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## SYNAPSE FORMATION BETWEEN IDENTIFIED MOLLUSCAN NEURONS: A MODEL SYSTEM APPROACH

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### 1. SUMMARY

From simple reflexes to complex motor patterns and learning and memory, all nervous system functions hinge upon the precise synaptic connectivity that is orchestrated during early development. The synaptogenic program does not stop with the cessation of development, rather it continues well into adulthood, forming the basis for synaptic plasticity that underlies learning and memory. Despite extensive advances in the field of neurodevelopment, the precise cellular and molecular mechanisms of synapse formation between central neurons remain largely unknown, due primarily to the complexity of developing mammalian brain and the rate at which the synaptogenic program proceeds (20,000–30,000 synapses/10 min). Here we report on the utility of various molluscan models whereby various steps underlying synapse formation can be investigated at the level of individual neurons and synapses. In these models, synaptogenesis can be examined both *in vivo* during regeneration and following single-cell transplantation, as well as *in vitro* through a variety of cell culture approaches.

### 2. INTRODUCTION

Imagine  $10^{15}$  neurons – all wired and interconnected through synapses into one motherboard – the brain, which in turn determines all that we do throughout our lives. Imagine also all those synaptic connections in different areas of this organ, being concurrently active, either transmitting or receiving information in a highly ordered manner. Also imagine the immaculate orchestration that would be required to connect this organ appropriately and the dire consequences if this wiring were to

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go haywire. Considering all this, it is then not so difficult to envisage challenges confronting our resolve to break the connectivity code of mammalian nervous system – let alone understand its functionality. The sheer numbers of mammalian neurons, their smaller sizes, and the rate at which the synaptic connectivity proceeds, all render it rather impossible to study synapse formation between defined sets of pre- and postsynaptic neurons. Invertebrates, on the other hand, and particularly mollusks, are endowed with relatively simple nervous systems, consisting of some 20,000–30,000 neurons. The molluscan neurons are often behaviorally well defined and have large somata that are readily identifiable on the basis of their size (50  $\mu\text{m}$ –1 mm), position, and coloration. Moreover, injured adult molluscan neurons have the innate propensity to recapitulate their developmental patterns of connectivity with remarkable accuracy<sup>1</sup>. Although the snail models lag behind their fly and worm counterparts regarding the genetic know-how of synaptic connectivity, they are nevertheless amenable to direct electrophysiological analysis at the level of single pre- and postsynaptic neurons.

### 3. SYNAPSE FORMATION: LESSONS LEARNED FROM VARIOUS MOLLUSCAN MODELS

Among the molluscan species used extensively to define cellular and synaptic mechanisms of neurite outgrowth, target cell selection, and specific synapse formation, are the pond snails *Lymnaea stagnalis*<sup>2,3</sup> and *Helisoma trivolvis*<sup>4</sup>, the land snail *Helix pomatia* and the sea hare *Aplysia californica*<sup>5,6</sup>. This chapter specifically focuses on various *in vivo* and *in vitro* techniques that are being used to reveal cellular and molecular mechanisms underlying synaptic connectivity.

#### 3.1. *In Vivo* Regeneration and Synaptic Connectivity

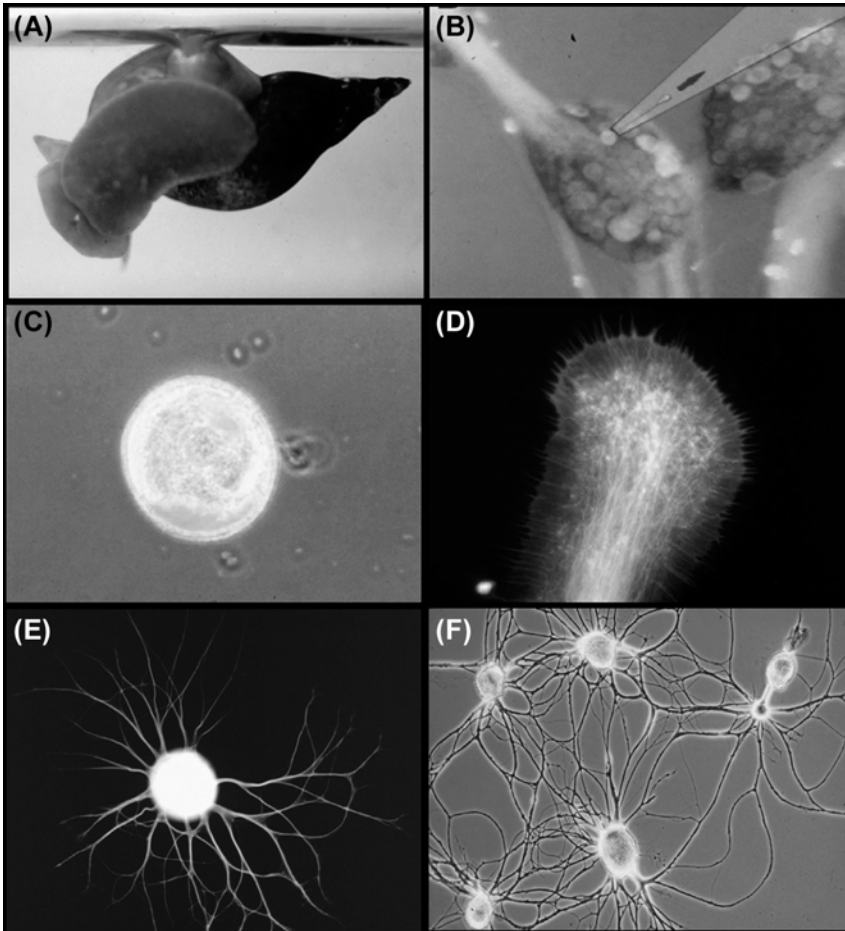
In contrast with vertebrate and other invertebrate species (*Drosophila* and *C. elegans*) where the mechanisms underlying specific synapse formation are generally investigated in developing animals, the adult mollusks with their inherent regenerative capacity are most favored for similar studies. Behaviorally defined, injured neurons from a variety of molluscan species such as *Melampus*<sup>7</sup>, *Lymnaea*<sup>8</sup> (Figure 2.1A; Colorplate 2) *Helisoma*<sup>9,10</sup>, and *Aplysia*<sup>11</sup> have been shown to regenerate their axonal projections, not only *in vitro*, but also in the intact animals. In most instances, regeneration from injured central neurons is complete and results in functional recovery<sup>7</sup>. However, the mechanisms underlying specific synapse formation could still not be elucidated in these intact preparations, due mainly to the intricacies of the intact brain and the lack of knowledge regarding various intrinsic and extrinsic factors that facilitate synapse formation *in vivo*. A variety of *in vitro* cell culture techniques were thus developed that enabled the extraction of uniquely identifiable neurons from the CNS/central ring ganglia. These approaches have since revealed that individually isolated neurons not only regenerate their neuritic processes in cell culture, but also develop specific synapses which are similar to those seen *in vivo*. This innate propensity of molluscan neurons to reconnect *in vitro* has enabled a number of laboratories to define various steps and mechanisms underlying synapse formation.

### 3.2. The Molluscan Cell Culture Techniques

Large molluscan neurons, such as those of *Lymnaea* (Figure 2.1A; Colorplate 2), are often clearly discernable in the central ring ganglia, even under a dissection microscope. Neuronal identification is facilitated by their size, coloration (white to red), and unique position within the ganglia. Following enzymatic treatment, which softens the connective tissue surrounding the ganglia, and subsequent mechanical removal of the inner sheath that encapsulates the neurons, individual somata can be extracted by applying gentle pressure through a suction pipette attached to a micromanipulator (Figure 2.1B). The isolated neurons (somata with their intact axon stumps) are then plated either on plastic or poly-L-lysine-coated glass coverslips attached to tissue culture dishes (Figure 2.1C). Whereas *Lymnaea* and *Helisoma* brain conditioned medium (CM – contains trophic factors), prepared by incubating central ring ganglia (2 brains/ml) in defined medium (DM) over a period of several days, is used to induce neurite outgrowth from their respective neurons, most *Aplysia* neurons are first cultured in defined medium (DM – does not contain trophic factors), with hemolymph (*Aplysia* blood) added later to promote sprouting. Within hours of neuronal plating in growth permissive medium, growth cones (Figure 2.1D) emerge either from the axon stump or from the soma itself (Figure 2.1E). Although these molluscan growth cones are much larger in size (50–100  $\mu$ M), they are structurally and functionally similar to their vertebrate counterparts. When cultured in CM, molluscan neurons exhibit extensive outgrowth (Figure 2.1F) and develop synapses that are similar to those seen *in vivo*.

### 3.3. *In Vitro* Reconstruction of Neuronal Networks

Several studies have shown that specific synaptic connections between pairs of pre- and postsynaptic neurons can reform in cell culture. True synapse specificity is, however, best tested at the network level, where neurons are concurrently challenged with multiple partners. It is also imperative to demonstrate that functionally defined networks of neurons are able to generate patterned activity in a manner similar to that of *in vivo*. The molluscan models were the first in which networks of functionally defined circuits were reconstructed in culture. Specifically, the neural network mediating gill withdrawal reflex in *Aplysia* was reconstituted in cell culture where the neurons not only re-established specific connections with their select partners but also exhibited synaptic plasticity that underlies learning and memory in the intact animals<sup>12</sup>. Similarly, the *Lymnaea* preparation was the first in which a three-cell network, comprising the respiratory central pattern generator (CPG) was reconstructed in culture. The co-cultured respiratory neurons not only re-established their specific synapses *in vitro*, but they also generated rhythmical patterned activity that was similar to that seen *in vivo*<sup>13</sup>. This *in vitro* reconstructed CPG has since been used to define fundamental mechanisms of synapse formation as well as respiratory rhythm generation. These studies underscore the importance of molluscan models for elucidating mechanisms underlying not only network connectivity but also how such interconnected neurons generate rhythmical patterns in a manner similar to those seen in the intact brain.



**Figure 2.1.** Cell Culture Techniques. (A) The pond snail *Lymnaea stagnalis*. (B) A cell extraction pipette is positioned to isolate individual neuronal somata. The arrow depicts neuronal extraction in progress. (C) An acutely isolated *Lymnaea* neuron in culture. (D) A *Lymnaea* growth cone fluorescently labeled with actin (red) and tubulin (green) antibodies. (E) An isolated neuron exhibiting neurite outgrowth overnight. (F) Interspecies synapse formation between *Lymnaea* and *Helisoma* neurons. A neuronal “hybrid” network reconstructed from two different snail species. Original copyright notice: Figure 2.1A reproduces Figure 3A on page 543 in *J Comp Physiology A*, volume 169, from the article by Syed, N.I., Harrison, D., and Winlow, W. (1991) Respiratory behavior in the pond snail *Lymnaea stagnalis*. Figures 2.1B, C and E reproduce Figures 2B, C (page 363), and 4C (page 372), respectively, of the book on *Modern Techniques in Neuroscience Research* (1999) Eds: Windhorst, U. and Johansson, H. Authors: Syed, N.I., Zaidi, H., and Lovell, P. Chapter 12: *In vitro* reconstruction of neuronal networks: a simple model system approach. All figures are reproduced with kind permission of Springer Science and Business Media. See Colorplate 2.

### 3.4. Transmitter–Receptor Interactions: A Mechanism for Synapse Specificity

When co-cultured with their synaptic partners, most molluscan neurons recapitulate their patterns of synaptic connections<sup>1</sup>. Intracellular recordings, concomitant with time lapse imaging of growth cones, revealed that synapses form within an hour of contact between pre- and postsynaptic neurons<sup>14</sup>. The process of specific target cell selection and subsequent synapse formation was shown to be facilitated by transmitter–receptor interactions between the approaching growth cones. Perturbation of either presynaptic transmitter release (dopamine) or postsynaptic neurotransmitter receptors, affected target cell selection but did not completely block synapse formation. It is important to note that transmitter release from the growth cone of an identified presynaptic *Lymnaea* neuron (Right Pedal Doral 1 = RPeD1) not only attracted growth cones from synaptic partners, but it also repelled nonpartner growth cones, thus avoiding synapse formation with inappropriate (synapses that do not exist *in vivo*) targets<sup>14</sup>. More recently, blocking cholinergic receptors during soma–axon pairing in *Lymnaea* was also shown to perturb synapse formation in cell culture<sup>15</sup>. It therefore seems safe to suggest that transmitter–receptor interactions between regenerating molluscan neurons play important roles in target cell selection and synapse formation, which in turn may define the early patterns of synaptic connectivity.

### 3.5. Synaptic Hierarchy: A Putative Mechanism for Determining Synapse Specificity

During early synapse formation, target cell contact is known to bring about specific changes in presynaptic transmitter release, and these changes can range from coupling of the secretory machinery to action potentials in snails<sup>16</sup>, to switching of transmitter phenotype in rats<sup>17</sup>. The *Lymnaea* model has uncovered yet another novel interaction that occurs between reciprocally connected neurons during early synapse formation. Specifically, the two respiratory neurons visceral dorsal 4 (VD4) and RPeD1 paired in culture establish mutual inhibitory synaptic connections within 24 h. However, when examined during early stages of synapse formation (12–18 h), the cell VD4 wins over RPeD1 by being the first to establish inhibitory synapses with RPeD1. This is achieved through VD4-induced suppression of transmitter release from RPeD1. This suppression is transient and it involves peptide release from the VD4<sup>18</sup>. Once VD4 has fully established synapses with RPeD1, its suppressive control over RPeD1's secretory machinery is lifted, thus enabling RPeD1 to release transmitter and hence establish its inhibitory synapses with VD4. This study provides a unique example of “synaptic hierarchy” whereby neurons such as VD4 which control higher order behaviors (such as cardio-respiratory) outcompete other neurons for target occupancy. Together, these studies from *Lymnaea* show that transmitter–receptor interactions can serve many important developmental roles in mollusks, ranging from target cell selection, synapse formation, to establishing the synaptic hierarchy.

### 3.6. Interspecies Synaptogenesis between Molluscan Neurons

The molluscan preparations were the first in which it was demonstrated that the mechanisms underlying specific synapse formation are conserved across

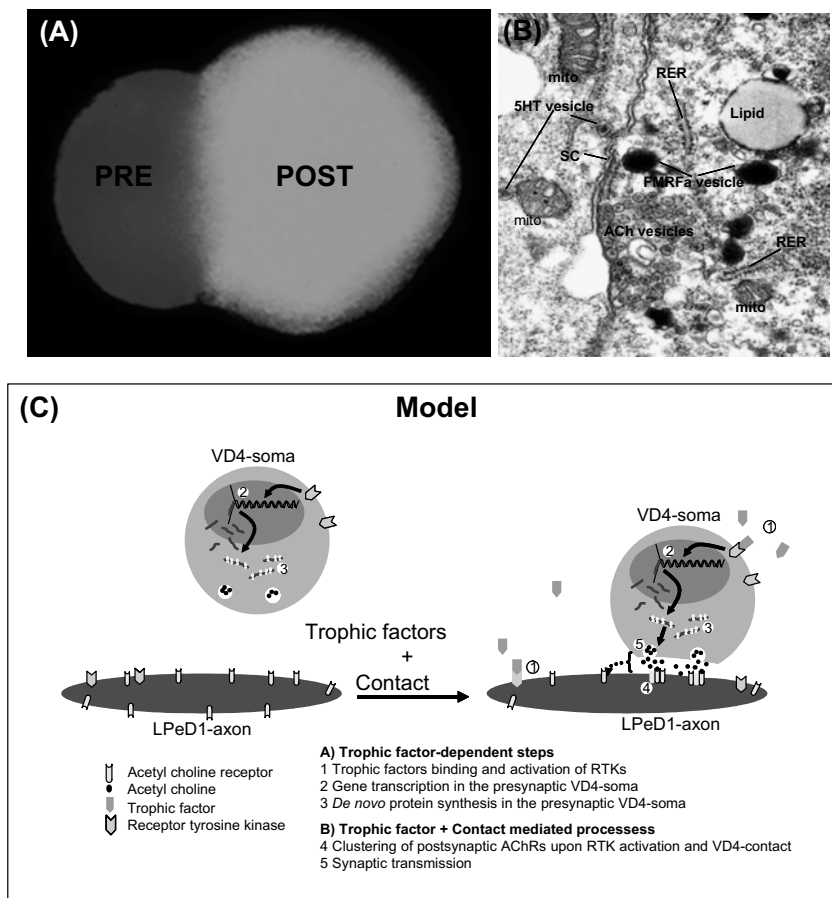
different snail species. Specifically, the giant dopaminergic neurons located in the right and left pedal ganglia of the snails *Lymnaea* and *Helisoma*, respectively, are considered homologs and innervate a variety of target cells in their respective species. To test whether the giant dopamine cells were indeed homologs, the *Lymnaea* dopamine cell (RPeD1) was paired with the postsynaptic targets of the *Helisoma* dopamine cell. The cells were allowed to exhibit outgrowth, which resulted in the formation of synapses between *Lymnaea* and *Helisoma* neurons<sup>19,20</sup> (Figure 2.1F). This study involving “neuronal hybrids” not only provided unequivocal evidence regarding the homologous nature of these cells but also demonstrated that the mechanisms of target cell selection and specific synapse formation are most likely conserved across the two related molluscan species. Therefore, it could be suggested that specific presynaptic neurons serving similar functions in a variety of molluscan species, may follow a common synaptogenic program to an extent that neuronal mixing and matching does not interfere with the mechanisms regulating synapse specificity in two different snails.

### 3.7. The Soma–Soma Synapse Model

Even in simpler model systems such as mollusks, synapses that develop between neurites are located at some distance from the somata and are thus inaccessible for direct morphological and electrophysiological analysis. Moreover, the precise timing and the numbers of synapses in these preparations are also difficult to define fully. Therefore, molluscan preparations were explored further to develop synapses between the somata, in the absence of outgrowth – an approach that was pioneered in leech<sup>21,22</sup> and subsequently refined in the snail *Helisoma*<sup>23</sup>. Specifically, Haydon<sup>23</sup> used identified neurons from *Helisoma* and juxtaposed their somata in culture. Indeed, chemical synapses developed between the somata in the absence of neurite outgrowth. Although the synapses developed between soma–soma paired *Helisoma* neurons did not exist in the intact brain, this model did nevertheless provide important insights into mechanisms of synapse formation, at a resolution that had not been attained before. Feng and colleagues<sup>24</sup> paired functionally well-defined, pre- and postsynaptic neurons from *Lymnaea* in a soma–soma configuration. The soma–soma paired *Lymnaea* neurons (Figure 2.2A; Colorplate 3) developed appropriate inhibitory, cholinergic connections overnight, and these synapses were target cell specific and both structurally (Figure 2.2B) and electrophysiologically similar to those seen *in vivo*<sup>24</sup>. In addition, voltage-induced,  $\text{Ca}^{2+}$  hotspots were shown to develop between soma–soma paired cells during synapse formation and these  $\text{Ca}^{2+}$  specializations were both target cell and contact site specific<sup>25</sup>. Using FM1-43 dye, which is taken up at the active synaptic site through endocytosis, the presynaptic secretory machinery was demonstrated to be specialized at the contact site between the paired cells<sup>26,27</sup>. Together, these data show that soma–soma synapses are both structurally and functionally similar to their neurite–neurite and *in vivo* counterparts, and are thus suitable for further studies on synapse formation.

### 3.8. Trophic Factors, Synapse Formation, and Synaptic Plasticity

*In vivo*, the processes of neurite outgrowth and synapse formation rely upon the availability of various trophic factors. Because neurite outgrowth precedes synapse formation, the involvement of growth factors in synaptogenesis, independent of neurite outgrowth, cannot be studied directly.



**Figure 2.2.** Mechanisms Regulating Synapse Formation in Various Model Preparations. (A) *Lymnaea* presynaptic neuron (visceral dorsal 4 – VD4 – fluorescently labeled with red dye, sulforhodamine) and postsynaptic neuron (left pedal dorsal 1 – LPeD1 – injected with Lucifer yellow) were soma–soma paired. In this configuration, most molluscan neurons develop appropriate excitatory and inhibitory synapses similar to those seen *in vivo*. (B) An electron micrograph showing the nature of synaptic contacts between soma–soma paired neurons. Vesicles dock at presynaptic site juxtaposed against the postsynaptic cell (Figure courtesy of Dr. Matthias Amrein, University of Calgary). (C) Model depicting steps and mechanisms underlying trophic factor and target cell contact-induced synapse formation between *Lymnaea* neurons in a soma–axon configuration. The model predicts that both target cell contact and extrinsic trophic support are required for appropriate, excitatory synapse formation. See Colorplate 3.

However, the soma–soma model permitted the study of trophic factor’s effects on synapse formation in the absence of neurite outgrowth. When paired in DM, inhibitory synapses between the identified neurons VD4 and RPeD1 developed<sup>24</sup>. However, attempts to reconstruct excitatory synapses between VD4 and its other partner left pedal dorsal 1 (LPeD1) failed in DM. Under these experimental conditions, the neurons established inhibitory synapses, which were inappropriate



and do not exist *in vivo*. In contrast, pairing in CM enabled appropriate excitatory synapses to develop between VD4 and LPeD1. This trophic factor-induced formation of excitatory synapses was mediated through receptor tyrosine kinases<sup>28,29</sup>. Similarly, the addition of trophic factors to pairs that developed inappropriate inhibitory synapses in DM resulted in a switch to appropriate excitatory synapses. This synapse switching also required receptor tyrosine kinase activity<sup>29,30</sup>. These findings have since been confirmed in the land snail *Helix* where appropriate, excitatory synapses were shown to rely upon the availability of specific trophic factors as well<sup>31</sup>. Utilizing novel synapses between soma–axon pairs (presynaptic soma paired with a somaless postsynaptic axon) from *Lymnaea*, it was subsequently shown that the trophic factor-induced excitatory synapse formation involves mobilization of excitatory, postsynaptic acetylcholine receptor from extrasynaptic to synaptic sites<sup>32</sup>. Together, these studies underscore the importance of trophic factor-mediated signaling in synapse formation and synaptic plasticity.

The precise identity of synapse-specific trophic molecules in *Lymnaea* is yet to be determined. However, the growth-promoting effects of human EGF (hEGF) on *Lymnaea* neurons led to the search for an EGF homolog in *Lymnaea*<sup>33</sup>. The *Lymnaea* albumen gland was found to be a rich source of *Lymnaea* EGF (L-EGF) and, when added to neurons *in vitro*, L-EGF exerted distinct growth-promoting effects on select neurons<sup>33</sup>. Addition of L-EGF to DM containing soma–soma<sup>28</sup>, and soma–axon pairs<sup>32</sup> resulted in the formation of excitatory synapses, which were similar to those seen in CM. These results strongly indicate that L-EGF may be a major component of the CM-derived trophic factors that mediate excitatory synapse formation between paired neurons. However, L-EGF induces synapse formation between only 40% of the paired neurons<sup>28</sup>, thus the search continues for the remaining complement of synapse-specific factors present in CM.

### 3.9. Synaptogenic Program Suppresses Neurite Outgrowth

It is generally believed that contact between growth cones from pre- and post-synaptic neurons results in the cessation of neurite outgrowth and the formation of specific synaptic contacts. It is therefore generally believed that neurite outgrowth and synapse formation may be two reciprocally inhibitory programs. Nowhere else is it better illustrated than in the soma–soma model. Single identified neurons plated in CM exhibit extensive sprouting, whereas identical neurons maintained in CM and paired with a specific synaptic partner failed to extend processes<sup>34</sup>. This suppression of neurite outgrowth from soma–soma paired cells is however transient and as the synapse matures fully, the neurons begin extending process in a manner similar to their single counterparts<sup>34</sup>. The snail model thus provides a clear evidence for direct, inhibitory interactions between neurite outgrowth and synapse formation.

### 3.10. Synapse-Specific Protein Synthesis, Gene Induction, and Synaptic Plasticity

#### 3.10.1. Gene Transcription

Because soma–soma paired neurons failed to extend neurites as compared to their single unpaired counterparts, a search began to identify genes in both single and paired neurons that might be differentially regulated under these two different



experimental conditions. Quantitative polymerase chain reaction (QPCR) techniques were utilized and this approach resulted in the identification of a molluscan homolog of the multiple endocrine neoplasia type 1 (*MEN1*) tumor suppressor gene, which encodes the transcription factor *menin*. This specific gene was found to be upregulated in soma–soma paired neurons during synapse formation<sup>35</sup>. To demonstrate the significance of *menin* in synapse formation, *MEN1* mRNA was selectively knocked down with antisense in paired neurons *in vitro*. *MEN1* perturbations completely blocked both excitatory and inhibitory synapse formation. Interestingly, cell-specific knock-down of *MEN1* mRNA revealed that *menin* expression was required only in the postsynaptic neuron<sup>35</sup>. The *MEN1* gene is the first such gene that has been shown to be essential for synapse formation between *Lymnaea* neurons. Consistent with the notion that neurite outgrowth and synapse formation are mutually exclusive, the BERP gene that is thought to be involved in neurite outgrowth in vertebrates<sup>36</sup> was found to be upregulated in single, regenerating *Lymnaea* neurons and downregulated in soma–soma paired cells. Therefore, the BERP gene may regulate neurite outgrowth from *Lymnaea* neurons<sup>37</sup>, though its inhibitory effects on the synaptogenic program remain to be determined.

### 3.10.2. Protein Synthesis

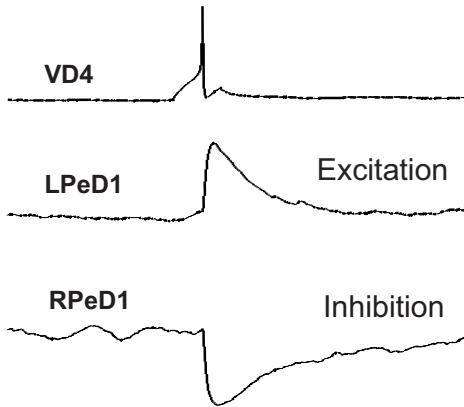
In addition to transcription of specific genes, synapse formation between soma–soma pairs also requires *de novo* protein synthesis<sup>24,28,29</sup>. However, the precise site where this protein synthesis is required (pre- versus postsynaptic cell) remained unknown. A step toward resolving this issue was taken by Meems and colleagues<sup>32</sup> who used soma–axon pairs, consisting of a presynaptic cell body paired with a postsynaptic axon severed from its respective cell body. The removal of postsynaptic cell body alone did not affect synapse formation between the pairs. However, when presynaptic somata were removed, the synapses failed to develop in cell culture<sup>32</sup>. These data suggest that during early synapse formation between soma–axon pairs, the gene transcription and protein synthesis is required in presynaptic soma but not the postsynaptic axon. All that is needed of the postsynaptic axon is a redistribution of neurotransmitter receptors<sup>32</sup>. Specifically, presynaptic target cell contact facilitates the mobilization of postsynaptic cholinergic receptors from extrasynaptic to synaptic sites, independent of gene transcription or protein synthesis, although it does require trophic factors (Figure 2.2C; Colorplate 3). Taken together these studies underscore the importance of trophic factors and site-specific gene transcription and protein synthesis in synapse formation.

### 3.10.3. Synaptic Plasticity and Synapse Formation

In *Lymnaea*, the synaptic plasticity-induced formation of long-term memory (LTM) in the intact animals is both transcription and translation dependent<sup>38</sup>. Interestingly, the locus for this transcription and translation-dependent process is confined to one single neuron termed RPeD1. Ablation of its cell body in the intact animals completely blocks memory formation<sup>39</sup>. Similarly, long-term facilitation (LTF), which is thought to be the underlying mechanism for LTM in *Aplysia*, has also been extensively studied and a variety of molecules and underlying mechanisms identified. One of the key issues is to define the precise locus for new protein synthesis during plasticity. For instance, how is a single synaptic connection subjected to modification in a neuron that has multiple synaptic connections? To

address this question, a single bifurcated sensory neuron with two branches was allowed to make synapses *in vitro* with two spatially separated motor neurons. In this configuration individual synapses could be selectively exposed to plasticity-inducing stimuli. LTF was induced by repeated stimulation with the neurotransmitter serotonin (5-HT) at one of the synaptic sites, whereas the other synapses were left untreated. This paradigm revealed modification only of the treated synapse and indicated that a single neuron can undergo branch-specific LTF. Furthermore, this branch-specific, synaptic modification is dependent upon gene transcription and local (at the synapse) protein synthesis. Interestingly, protein synthesis was only necessary in the presynaptic but not the postsynaptic terminals<sup>40</sup>. While altered gene transcription likely serves all synaptic connections formed by any given neuron, the local protein synthesis occurs only at synapses that are subjected to plasticity-specific stimuli. The products of altered gene expression and local protein synthesis thus collectively account for the changes that underlie LTF. Consistent with this idea are studies in which cell bodies from *Aplysia* neurons were removed after synapse formation to determine the contributions of local protein synthesis in synaptic plasticity. The synapses were exposed to LTF-inducing stimuli and changes in synaptic efficacy at synapses either with or without presynaptic somata were compared, showing that somaless axons exhibit LTF similar to their intact counterparts<sup>41</sup>. However, the LTF induced in the somaless configuration appeared to be transient. It was thus suggested that local protein synthesis in axons accounts only for initial changes in synaptic efficacy, whereas gene transcription was required for the maintenance of LTF<sup>41</sup>. These results underscore the importance of gene transcription and protein synthesis in synapse formation as well as in long-term changes in synaptic efficacy.

The search for the molecular machinery that contributes to gene transcription and protein synthesis-dependent LTF has led to the identification of a variety of well-known kinases. In *Helix* for instance, synapsin (a synaptic vesicle-associated phosphoprotein) was found important for increased efficiency of neurotransmitter release in neurons that were otherwise cultured under low-release conditions<sup>42</sup>. Furthermore, a *Helix* synapsin ortholog cloned from *Aplysia* (ApSyn) was mutated on its phosphorylation sites in search of ApSyn substrates that are involved in synaptic plasticity. ApSyn appeared to be an excellent substrate for cAMP-dependent protein kinase. Injection of wild-type ApSyn in identified *Helix* neurons cultured under low-release conditions resulted in increased neurotransmitter release, whereas injection of mutant ApSyn failed to do so<sup>43</sup>. These data indicated that cAMP-dependent protein kinase may be an essential player involved in the induction of synaptic plasticity. Earlier, *Aplysia* protein kinases A and C were shown to be essential for the formation of 5-HT-induced LTF<sup>44-47</sup>, whereas more recently mitogen-activated protein kinase (MAPK) has also been implicated in the induction of increased synaptic efficacy<sup>48,49</sup>. Moreover, MAP kinase was shown to translocate to the nucleus<sup>50</sup>, suggesting that the site for MAPK action could reside within the nucleus, where it may be involved in transcribing various mRNAs and the translation of their encoded proteins. Taken together, a variety of kinases appear to be involved in the cellular and molecular changes underlying synaptic efficacy, although the exact order of these events remains to be elucidated.



**Figure 2.3.** Synapse Specificity Between Multiple *Lymnaea* Neurons. (A) Presynaptic neuron VD4 paired simultaneously with its inhibitory (RPeD1) and excitatory (LPeD1) partners recapitulates appropriate synapses in cell culture. An action potential in VD4 simultaneously induces 1:1 excitatory (LPeD1) and inhibitory (RPeD1) postsynaptic potentials (PSPs) in synaptic targets. Both synaptic responses involve cholinergic transmission between VD4 and its target neurons.

### 3.11. Regulation of Synapse Number and Synaptic Scaling

An important unanswered question in the field of neurodevelopment and regeneration is the following: How is synapse number and efficacy regulated? That is, how does a neuron “know” that it has acquired its full complement of synapses that would be required for functionality? It is hypothesized that the synaptic efficacy is regulated globally and enhances synaptic output nonselectively<sup>51,52</sup>. Alternatively, synaptic efficacy could also be modulated locally and selectively at specific synaptic sites<sup>40,53</sup>. Similarly, the number of synapses that any given neuron establishes could also be regulated either globally or locally. To this end, single isolated LPeD1-axons were paired with two identical presynaptic VD4 neurons. Electrophysiological recordings demonstrated that in this configuration only one VD4 formed synaptic connections with the isolated axon<sup>15</sup>. Since the isolated axon is devoid of its cell body, the synapse numbers are likely regulated locally by the axon itself. To address this issue further, we isolated a single, presynaptic neuron from the *Lymnaea* central ring ganglia and challenged it with two identical postsynaptic targets. Specifically, a single VD4 neuron was simultaneously soma-soma paired with two postsynaptic LPeD1 neurons. In the intact brain VD4 connects with only one LPeD1. Interestingly, under these experimental conditions only one postsynaptic cell received innervation from VD4 during the first 12–18 h of cell pairing. In contrast, when VD4 was paired with two different postsynaptic neurons, LPeD1 and RPeD1, it formed excitatory and inhibitory synapses, respectively, with both neurons (Figure 2.3). Similarly, when the LPeD1–VD4–LPeD1 triplet was examined during early stages of synapse formation (4 h), both LPeD1 cells were innervated by VD4. However, the efficacy of each individual synapse under these experimental conditions was a fraction of the monosynaptic strength exhibited among pairs and involved the cAMP–PKA-dependent pathway. Indeed, experimental activation of the cAMP–PKA pathway resulted in reduced synaptic efficacy, whereas inhibition of this cascade generated hyperinnervation and an enhancement of synaptic strength<sup>54</sup>. These data show that cAMP–PKA-dependent signaling plays a novel role in controlling synaptic efficacy and thus regulating single or multiple innervations. In mollusks, this may thus serve as one

of the mechanisms that ensure a balance between neuronal input and output capacities.

### **3.12. In Vivo Synapse Formation and Behavioral Recovery Following Single-Cell Transplantation**

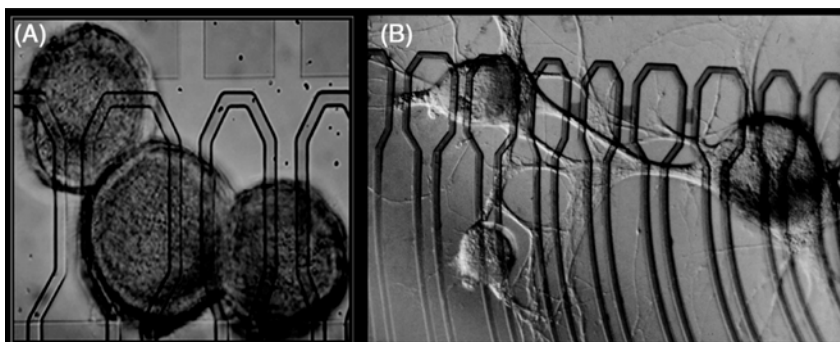
It can always be argued that the *in vitro* approach only offers an artificial environment, which bears little resemblance to the *in vivo* milieu that is pivotal for normal development. To determine whether the data obtained *in vitro* can be extrapolated to derive conclusions about fundamental principles governing synapse formation *in vivo*, single-cell transplantation techniques were developed in *Lymnaea*. Specifically, ablation of the single respiratory neuron VD4 in the intact animal rendered the snail unable to exhibit normal respiratory behavior<sup>55</sup>. This behavioral deficit was however restored by transplanting a VD4 neuron from a donor animal. The transplanted neuron subsequently recapitulated its pattern of synaptic connectivity and restored functional contacts with its target cells as well. Interestingly, when a VD4 was transplanted into the host in the presence of its native VD4, the newly transplanted cell failed to innervate its appropriate target and began making contacts with inappropriate target cells. These findings have been confirmed further by transplanting RPeD1 into the right parietal ganglia<sup>56</sup>, a location that is different from its native habitat (right pedal ganglia). Consistent with our previous study, the transplanted neuron would only connect with its targets if they were deprived of synaptic input from the host cell. Together, these data suggest that the neuronal ability to regenerate and recognize appropriate target neurons in cell culture involves fundamental mechanisms that are likely operative in the intact brain.

## **4. THE FUTURE OF MOLLUSCAN MODELS: FROM PROTEINS AND GENES TO SILICON CHIPS**

The ability to manipulate single molluscan neurons holds tremendous potential toward the identification and characterization of various genes and their encoded proteins regulating synapse formation. One possibility for future exploration will be the identification and characterization of a full complement of both pre- and postsynaptic proteins involved in synapse formation. This functional proteomics approach has already enabled the identification and characterization of a whole array of proteins within specific compartments of select mammalian neurons<sup>57,58</sup>. Although these mammalian neurons hold tremendous potential for identifying synapse-specific proteins, the molluscan neurons due to their larger somata, are perhaps better suited for such functional proteomics approaches. For instance, Jimenez and colleagues<sup>59</sup> have recently successfully identified and characterized a full complement of neuronal neuropeptides at the level of a single cell. This approach can now be employed to identify various proteins during synapse formation – at the level of single pre- and postsynaptic neurons. These proteins can then be manipulated experimentally to determine their exact involvement in synapse formation and synaptic plasticity.

Finally, a new approach to investigate connectivity of multiple neurons concurrently and noninvasively is the utilization of silicon chip technologies. The neuron and silicon chip interfacing allows noninvasive examination of large neuronal ensembles during synapse formation (Figure 2.4). Specifically, the

*Lymnaea* model was recently used to successfully interface individual neurons with silicon chips and a bidirectional communication was established between brain cells and this electronic device<sup>60</sup>. This approach now provides an unprecedented opportunity to examine the role of activity-dependent mechanisms in synapse formation and synaptic plasticity at a resolution that has never been attained before.



**Figure 2.4.** Synapse Formation as Revealed Through Brain–Chip Neuron Interfacing. (A) Identified *Lymnaea* neurons can be successfully interfaced with silicon chips (Kaul, Syed, Fromherz, unpublished data). The cultured cells can either be soma–soma paired (A) or allowed to extend neurites to develop networks (B). This approach has been used to reconstruct specific synapse on the chip which was subsequently used to stimulate and record synaptic activity and plasticity.

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