal parts of a single isolated axon exhibited identical responses to exogenous ACh \( (n = 7) \) (Fig. 6C). Next, axons paired overnight in DM with VD4 soma were tested for their responsiveness to ACh. As shown earlier, no synapses were detected between the pairs in DM \( (n = 25) \) (Fig. 6D1, inset). Eight of eleven pairs tested under this experimental condition exhibited subthreshold depolarizing responses to exogenously applied ACh at the contact site between VD4 and LPeD1 axon. However, the ACh-induced depolarizing responses rarely led to action potentials in the LPeD1 axon (Fig. 6D1). Identical pressure pulses of ACh to noncontact sites, on the other hand, resulted in a burst of 10–12 action potentials in LPeD1 (Fig. 6D2).

Taken together, these data demonstrate that VD4 contact,
only in the presence of CM, alters the responsiveness of the LPeD1 axon to exogenously applied ACh. These results thus underscore the importance of trophic factors in mediating the cell–cell interactions during synapse formation.

DISCUSSION

This study has demonstrated that cell signaling and extrinsic trophic factors act in concert to bring about specific changes in both pre- and postsynaptic partners during synaptogenesis. In the case of the presynaptic neuron, these changes invoke both the genetic and protein synthetic machinery, whereas the postsynaptic partner does not require gene transcription or de novo protein synthesis. However, a trophic factor–mediated step is required in the postsynaptic axon to allow a redistribution of cholinergic receptors at the synapse.

Both pre- and postsynaptic neurons are deemed ready for synapse formation prior to contact with their synaptic partners (Haydon and Drapeau 1995). However, cell–cell signaling, which often requires new protein synthesis, plays a pivotal role in the maturation and consolidation of newly formed synapses. Indeed Martin et al. (1997) have shown that the precise site for the synthesis of synapse/plasticity-specific proteins is the presynaptic cell. Consistent with these studies are our data, which have shown that, although synapses can be maintained between isolated pre- and postsynaptic axons for several days, the presynaptic but not the postsynaptic soma is required for new synapse formation. Furthermore, as shown earlier, although the isolated Lymnaea axons are capable of protein synthesis (van Minnen et al. 1997; van Minnen and Syed 2001), new proteins are, however, not required in the postsynaptic LPeD1 axon for synapse formation. Because treatment of the presynaptic cell with either transcription or translation inhibitors both blocked synapse formation, our data do nevertheless support the hypothesis that both transcription and translation machinery required for excitatory synapse formation between Lymnaea neurons involves the presynaptic cell.

Although the requirement of presynaptic protein synthesis for synapse formation in Lymnaea is consistent with similar roles in synaptic plasticity in Aplysia, these studies differ from a recently published paper by Schacher and Wu (2002), who have shown that neither presynaptic nor postsynaptic soma is required for new synapse formation. A potential explanation for the discrepancy between these studies may be that, in our experiments, axons were severed from their soma immediately after neuronal extraction, whereas Schacher and Wu (2002) allowed axons to grow first for at least 2 days before their somata were removed. It is therefore plausible that the Aplysia isolated axons may have had the opportunity to transport and harbor various mRNA and their encoded proteins prior to soma removal. Thus these transported molecules may have subsequently been used to facilitate synaptic transmission in the Aplysia model.

Cell–cell contacts between synaptic partners bring about specific changes in their corresponding partners and these range from clustering of postsynaptic receptors (Lin et al. 2001) at the synapse to redistribution of prepackaged, presynaptic assembly (Ahmari et al. 2000). The data presented in this study show that cell–cell contact/signaling alone is not sufficient to bring about specific changes required for synaptogenesis at the soma–axon synapse. Specifically, because synapses failed to develop in DM, our results demonstrate that CM-derived trophic factors, in addition to cell–cell contact, are required for the development of excitatory synapses between VD4 soma and LPeD1 axon. Moreover, because paired LPeD1 axon’s responsiveness to exogenously applied ACh was altered only in CM, our data suggest that the trophic factor–mediated cell–cell signaling is required for excitatory synapse formation.

FIG. 7. A model depicting steps involved in CM and VD4 contact-mediated excitatory synapse formation between Lymnaea neurons. Trophic factors may act on the presynaptic neuron VD4 via receptor tyrosine kinases and activate a gene transcription and protein synthesis–dependent priming of presynaptic machinery (such as targeting of Ca²⁺ channels, see Feng et al. 2002) (Steps 1–3). Postsynaptic contact in the presence of trophic factors may subsequently localize the presynaptic structures to the synaptic site. VD4 contact (or initial synaptic transmission–step 5) with LPeD1 axon in CM may also subsequently mobilize postsynaptic ACh receptors to the synaptic site (step 4).
between *Lymnaea* neurons. Interestingly, we observed opposite cholinergic responses in LPeD1 axons that were paired with VD4 in either DM or CM. Specifically, while a LPeD1 axon paired in CM with VD4 showed an enhanced response to ACh at the synaptic versus extrasynaptic site, a reverse relationship was observed in DM (Fig. 6). The possibility that ACh acting on presynaptic nicotinic receptors may have induced additional, highly localized transmitter release in CM, thus generating an enhanced postsynaptic response, is highly unlikely, because ACh inhibits the activity of presynaptic neuron VD4 (not shown). These data thus suggest that, in CM, the AChR may selectively redistribute toward the synaptic site, whereas, in the absence of trophic factors, these receptors either remain spread throughout the entire length of the axon or move away from the contact site. This assumption is consistent with our data, which showed that only 12% of excitatory synapses had developed in DM (Fig. 4). Since the AChRs are also expressed at the axon in DM, it is therefore possible that, in some instances, the VD4 contact on a receptor-rich area on the axon may have been sufficient for functional synaptic transmission. These data therefore extend our previous finding in which trophic factors (including L-EGF) were shown to be necessary for synapse formation between soma–soma paired cells (Hakamakawa et al. 1999).

Isolated axons maintained in DM had similar electrophysiological properties (resting membrane potential, spike threshold, and amplitude) to those of their CM counterparts (data not shown) and responded to exogenously applied ACh in an identical manner. We thus feel that it is highly unlikely that the axonal viability in DM may have been a contributing factor in their preclusion from the synaptogenic program under these experimental conditions. Our data, on one hand, underscore the importance of trophic factors in synaptogenesis and, on the other hand, suggest that the trophic factor–mediated synapse formation may be independent of their effects on axonal viability.

Because new protein synthesis in postsynaptic axons was not required for synapse formation, it is unlikely that an enhanced responsiveness to exogenously applied ACh at the synaptic site of isolated axon involves synthesis and insertion of new receptors. Alternatively, extrinsic trophic factors may mediate protein synthesis–independent changes in nAChR subunit composition or receptor function (Le Novère et al. 2002). This notion would be consistent with earlier studies in which target-derived signals differentially regulated nAChR subunit expression, and this regulation occurred at both synaptic and nonsynaptic sites (Devay et al. 1999). Moreover, these changes were also shown to involve alteration in the subunit composition of AChR complexes. This study concluded that either alteration in the expression level and/or assembly of specific nAChR subunits might underlie observed changes in the physiology of synaptic and nonsynaptic AChR (Devay et al. 1999). Similarly, clustering of transmitter receptors at the contact site may also enhance the functional strength of synapses. For instance, contact with synaptic partner induces clustering of alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors, which otherwise remain diffusely distributed (O’Brien et al. 1997). Whether a similar change in either receptor subunit composition or AChR clustering results in the differential regulation of cholinergic response in *Lymnaea* remains to be determined.

In this study, we have shown that, although gene transcrip-

ion and the protein synthesis–dependent steps are important for synapse formation between *Lymnaea* neurons, these proteins are either transcribed or inserted only in the presence of trophic factors. Moreover, it is also possible that the trophic factor–mediated events occur in parallel with other transcription- and translation-dependent mechanisms. Based on our data presented in this study, we propose the following model (Fig. 7).

Trophic factors may prime the presynaptic cell for synaptogenesis by upregulating both regeneration and synapse formation–specific genes and their encoded proteins. On contact, synapse-specific proteins (such as Ca2+ channels, see Feng et al. 2002) are selectively targeted to the presynaptic endings. Either trophic factor–induced priming of the presynaptic machinery or a continued action of trophic factor on the axon directs postsynaptic AChRs to the site of presynaptic contact. Although the latter step is independent of new protein synthesis, it does nevertheless rely on extrinsic trophic factors. To understand the precise mechanisms of AChR clustering at synaptic sites, we attempted to label AChRs with α–bungarotoxin (α-BTX). Although α-BTX labels AChRs in *Lymnaea* glial cells (Smit et al. 2001), it failed to label AChRs on LPeD1. This suggests that the ACh receptors expressed on LPeD1 are insensitive to α-BTX and are thus different from those located on the glial cells.

Taken together, our data underscore the importance of trophic factors in the maturation of newly formed synapses. The trophic factor–induced effects involve gene transcription and de novo protein synthesis in the presynaptic soma and these effects are mediated via receptor tyrosine kinases. These data also suggest a synergistic action of cell–cell signaling and trophic factors, which brings about specific changes in both pre- and postsynaptic neurons during synapse formation.

The authors thank Dr. David Proud for critical comments on an earlier version of this manuscript. Excellent technical support by W. Zaidi is also acknowledged. *Lymnaea*-EGF was kindly provided by W. Wilderling, University of Calgary, Faculty of Medicine, Department of Physics and Biophysics. N. I. Syed is an Alberta Heritage Foundation for Medical Research (AHFMR) scientist and a Canadian Institutes of Health (CIHR) investigator. D. W. Munno is a recipient of an AHFMR studentship. This study was made possible by a Netherlands Organization for Scientific Research/Earth and Life Sciences project grant to J. van Minnen (The Netherlands) and was supported by CIHR (Canada).

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