VISUALIZATION OF CELLULAR MECHANISMS REGULATING DIFFERENTIAL NEURONAL SYNAPSE FORMATION

A Thesis

by

JOSHUA PAUL NEUNUEBEL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

August 2004

Major Subject: Biology

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Approved as to style and content by:

Mark J. Zoran (Chair of Committee)	Vincent M. Cassone (Member)
Robert C. Burghardt (Member)	Vincent M. Cassone (Head of Department)

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ABSTRACT

Visualization of Cellular Mechanisms Regulating Differential Neuronal Synapse Formation. (August 2004)

Joshua Paul Neunuebel, B.S., Texas A&M University

Chair of Advisory Committee: Dr. Mark J. Zoran

Over thirty years ago electrical coupling was observed in embryonic cells prior to chemical communication. This temporal relationship of electrical coupling preceding functional chemical neurotransmission occurs throughout neurogenesis, prompting the idea that gap junctional coupling synchronizes the synaptogenic establishment of functional neural networks. *Helisoma* neuronal pairs treated with trophic factors exhibit increased electrical coupling and subsequently delay the formation of inhibitory chemical connections. Studies in this thesis addressed the mechanism regulating this inverse relationship between electrotonic and chemical communication.

Synaptogenesis between two neurons from the *Helisoma* buccal ganglia, B110 and B19, were examined using alternative culturing conditions that were either exposed to or deprived of trophic factors. Incubating neuronal pairs in trophic factors induced transient electrical synapses and postponed the formation of chemical connections. In electrically coupled neuronal pairs, presynaptic secretory vesicles were recruited to the sites of presynaptic contact, but did not respond to calcium elevation (i.e., photolytic release of calcium from NP-EGTA) with neurotransmitter release. These and other studies demonstrated that transient electrical coupling does not disrupt calcium handling or postsynaptic responsiveness. Rather, electrotonic coupling delays chemical synaptic

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transmission by imposing a functional block between the accumulation of presynaptic calcium and the synchronized vesicular release of neurotransmitter.

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to my parents
who continue to inspire...

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ACKNOWLEDGMENTS

In a manner as sincere as possible, I would like to pay tribute to the plethora of friends and family that remained supportive throughout the entire longevity of graduate school. First and foremost, I must acknowledge my primary investigator. Dr. Mark Zoran, in my opinion, is arguably the best mentor that naïve students could have help guide them in research driven science. His ability to teach the essential techniques necessary to address the questions in science, explain the fundamental knowledge in the field, and provide instructions for a colorful writing style has helped mold many graduate students for successful scientific careers. Despite failing at his best attempts of cruelty by suggesting difficult experiments, Mark is an extremely fair and kind man. We even discussed the smaller points in life, such as baseball games between the Cubs and Astros. All in all, Mark is an extremely kind and patient man. I believe that every person that joined the Zoran lab surely benefited from his advice. Additionally, the guidance from my committee members, Vincent Cassone and Robert Burghardt, about technical experimental protocols and presentation styles need copious amounts of recognition.

Further thanks must be bestowed upon my lab mate, Veronica Martinez. Veronica and I formed a support group during the trying periods in the Zoran lab, but celebrated all the breakthroughs. Her advice was scholarly and exceptionally insightful. Without her, the duration of my Texas A&M graduate school education would have easily doubled. In addition, my beloved family deserves considerable recognition for generating a falsified interest in neuroscience as I described the trials and tribulations of my research. Whenever a reprieve from the lab was needed, I must thank my friends for their company at Mad Hatters, Zapatos, and the Fox. I guess that at this point in time it is

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safe to say these intermissions occurred relatively frequently; however, in our defense, we primarily discussed science. Finally, I must thank my exceptionally intelligent and beautiful girlfriend, Ramona Aldea, who continued to date me even during the period when I was obsessed with writing a flawless thesis. Thanks for everything!

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CHAPTER I

GENERAL INTRODUCTION

Electrical coupling, mediated by gap junctions, exists transiently between neurons and targets in both developing and regenerating nervous systems. These transient electrical synapses constitute a temporary mechanism of cellular communication, where signaling occurs when ions and small molecules pass through junctional pores into neighboring cells (Veenstra et al., 1995; Nicholson and Bruzzone, 1997; Bennett and Zukin, 2004). Electrotonically coupled cells are present during the development of the spinal cord. For example, *Xenopus* neurons maintain electrical connectivity until Na^+ spikes emerge (Spitzer, 1982). The developing mammalian neocortex transiently expresses gap junctions, which possibly coordinate the mitotic phases in groups of clonally-related cells (Bittman et al., 1997; reviewed in Sutor, 2002). In addition to embryonic development, transient electrical synapses exist following axotomy of mammalian PNS neurons, which suggests a role in regeneration (Chang et al., 2000).

Despite appearing in both developing and regenerating nervous systems, the role of these short-lived intercellular connections remains elusive. Since transient electrical coupling often occurs prior to the formation of chemically transmitting synapses, a role for gap junctional coupling in the synaptogenic establishment of functional neural networks has been suggested (reviewed in Kandler and Katz, 1995). A temporal pattern of electrical communication preceding chemical connectivity exists during the development of frog neuromuscular junctions (Allen and Warner, 1991), both vertebrate and invertebrate visual systems (Curtin et al., 2002; Penn et al., 1994), and mouse motor neurons (Personius et al., 2001). Several recent studies have implicated a functional interaction between these two forms of neurotransmission during synaptic development. First, chemical neurotransmission is inhibited in neonatal rats as electrical coupling increases (Mentis et al., 2002; Pastor et al., 2003). Second, trophic factor-induced enhancement of electrical connectivity delays the onset of functional chemical neurotransmission at regenerating synapses (Szabo et al., 2004).

This thesis follows the style and format of Neuron.

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Helisoma motor neurons B19 and B110, while regenerating synapses in cell culture, exhibit transient electrical connections that are maintained for several days. Intercellular electrical coupling is subsequently replaced by inhibitory chemical synaptic transmission at these synapses and the acquisition of functional neurotransmission is correlated with decline in gap junctional communication (Szabo et al., 2004). This inverse relationship between electrical and chemical transmission is virtually identical to the pattern of synaptogenesis observed between these neurons following axotomy *in vivo* and suggests that electrical synaptic mechanisms facilitate the exclusion of functional chemical neurotransmission at developing synapses. Since the nature of both electrical and chemical synapse formation has been extensively examined in *Helisoma* neurons (Bulloch et al., 1980; Bulloch and Kater, 1981; Hadley et al., 1982, 1983, 1985; Haydon and Kater, 1988; Zoran et al., 1996) and giant somatic synapses allow for precise spatial and temporal resolution of synaptic properties (Haydon and Zoran, 1989; Hamakawa et al., 1999; Szabo et al., 2004), I have used this system to test the hypothesis that electrical coupling mediates the functional exclusion of chemical neurotransmission at synaptic contacts formed between *Helisoma* neurons 110 and 19 in cell culture.

This thesis has been divided into four chapters. Following this brief introduction to the research topic (Chapter I), I will summarize the critical background literature (Chapter II) necessary to provide the reader with a fundamental knowledge of electrical and chemical neurotransmission and a historical perspective on research in synaptic transmission, especially as it applies to this master's research. I will also describe the *Helisoma* buccal nervous system and the relevant aspects of this animal's neurophysiology, cell biology, and neuroethology. Finally, I will indicate the relevance of this project to the field of medical science.

Chapter III embodies the bulk of my research, excluding several preliminary and functional experiments that will not be discussed in this venue. This section is written in scientific manuscript format, following the style of the journal Neuron, and is subdivided into conventional sections. The studies described in Chapter III have examined the potential role of electrotonic/biochemical coupling in the 1) regulation of voltage-dependent calcium accumulation, 2) shunting of presynaptic calcium by cell to cell diffusion, 3) regulation of postsynaptic receptor sensitivity, and 4) the developmental

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mobilization of the presynaptic secretory vesicles. My data suggest that transient electrical coupling does not disrupt these fundamental mechanisms of chemical neurotransmission, but rather mediates a functional block between the accumulation of presynaptic calcium and synchronized, vesicular release of neurotransmitter.

The final section (Chapter IV) is a general conclusion that addresses the role that transient electrical synapses might play in shaping a neural network and intertwines my results with current opinions in neural development.

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CHAPTER II

LITERATURE REVIEW

HISTORICAL DEBATE

Soon after Ramon Cajal and others had established that nervous systems consists of interconnected cells called neurons, research and argument became focused on how these neurons communicate with each other. One side, composed largely of pharmacologists, favored a chemical means of transmission. They were opposed by a group of electrophysiologists that supported the direct transfer of a presynaptic electrical signal into the postsynaptic cell. From 1910 to 1911, Henry Dale and George Barger discovered and synthesized structures from extracts of ergot fungus that mimicked autonomic nerve stimulation. Further evidence supporting the existence of chemical neurotransmitters came in 1920 when Dale's friend and collaborator, Otto Loewi, performed a landmark but controversial experiment. Loewi excised a frog heart with the vagus and accelerator nerves still attached, and then a second heart that lacked innervation. After stimulating the vagus nerve, the media from the first heart was transferred to the bathing solution of the second heart. This exogenous media caused a decrease in the contraction rate of the second heart, mimicking the effect that vagal stimulation had on the first. Loewi claimed that a chemical messenger was released from these nerves, which he called Vagusstoff. Electrophysiologists responded with the argument that the duration of time required to release a chemical neurotransmitter and generate a postsynaptic response could not account for the rapid signaling necessary in the central nervous system. Nonetheless, acetylcholine was soon shown to be the principal chemical neurotransmitter at vertebrate neuromuscular junctions (Dale and Dudley, 1929) and sympathetic and parasympathetic

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ganglia (Dale and Feldberg, 1934a, 1934b). Additionally, the level of hyperpolarization recorded with intracellular microelectrodes could not be induced electrically, but inhibition in the CNS resulted from chemical transmission (Brock et al., 1951). These studies helped convince skeptics that chemical neurotransmission was a viable form of communication, even those vocally-opposed to the idea such as Sir John Eccles. Yet the belief that the propagation of an eddy current from pre- to postsynaptic neurons was not lost.

In the late fifties, a wealth of information supported the notion that electrical synapses could mediate excitatory signals. Akira Watanabe revealed that electrotonic transmission occurs in cardiac ganglion neurons of *Squilla*. This form of electrical coupling was proposed to synchronize neuronal firing as the signal passed through a cytoplasmic continuity (Watanabe, 1958). Another example of electrical transmission, the quintessential finding, was observed in the crayfish giant fiber system in which an excitatory current mediates a rapid escape reflex (Furshpan and Potter, 1959). The first example of electrical synapses in a vertebrate model system, discovered by Michael Bennett, Stanley Crain, and Harry Grundfest, were located in the supramedullary neurons of the pufferfish (reviewed in Bennett, 1997). Clearly, evidence for dual forms of neuronal communication exists in the nervous system, but these signaling processes serve different functions throughout neural networks. A summary of the structure and function of each follows.

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ELECTRICAL SYNAPSES

After the discovery of intracellular gap junctional communication in the crayfish (Furshpan and Potter, 1959), critics claimed that electrical coupling was limited to lower invertebrates needing to synchronize their reflexes; however, this was not the case. In 1971, Baker and Llinas revealed electrotonic coupling in the mesencephalic trigeminal nucleus of the mammalian midbrain/brainstem, which gave rise to an explosion of discoveries exposing the vast coupling within the nervous system. For example, observation of extensive coupling occurred in the forebrain, in the regions of the external plexiform (Landis et al., 1974), internal granular layers (Reyher et al., 1991), piriform cortex (De Zeeuw et al., 1997), neocortex (Sloper, 1972), hippocampus (MacVicar and Dudek, 1981), dentate gyrus (MacVicar and Dudek, 1982), dorsal septal nucleus (Phelen et al. 1993), nucleus accumbens (O'Donnell and Grace, 1993), striatum (Cepeda et al., 1989), and the Islands of Calleja (Ribak and Fallon, 1982). Additionally, electrical synapses exist in the paraventricular and supraoptic nuclei of the diencephalons (Andrew et al., 1981) and the cerebellar cortex (Sotelo and Llinas, 1972), substantia nigra (Grace and Bunney, 1983), locus coeruleus (Travagli et al., 1995), and inferior olive (Llinas et al., 1974). Gap junctional intracellular communication is not limited to the brain, but also extends into other areas of the nervous system. Both the retina and spinal cord possess the ability to communicate in this manner (Vaney, 1994; Rash et al. 1996). In 1967 Michael Bennett used the pufferfish to demonstrate that connexins were the ultrastructural component of electrical transmission (Bennett et al., 1967a, 1967b), which became an excellent model system to study the structure and method of communicating in electrical synapses.

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Gap junctions provide the physical bases for the formation of regulatory syncytia between juxtaposed cells with respect to ions and small molecular weight molecules. The vertebrate gap junctional composition consists of a hemichannel (connexons), which contains six oligomerized connexins in the membrane of a cell, pairing with the connexons of an adjacent cell (Laird, 1996; Kumar and Gilula, 1996; Alexander and Goldberg, 2003). In invertebrates, innexin proteins oligomerize forming the hemichannels (Phelan and Starich, 2001), but maintain similarity to vertebrate gap junctional proteins both in topology and function (Phelan et al, 1998; Landesman et al., 1999; Stebbings et al., 2000). For vertebrates, connexins are a family of proteins indispensable in pore formation. These proteins consist of four hydrophobic domains spanning across the cellular membrane (reviewed in Harris, 2001). The amino-and carboxy-terminals, in addition to the loop linking the second and third membrane spanning regions, are accessible from the cytoplasm (Milks et al., 1988; Yancey et al. 1989; Laird and Revel, 1990; Yeager and Gilula, 1992). Whereas, the domains between first and second and between the third and fourth are accessible from the extracellular space (Goodenough et al., 1988; Milks et al., 1988; Laird and Revel, 1990). In Cx43, each hydrophobic segment is an alpha helical structure revealed from electron cryo-microscopy (Unger et al., 1997). By analogy, the corresponding domains in the other connexins are presumed to be α-helical, while the only significant difference in this family of proteins results from variations in the length of the carboxy-terminal (Harris, 2001; Table 1). Different permutations of these proteins oligomerize creating half the gap junctional channel.

Structurally, gap junctional channels are quite diverse. For example, homomeric,

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Table 1. Distribution of Connexin Expression in the Central Nervous System

Cellular types	Connexin	Reference
Astrocytes	Cx26	Alvarez-Maubecin et al., 2000
	Cx30	Nagy et al., 1999
	Cx40	Dermietzel et al., 2000
	Cx43	Dermietzel et al., 1989
	Cx45	Dermietzel et al., 2000a
	Cx46	Dermietzel et al., 2000b
	Cx47	Rouach et al., 2002
Oligodendrocytes	Cx29	Sohl et al., 2001
	Cx32	Dermietzel et al., 1989
	Cx36	Parenti et al., 2002
	Cx45	Dermietzel et al. 1989
Neurons	Cx26	Dermietzel et al., 1989
	Cx32	Dermietzel et al., 1989
	Cx36	Condorelli et al., 1998
	Cx37	Chang et al., 1999
	Cx40	Chang et al., 1999
	Cx43	Nadarajah et al., 1996
	Cx45	Chang et al., 1999
	Cx47	Teubner et al., 2001
Neuronal	Cx26	Bittman and LoTurco, 1999
Precursors	Cx33	Rozental et al., 1998
	Cx40	Rozental et al., 1998
	Cx43	Rozental et al., 1998

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heterotypic, and heteromeric junctional channels are three different motifs. For homomeric junctional channels, both connexon arrangements consist of a single connexin isoform (Harris, 2001). These conduits have radial symmetry around a central pore that is approximately 40 Å at the cytoplasmic lumen, tapers to roughly 15 Å, and then expands to 25 Å within the extracellular vestibule (Yeager and Nicholson, 1996). These dimensions permit molecules under 2 kDa to diffuse into a neighboring cell (Phelan and Starich, 2001). The two hemichannels are not aligned so that homologous connexin monomers are positioned directly above each other, but instead one channel is rotated 30 degrees; thus, staggering the proteins so that each monomer is directly positioned between two monomers in the apposing hemichannel (Unger et al., 1997; Perkins et al., 1998). Heterotypic junctional channels have one hemichannel containing only a single connexin isoform and the other channel has a different connexin isoform repeat (i.e., channel one contains six Cx38s and the second hemichannel contains six Cx41s). Heteromeric hemichannels are hemichannels composed of multiple different connexins (Harris, 2001). These structural deviations provide cells with opportunity to regulate channel physiologies and govern the permeability to ions and other messengers propagating through the porous junction.

Electrotonic transmission transpires when a presynaptic neuron depolarizes and the influx of ions diffuse into the postsynaptic cell. Similar to chemical synapses, voltage-gated ion channels must produce enough current to surpass the threshold necessary to generate an action potential, and also generate sufficient ionic current to produce a change in postsynaptic membrane potential. Based on the experiments of Furshpan, Potter, and Watanabe, electrical transmission mediates two different functions:

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(1) transmitting excitation from an active axon to a postsynaptic cell, and (2) synchronizing activity of cell bodies, in which coupling is both excitatory to the less depolarized cell and inhibitory to the more depolarized cell, since current flowing to depolarize one cell is making the other cell less depolarized (Bennett, 1997). The passage of eddy current into the postsynaptic neuron requires a high degree of regulation that is conducted by phosphorylating or dephosphorylating the carboxy-terminal region of connexins. Many of the connexins not only contain consensus phosphorylation sequences for protein kinases, but experiments also showed that this enzymatic activity occurred on the carboxy-terminal of assorted connexins (Lampe and Lau, 2000). The crucial influence of the carboxy-terminal region on channel function was illustrated when a truncated Cx43 mutant in *Xenopus* oocytes exhibited different permeability and electrophysiological properties than those formed by wild-type Cx43 (Dunham et al., 1992). Differential effects on the gating of gap junctional communication occur resulting from variations in both the method and object of phosphorylation. When phosphorylating Cx32 and subsequently increasing gap junctional communication, T84 cells physiologically respond with increases in fluid secretion (Chanson et al., 1996). In pancreatic cells expressing Cx45, an increase in electrotonic communication occurred only in the presence of a functional cAMP-gated chloride channel (Chanson et al., 1999). Gap junctional communication increases after some connexins are phosphorylated; however, this is not the case for all connexins. Cx43 channels reconstituted into lipid vesicles were phosphorylated by purified MAP kinase and the permeability of the liposomes decreased significantly (Kim et al., 1999). The phosphorylation of Cx43 by PKC, stimulated by the phorbol ester tumor promoter (TPA), decreased dye coupling in

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rat epithelial cells; thus, implying that TPA disrupts gap junctional communication after protein phosphorylation (Lampe et al., 2000). Additionally, EGF treatment of rat liver epithelial cells resulted in a transient disruption of gap junctional communication that correlated with a marked increase in phosphorylation of Cx43 (Lau et al., 1992). Obviously, gap junctional communication is a tightly regulated process necessary for synchronizing neural networks both during and after development.

ELECTRICAL SYNAPSES IN DEVELOPMENT

Intercellular coupling is prevalent during vertebrate embryogenesis. For example, cells in the neural fold of amphibians are permanently coupled to other neural cells and evanescently coupled to surrounding ectodermal cells (Warner, 1973). *Xenopus* neurons are extensively coupled during the differentiation of the neural tube into the spinal cord; however, coupling decreases following this developmental progression (Spitzer, 1982). This pattern of transient coupling preceding chemical connectivity is also common throughout the development of the rat brain (Connors et al., 1983; Christie et al., 1989; Walsh et al., 1989; Walton and Navarrete, 1991). The timing of this phenomenon fluctuates in different neuronal populations, insinuating that in different systems, electrotonic transmission contributes to different functions or interferes with new neuronal processes (Kandler and Katz, 1995; Table 2). Uncoupling occurs simultaneous to synaptogenesis in rat spinal cord neurons (Walton and Navarrete, 1991), cerebral cortex (Connors et al., 1983; Peinado et al., 1993), and locus coeruleus (Christie et al., 1989). Furthermore, the chick otocyst sensory epithelium exhibits ephemeral coupling before and during early synapse formation (Ginzberg and Gilula, 1979). Invertebrates

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Table 2. Expression of Transient Electrical Coupling

System	Time	Location	Possible Function	References
Frog	Development	spinal cord	synchronizes motor neurons	Spitzer, 1982
Frog	Development	nmj	establishing a functional neural networks	Allen and Warner, 1991
Grasshopper	Development	DUM neurons	coordinates differentiation	Goodman and Spitzer, 1979
Rat	Development	spinal cord	synchronizes motor neurons	Walton and Navarrete, 1991
Rat	Development	cerebral cortex	???	Conors et al., 1983
Rat	Development	cerebral cortex	temporal coordination of neuronal ensembles during circuit formation	Peinado et al., 1993
Rat	Development	locus coeruleus	synchronize subthreshold activity	Christie et al., 1989
Rat	Development	retina	synchronize biochemical activity	Penn et al., 1994
Rat	Development	neostriatum	contributes to the differentiation and growth of neurons	Walsh et al., 1989
Rat	Regeneration	motor neurons	may mediate signaling until reestablishing synaptic connections	Chang et al., 2000
Cat	Regeneration	motor neurons	may mediate signaling until reestablishing synaptic connections	Chang et al., 2000
Snail	Regeneration	motor neurons	Delays chemical neurotransmission	Szabo et al., 2004

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also display similar patterns of electrical synapse formation during development. For instance, downregulating gap junctional communication in DUM neurons of the grasshopper correlates with the appearance of Na^+ spikes (Goodman and Spitzer, 1979). In *Helisoma*, both *in vitro* and *in vivo*, transient electrical coupling occurs prior to the onset of chemical neurotransmission (Szabo et al., 2004). The demonstration of specific neuronal coupling patterns during synaptogenesis raises the possibility that gap junctions can create functional communication compartments within neuronal populations, which might guide the subsequent development of organized synaptic connections (Kandler and Katz, 1995). Transient electrical coupling might precede chemical communication in many systems, but the mechanism prompting the switch remains enigmatic.

CHEMICAL SYNAPSES

Chemical neurotransmission depends on synaptic vesicles fusing with the presynaptic membrane and exocytosing their vesicular contents following an influx of calcium. Synaptic vesicles consist of two functionally distinct domains referred to as the reserve and readily-releasable pools. These two pools differ in the strength of stimulation necessary for release, location in the neuron, and the relative sizes of the clusters. Vesicles in the reserve pool, approximately 100-200 nm from the active zone, cluster together and bind to actin utilizing synapsin proteins (Brodin et al., 1997; Pieribone et al., 1995; Takei et al., 1995; Hilfiker et al., 1999). This synapsin-dependent pool requires a high frequency (18-20 Hz) stimulation to generate transmitter release; ablating the pool by injecting synapsin antibodies causes release to occur during low frequency stimulation (Pieribone et al., 1995). Using bafilomycin A_1 to prevent the reuptake of transmitter, data

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support the notion that the readily-releasable pool recruits synaptic vesicles from the larger reserve pool following high frequency tetanic stimulation (Kuromi and Kidokoro, 2003). On the other hand, the readily releasable pool differs considerably from the reserve pool and other fusion-competent vesicles in different neurons. At mammalian hippocampal synapses, application of a hypertonic solution generates vesicle exocytosis from the readily-releasable pool (Rosenmund and Stevens, 1996); whereas, goldfish retinal bipolar neurons require a short pulse of strong depolarizing current for neurotransmission (von Gersdorff et al., 1996). The readily-releasable vesicles at the *Drosophila* neuromuscular junction are defined as the endo-exo cycling pool and released either by injecting a depolarizing current or through high K^+ depolarization (Kuromi and Kidokoro, 1998). In essence, the readily-releasable pool consists of a small number of synaptic vesicles that are capable of fusing with the adjoining membrane and is secreted in an activity-dependent fashion.

The complicated process of releasing chemical neurotransmitters from the active zone requires precise temporal and spatial coordination of the synaptic proteins that govern calcium-dependent exocytosis. Synaptic vesicles undergo a five step process during neurotransmission, which is regulated through a series of phosphorylation/dephosphorylation enzymatic pathways, and consists of vesicular mobilization, priming, docking, fusion, and endocytosis (reviewed in Li and Chin, 2003; Table 3, Table 4). Vesicles from the reserve pool are recruited after detaching from the actin cytoskeleton following the phosphorylation of a linker protein, synapsin I (Doussau and Augustine, 2000). A decreased affinity for both actin filaments and synaptic vesicles results from Ca2+/calmodulin-dependent protein kinase II (CaM kinase II) inducing a

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Table 3. Enzymatic Phosphorylation of Synaptic Proteins

Name	Enzyme	Reference
Mobilization		
Synapsins		
I	CaMKI, PKA, MAPK, CaMKII	Greengard et al., 1993; Jovanovic et al., 1996
II	CaMKI, PKA	Greengard et al., 1993
Docking		
Rab 3	???	
Rabphilin	CaMKII, PKA	Fykse and Sudhof, 1995
PRA1	???	
Calmodulin	???	
Exocyst	???	
Priming		
RIM	???	
Synaptobrevin	CaMKII, PKC	Bennett et al., 1993
SNAP-25	CaMKII, PKC	Hirling and Scheller, 1996
Syntaxin	CK2	Nielander et al., 1995; Shimazaki et al., 1996
Munc18/nSec1	PKC, Cdk5	Fujita et al., 1996; Shuang et al., 1998
Complexins	???	
Munc13	???	
Fusion		
Synaptotagmin	CK2, CaMKII	Bennett et al., 1993; Popoli, 1993
Endocytosis		
NSF	CaMKII	Hirling and Scheller, 1996
SNAP	CaMKII, PKA	Hirling and Scheller, 1996
Staring	???	

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Table 4. Synaptic Proteins Affiliated With Chemical Neurotransmission

Name	Functions	Reference
Mobilization		
Synapsins	phosphorlation permits vesicles to move into active zone	Doussau and Augustine, 2000
Docking		
Rab 3	traffics vesicles to active zones; couples exocytosis and endocytosis	Nonet et al., 1997; Geppert et al., 1997; Coppola et al., 2001b
Rabphilin	GTP-bound Rabs effector protein; regulates exocytosis and endocytosis	Burns et al., 1998; Coppola et al., 2001a
PRA1	Rab3 effector protein; involved in inserting synaptobrevin into vesicles; targeting	Martincic et al., 1997; Gougeon et al., 2002
Calmodulin	effector protein; Rab3 and synaptic vesicle dissociation	Park et al., 1997
Exocyst	large conglomerate of proteins that aid in vesicle docking	Hsu et al., 1999
Priming		
RIM	coordinates the components that regulate transmission	Hibino et al., 2002
Synaptobrevin	SNARE integral membrane protein located on synaptic vesicles	Trimble et al., 1988
SNAP-25	part of SNARE complex localized to target membrane	Oyler et al., 1989
Syntaxin	part of SNARE complex bound to target membrane; binds synatotagmin & N-type Ca^{2+} channel	Bennett et al., 1992
Munc18/nSec1	prevents closed conformation of syntaxin from interacting with synaptobrevin and SNAP-25	Dulubova et al., 1999
Complexins	regulate fast Ca^{2+}-triggered release,	Reim et al., 2001
Munc13	switches syntaxin to open conformation	Betz et al., 1997
Fusion		
Synaptotagmin	calcium sensing protein that mediates vesicle fusion	Mackler et al., 2002
Endocytosis		
NSF	disassembles SNARE complex; regulates number of readily releasable vesicles	Sollner et al., 1993
SNAP	regulates number of readily releasable vesicles	Tolar and Pallanck, 1998
Staring	regulates degradation of syntaxin 1 through ligase activity	Chin et al., 2000
Cytoskeleton		
Bassoon	scaffolding protein; helps assemble machinery	Dresbach et al., 2001
Piccollo	scaffolding protein; helps assemble machinery	Dresbach et al., 2001

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conformational change in the protein (Benfenati et al., 1990; Schiebler et al. 1986; Bahler and Greengard, 1987). After separation, the vesicles become competent to migrate to the active zone and dock with the presynaptic membrane.

Synaptic vesicles in the docking stage of transmission enter the active zone and join the readily-releasable pool. This cytomatrix of the active zone contains a high density of a derivative of the cortical cytoskeleton and presumed to direct vesicles to the plasma membrane (Dresbach et al., 2001). Rab3 and exocyst, two key players in docking, regulate vesicular guidance. When GTP binds to Rab3, a structural change occurs that permits the protein to bind to synaptic vesicles and guide them to the membrane (Pfeffer, 2001). Data gathered from *C. elegan* Rab3 loss-of-function mutants support the notion that Rab3 functions as a guidance molecule, since synaptic vesicles accumulate in the axon and levels decrease at the synaptic bouton (Nonet et al., 1997). Exocyst, a large conglomerate of proteins, initiates the tethering of vesicles to the membrane (Pfeffer, 1999). Once in the proper position, the vesicles enter a priming stage.

Exocytosis requires fusion competent vesicles to make contact with the presynaptic membrane, preparing the vesicle for release. Soluble N-ethylmaleimide-sensitive fusion protein receptors (SNAREs), three structures comprising the core of the exocytic machinery, are necessary for this process. In the SNARE hypothesis, the vesicle bound synaptobrevin (v-SNARE) forms a stable four-helix bundle with the target membrane receptors, syntaxin (t-SNARE) and SNAP-25, and effectively pulls the two membranes into close proximity (Sollner et al., 1993; Sutton, 1998). SNAP-25 links the two integral membrane receptors to each other, but conformational changes in syntaxin

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regulate the process. In isolation, the amino-terminal of syntaxin folds onto the carboxy-terminal, which increases the affinity for Munc18, an inhibitory protein (Dulubova et al., 1999). This structural modification prevents interaction with the core complex until Munc13 causes t-SNARE and Munc18 to dissociate (Betz et al., 1997). After the SNARE complex associates, the vesicles are fusion competent and await a signal to initiate the release of chemical neurotransmitter.

The rapid process of chemical communication, approximately 200 μs from Ca^{2+} influx to postsynaptic receptor response, requires a rapid kinetic response in the sequence of events governing exocytosis. Following membrane depolarization in mammalian systems, voltage gated calcium channels switch to the open conformational state, thus creating an influx of calcium (Hanlon and Wallace, 2002). Surprisingly, calcium entry in *Lymnaea* and other invertebrates depends on a splice variant of the calcium channel that interacts with the adaptor proteins Mint-1 and CASK (Spafford et al., 2003). Synaptotagmin, the calcium sensing protein, possess a weak affinity for syntaxin before calcium ions enter the cell; however, ionic influx results in a tighter binding between the two proteins (Koh and Bellen, 2003). Additionally, calcium increases the affinity between synaptobrevin and the core complex (Hu et al., 2002). The compact configuration of the four proteins causes the two membranes to fuse, creating a pore that permits the preliminary release of neurotransmitters, and eventually, the neuronal membrane incorporates the entire vesicular membrane (Koh and Bellen, 2003). After exocytosis, NSF and SNAP disassemble the components of the synaptic vesicles and the cycle of chemical neurotransmission is repeated (Weber et al., 2000).

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MIXED SYNAPSES

Active neuronal communication does not exclusively result from either chemical or electrical transmission, but in many instances dual forms of communication at the same synapses are prevalent. Mixed synapses reside in many invertebrate nervous systems (Maniya et al., 2003), in the spinal cord of both mammalian (Rash et al., 1996) and primitive fishes (Christensen, 1983). Additionally, electrotonic and chemical postsynaptic potentials exist in Mauthner cells of the goldfish (Lin and Faber, 1988). These composite, mixed synapses function as a single synaptic unit and are thought to act to synchronize the firing of neurons within a network and subserve rhythm generation. The electrical component of the synapse might provide a less use-dependent communication; whereas, the chemical component might show facilitation and depression of synaptic transmission.

HELISOMA MODEL SYSTEM

The buccal ganglia and associated structures in feeding behavior have been extensively examined to determine the physiological properties regulating the function of neural networks (Bulloch and Kater, 1981; Bulloch et al., 1980; Hadley et al., 1982, 1983, 1985; Haydon and Kater, 1988; Zoran et al., 1996). The neuronal model predicted to pattern feeding requires triphasic neuronal activity (Quinlan and Murphy, 1991, 1996; Quinlan et al., 1995, 1997). These phases include protraction (phase 1), retraction (phase 2), and hyper-retraction (phase 3), in which neurons are either inhibited or excited (reviewed in Murphy, 2001). In neuron B110, excitation occurs during both retraction and hyper-

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retraction of the radular feeding structure. This motorneuron peripherally innervates the SLrT muscle via extending axonal projections through the ventrobuccal and laterobuccal nerves. Another neuron, B19, also has axonal branches in the ventrobuccal nerves that innervate the SLrT; however, excitation occurs during the hyper-retraction phase. Despite innervating the same peripheral muscle, neurons B110 and B19 do not elicit direct neuronal communication after development; this is not the case during regeneration.

Helisoma motor neurons B19 and B110, while regenerating synapses in cell culture, exhibit transient electrical connections that are maintained for several days. Intercellular coupling is subsequently replaced by inhibitory chemical synaptic transmission at these synapses and the acquisition of functional neurotransmission is dependent on the suppression of gap junctional communication (Szabo et al., 2004). This inverse relationship between electrical and chemical transmission is virtually identical to the pattern of synaptogenesis observed between these neurons following axotomy *in vivo* and suggests that electrical synaptic mechanisms facilitate the exclusion of functional chemical neurotransmission at developing synapses. A bidirectional electrical connection forms between neurons B19 and B110 two days following commissural crush, and is only faintly detectable by the fourth day (Szabo et al., 2004). As electrical coupling declines, a unidirectional chemical connection from neuron B110 onto neuron B19 emerges (Szabo et al., 2004). By 5 weeks, these connections are lost (Szabo et al., 2004). This *in vivo* phenomenon is recapitulated in cell culture by manipulating cells into contact and then exposing the cell pairs to trophic factors. These trophic factors induce temporary electrotonic coupling between neurons, but the ability to generate an action-potential

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evoked change in the postsynaptic membrane is impeded. Consequently, it was hypothesized that electrical coupling mediated the functional exclusion of chemical neurotransmission at synaptic contacts formed between *Helisoma* neurons B110 and B19 in cell culture.

HYPOTHESES

One of the implications of transient electrical coupling during development is that specific chemical synapses, perhaps at discrete locations, might be delayed in their formation. Three plausible circumstances could explain the mechanism in which electrical coupling impedes chemical neurotransmission. First, gap junctional proteins might spatially prevent neurotransmitter machinery from accessing the presynaptic membrane. Second, gap junctions might functionally exclude the components necessary to generate neurotransmitter secretion. Finally, the presence of electrical synapses may exclude the development of secretory machinery.

The ultrastructural changes to chemical synapses in the presence of junctional coupling remain unclear; however, the structural compositions of neurons and glia in fully developed buccal ganglia have been resolved. The axonal processes of neuron B19 contain gap junctions (Berdan et al., 1987). Gap junction formation varies depending on cell type, but it arises from the insertion of hemichannels into the plasma membrane utilizing either polymerized actin or molecular motors transporting vesicles along intact microtubules (Johnson et al. 2002; Lauf et al. 2002). These channels continue to accumulate at the periphery of existing gap junctions and contribute to gap junctional growth; thus forming gap junction plaques (Gaietta et al. 2002; Lauf et al., 2002).

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Therefore, we hypothesize that the junctional machinery present in the synaptic membrane could interfere with the incorporation of presynaptic secretory machinery into this same area (i.e., a physical exclusion hypothesis). That is, the spatial requirements of gap junctional plaques might exclude the placement of chemical synaptic machinery; thus, impeding the release of chemical neurotransmitter.

A second mechanism explaining the inverse relationship between the increase in electrotonic coupling and the inhibition of chemical connectivity might result from functionally excluding molecules necessary for synaptic transmission. This idea suggests that gap junctions would function as a conduit for calcium ions. During action potential induced elevations, ions would readily diffuse down their concentration gradient from the presynaptic to postsynaptic cytoplasm. Elevating the concentration of calcium to a minimum of 1.0 μM is mandatory for generating cholinergic neurotransmitter release in *Aplysia californica* (Ohnuma et al., 2001); therefore, such a molecular sink would decrease local concentrations and reduce the probability of action potential-evoked neurotransmitter release. Indeed, morphologically mixed synapses and sites of colocalized chemical and electrical synaptic machinery, are not necessarily indicative of dual transmission (Lin and Faber, 1988; Rash et al., 1996).

A third mechanism explaining inhibition of chemical neurotransmitter release might involve the exclusion of secretion machinery through the transient disruption of crucial developmental events. In embryonic vertebrate neurons synaptic vesicle protein synthesis increases as a result of elevating mRNA levels and escalating the rate of translational initiation (Daly and Ziff, 1997). This suggests that regulation could occur not only in the nucleus, but also in the cytosol. Additionally, the distribution of synaptic

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proteins is initially confined to the soma and neurites, but then redistributed during the developmental progression of synaptogenesis (Basarsky et al., 1994). The exclusion hypothesis therefore proposes that signaling pathways directly conducted through gap junctions could inhibit the maintenance of the chemical synaptic machinery. Alternatively, intrinsic developmental programs orchestrating the formation of electrical and chemical synapses might involve mutually exclusive molecular cascades. Here, transcriptional or posttranscriptional regulatory pathways might select for the production of one type of synaptic machinery over the other.

RELEVANCE TO MEDICAL SCIENCE

Approximately 250,000 people in America currently suffer from debilitating spinal cord injuries, with 47% becoming paraplegic and 52% quadriplegic. Recovering the lost function of injured neurons and damaged networks is crucial for significant physical rehabilitation. Since transient electrical connections form following nerve injury in vertebrate spinal motor neurons and exist until proper chemical connectivity is reformed, it is likely that electrical coupling plays a fundamental role in regulating nerve regeneration. Determining the basic physiological mechanisms underlying the relationship between electrical and chemical synapses during regeneration is critical to our understanding of these cellular events.

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CHAPTER III

ELECTROTONIC TRANSMISSION AT DEVELOPING SYNAPSES DISRUPTS CALCIUM-DEPENDENT EXOCYTOSIS DESPITE ENHANCING VESICLE MOBILIZATION

Electrical coupling, mediated by gap junctions, exists transiently between neurons and targets in both developing and regenerating nervous systems. These transient electrical synapses constitute a temporary mechanism of cellular communication, where signaling occurs when ions and small molecules pass through junctional pores into neighboring cells (Veenstra et al., 1995; Nicholson and Bruzzone, 1997; Bennett and Zukin, 2004). Electrotonically coupled cells are present in the development of the spinal cord, for example, *Xenopus* neurons maintain electrical connectivity until Na^+ spikes emerge (Spitzer, 1982). The developing mammalian neocortex transiently expresses gap junctions, which possibly coordinate the mitotic phases in groups of clonally related cells (Bittman et al., 1997; reviewed in Sutor, 2002). In the developing vertebrate visual system, both ions and second messengers pass through gap junctions; thus, establishing the idea that these channels synchronize biochemical activity (Kandler and Katz, 1998). In addition to embryonic development, transient electrical synapses exist following axotomy of mammalian PNS neurons, which suggests a role in regeneration (Chang et al., 2000). Despite appearing in both developing and regenerating nervous systems, the role of these short-lived intercellular connections remains elusive.

Since transient electrical coupling occurs at many developing synapses prior to the onset of chemical neurotransmission, a role for gap junctional coupling in the

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synaptogenic establishment of functional neural networks has been suggested (reviewed in Kandler and Katz, 1995). A temporal pattern of electrical communication preceding chemical connectivity exists during the development of frog neuromuscular junctions (Allen and Warner, 1991), both vertebrate and invertebrate visual systems (Curtin et al., 2002; Penn et al., 1994), and mouse motor neurons (Personius et al., 2001). Chemical neurotransmission is inhibited in neonatal rats, when electrical coupling increases (Mentis et al., 2002; Pastor et al., 2003). Furthermore, trophic factor-dependent expression of electrical connectivity delays the onset of functional chemical neurotransmission at regenerating synapses in *Helisoma* (Szabo et al., 2004).

Helisoma motor neurons B19 and B110, while regenerating synapses in cell culture, exhibit transient electrical connections that are maintained for several days. Intercellular coupling is subsequently replaced by inhibitory chemical synaptic transmission at these synapses and the acquisition of functional neurotransmission is dependent on the suppression of gap junctional communication (Szabo et al., 2004). This inverse relationship between electrical and chemical transmission is virtually identical to the pattern of synaptogenesis observed between these neurons following axotomy *in vivo* and suggests that electrical synaptic mechanisms facilitate the exclusion of functional chemical neurotransmission at developing synapses. Since the nature of both electrical and chemical synapse formation has been extensively examined in *Helisoma* neurons (Bulloch and Kater, 1981; Bulloch et al., 1980; Hadley et al., 1982, 1983, 1985; Haydon and Kater, 1988; Zoran et al., 1996) and giant somatic synapses allow for precise spatial and temporal resolution of synaptic properties (Haydon and Zoran, 1989; Hamakawa et al., 1999; Szabo et al., 2004), we have used this system to test the hypothesis that

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electrical coupling mediates the functional exclusion of chemical neurotransmission at synaptic contacts formed between *Helisoma* neurons 110 and 19 in cell culture. In the present studies, we have examined the potential role of electrotonic/biochemical coupling in the 1) regulation of voltage-dependent calcium accumulation, 2) shunting of presynaptic calcium by cell to cell diffusion, 3) regulation of postsynaptic receptor sensitivity, and 4) the developmental mobilization of the presynaptic secretory vesicles. Our data suggest that transient electrical coupling does not disrupt these fundamental mechanisms of chemical neurotransmission, but rather mediates a functional block between the accumulation of presynaptic calcium and synchronized, vesicular release of neurotransmitter.

EXPERIMENTAL PROCEDURES

Animals

Laboratory stocks of American pond snails, *Helisoma trivolvis*, were cultured in twenty gallon aquaria at 22ºC. Snail cultures were maintained on a 12 hour light / 12 hour dark photoperiod and fed daily on a combination of trout chow and lettuce.

Cell Culture

Two motor neurons, B110 and B19, were isolated from buccal ganglia based on location, pigmentation, and morphology as previously described (Zoran et al., 1991; Poyer and Zoran, 1996). Cells were placed in conditioned medium (CM) for three days, which allowed for absorption of original axons and the formation of spherical somata. CM was prepared by incubating two ring-ganglia per 1 mL of defined medium (DM) in sigmacoated (Sigma, St. Louis, MO) glass culture dishes. After three days, neurons were

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paired and transferred to 35 mm plastic culture dishes (No. 1008 Falcon) containing 2 mL of medium for 24 hours. The plastic culture dishes were made non-adhesive through pretreatment with a 0.5% solution of bovine serum albumin (BSA).

Electrophysiology

Electrophysiological experiments were conducted using intracellular, current-clamp techniques, while viewing and imaging preparations with an Olympus IX70 inverted microscope. Dual glass electrodes (Borosil 1.5 mm, FHC), filled with 1.5 M KCl (10-20 MΩ), were used for simultaneous recordings (pre-and postsynaptic) of neuronal pairs. Cell pairs were plated on poly-L-lysine (Sigma, St. Louis, MO) coated culture dishes (plastic or coverglass bottom) containing DM. The neuronal membrane potential was held at approximately -75 mV before base current injection. Electrical coupling was measured by injecting a hyperpolarizing step current using a Grass S48 stimulator and bridge-balanced electrometers (Model 5A, Getting Instruments, Inc.). Electrophysiological input signals were converted from analog to digital by MacLab software (ADInstruments) and a Macintosh computer. The digitalized data were archived and later analyzed with MacLab Chart software. Electrical coupling was calculated using the following formula:

$$ECC = (\Delta Vm \text{ postsynaptic cell}) / (\Delta Vm \text{ presynaptic cell}).$$

Digital Microscopy

Neurons were viewed at 40X magnification for differential interference contrast microscopy (DIC; objective, NA=0.6) or ratiometric calcium imaging (Fura-2 oil objective, NA=0.65). For Fura-2 experiments, excitation light was emitted from a computer-controlled Lambda DG4 monochromator (Sutter Instrument Company). The

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excitation light path included passage through 1.0 or 1.3 neutral density transmission filters and the excitation filters (340 and 380 nm). A dichroic mirror (400 nm) and emission filter (510 nm) were used for collection of emitted fluorescence. A digital CCD camera (OreaER; Hamamatsu) collected 2 X 2 binning images at variable exposures.

Intracellular Pressure Injections

Sharp glass pipettes, with a tip diameter ranging from 0.5-1.0 μm, were created from capillary tubes (Borosil 1.5 mm, FHC) and either filled with internal solutions of fluorescent calcium indicators or NP-EGTA preloaded with calcium. Solutions were pressure injected with a Picospritzer II (General Valve). The duration of the injection pulses were 5-10 msec at 20-30 psi. Solutions were injected until the diameter of the cell detectably increased by approximately 5-10%. Therefore, the final cytosolic concentrations were lower than that contained in the pipette.

Calcium Imaging

Cells were loaded for one hour with the cell permeable calcium-sensitive probe Fura-2 AM (Molecular Probes, Eugene, OR) at a final concentration of 5 μM in DM, or pressure injected (as previously described) with Fura-2, pentapotassium salt (Molecular Probes, Eugene, OR). The membrane permeable calcium dye was washed from the cells by transferring them through two, 30 min DM baths. After washing, the cells were plated on poly-L-lysine coated coverglass slides. Cell pairs injected with the cell impermeable version of Fura-2 were plated prior to loading.

Fura-2 fluorescence was viewed in the manner indicated earlier. SimplePCI software (Compix, Inc.) was used to determine F_{340}/F_{380} ratios for intracellular calcium concentrations from image pairs collected at rates of 2-4 pairs/second. A portion of the

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image devoid of cells, processes, or debris was sampled for background subtraction. Calcium concentrations were calculated using the ratio of Fura-2 excitation at 340 and 380 (Grynkiewicz et al., 1985) with the equation:

$$[Ca^{2+}] = K_d \times [R - R_{min} / R_{max} - R] \times (F_{max380} / F_{min380}).$$

$[Ca^{2+}]$ represents the intracellular free calcium concentration, K_d is the dissociation constant of calcium from the dye (224 nM), and R is the calculated ratio intensity. Zero and saturation calcium intensity ratios are denoted by R_{min} (0.251) and R_{max} (10.876), respectively. F_{max380} (210.8) is the fluorescence intensity measured at zero calcium, and F_{min380} (18.9) is the fluorescence intensity measured when the cells were saturated. These values were determined for this imaging system using cell free methods and Fura-2 calibration standards (Molecular Probes, Eugene, OR).

Calcium Manipulation

Flash photolysis of *o*-nitrophenyl EGTA (NP-EGTA, Molecular Probes, Eugene, OR), preloaded with calcium, was implemented to experimentally manipulate intracellular calcium concentrations. Presynaptic neurons were pressure injected (as previously described) with a solution that contained 40 mM NP-EGTA, 32 mM $CaCl_2$, and 10 mM Hepes at a pH of 7.4. Neurons were allowed to recuperate from the injection for 15-30 minutes. After obtaining resting calcium levels for ten seconds (as formerly mentioned), electronically controlled shutters (Prior) were opened for 2-3 seconds exposing cells to UV light emitted from an Olympus mercury vapor lamp. Ratiometric image pairs were captured before and after UV exposure for calcium estimations.

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UV-Evoked Neurotransmitter Release

Neurotransmitter release was estimated by calculating the total area under the postsynaptic potential (PSP) trace following UV photolysis of NP-EGTA. Area under the PSP curve was determined by analyzing images of postsynaptic electrophysiological recordings (Adobe Photoshop 6). A contiguous line was extended that connected the extrapolated baseline (resting potential) to the depolarizing postsynaptic membrane potentials. Image J (NIH Image) software was used to calculate the total area in pixels encompassed by this line. A relative neurotransmitter release index was determined for both CM and DM preparations, where an index of 1 equals 1000 pixels of area under a PSP curve.

Postsynaptic Receptor Sensitivity

Neuronal pairs were cultured in CM or DM for 24 hours and then transferred to a poly-L-lysine coated 1008 culture dish containing DM. Acetylcholine (10 μM; Sigma, St. Louis, MO), contained in a glass pipette (~1 μm tip) positioned 5-10 μm from the postsynaptic cell membrane, was focally applied to the B19 with 5 msec pulses provided by a Picospritzer II (30 psi). Tubocurarine chloride (3.75-10 μM; Sigma, St. Louis, MO) antagonized the effects of acetylcholine at these synapses.

Synaptic Vesicle Imaging

Synaptic vesicles were stained with FM 1-43 (Molecular Probes, Eugene, OR) to monitor vesicle recruitment during synapse formation. Prior to cell contact, nascent presynaptic neurons (B110s) were incubated for five minutes in a solution of high potassium DM (50 mM KCl) and 2.5 μM FM 1-43. Negative control cells were depolarized in high K^+ DM that lacked the synaptic vesicle stain. Following the depolarization-facilitated loading,

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the cells were washed in DM. The loading and wash procedure was repeated two additional times to insure pervasive vesicle staining. B110s were cultured, either isolated or with B19s, in the appropriate medium. After plating on coverglass dishes containing DM, fluorescence images were acquired using rhodamine optics (excitation: 530-550 nm; dichroic mirror: 570 nm; emission: 590 nm) and digital photography. Fluorescence intensity was determined for individual neurons. Regions of interest (ROI) were digitally selected and the background intensity was subtracted. An arbitrary point on a single cell was chosen for the first ROI (7 μm in diameter). Subsequently, every 30 degrees (clockwise and counter clockwise) an additional ROI was analyzed. Two groups consisting of the average of three points proximal to the synapse and three distally located ROIs were chosen to determine synaptic vesicle distribution in neuronal pairs. ROIs were spaced 30 degrees apart in each group.

Data Analysis

SPSS software (SPSS Inc.) was used to analyze experimental data sets. Mann-Whitney or Wilcoxon Signed tests were implemented for data analysis as indicated in the text. Values are displayed as the mean plus or minus the standard error of the mean (SEM). Statistical significance was defined as $p < 0.05$.

RESULTS

Giant somatic synapses, derived from the nervous system of *Helisoma*, exhibited differences in intercellular coupling when cultured with and without exposure to trophic factors in conditioned medium (Fig. 1). An electrical coupling coefficient (ECC) of 0.34 ± 0.05 was calculated for cell pairs cultured in CM, medium containing trophic factors.

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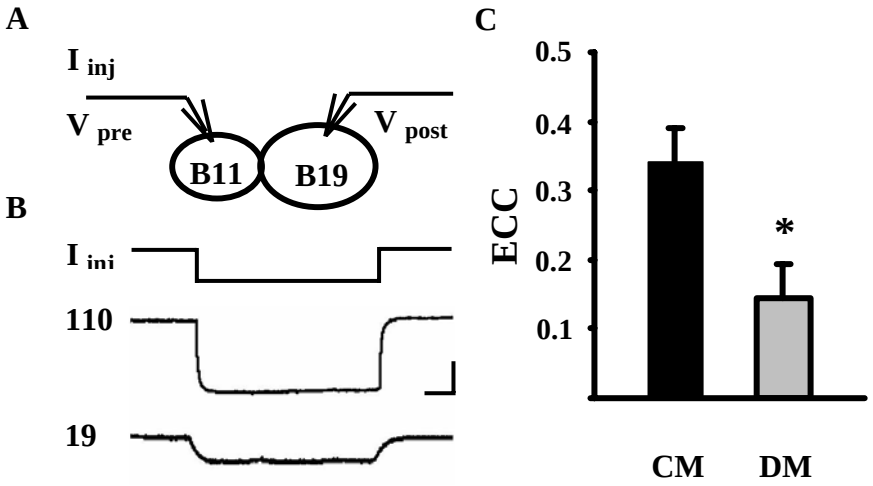

Figure 1.
Trophic factors promote electrical synapse formation. (A) This schematic diagram of neuronal somata pairs, B110 and B19, demonstrates the electrophysiological recording configuration for manipulation and assessment of presynaptic and postsynaptic membrane potentials. Hyperpolarizing current (I_{inj}) was injected into the presynaptic neuron B110 for the purpose of monitoring electrical coupling. (B) Representative recordings of electrical coupling in B110-B19 cell pairs cultured in CM. The onset and termination of the current injection pulse is indicated in the top trace. Membrane potentials were current-clamped for both neurons B110 (middle trace) and B19 (bottom trace) at approximately -75 mV prior to the hyperpolarizing current injection. Horizontal bar equals 1.0 sec; vertical bar equals 20 mV. (C) CM cell pairs (n = 18; black bar) exhibited a strong electrical coupling coefficient (ECC); whereas, DM pairs (n = 18; gray bar) were significantly less coupled (*, $p < 0.002$). Data represent mean ± SEM.

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In contrast, pairs incubated in DM, medium lacking trophic factors, possessed an ECC of 0.14 ± 0.05. These ECC values for CM and DM groups were significantly different ($p < 0.002$; $n = 18$ per group; Fig. 1C). In six cell pairs from each group, the presynaptic B110 was injected with the photolytic calcium cage, NP-EGTA, and then exposed to a brief UV light flash to elevate presynaptic calcium levels (Fig. 2A). An inverse relationship between electrical coupling and chemical neurotransmission was obvious in these cell pairs (Fig. 2B-E). Synapses with strong electrical coupling (i.e., CM pairs; Fig. 2B) did not respond to photolytic elevation in calcium with release of neurotransmitter (Fig. 2C). At synapses that lacked detectable electrotonic transmission (i.e., CM pairs; Fig. 2D), large PSPs were induced by UV flash (Fig. 2E). Photolytic release of calcium induced a significantly greater change in the postsynaptic potential (PSP) at DM-cultured synapses than at CM-cultured synapses ($p < 0.02$; Fig. 2F). Additional UV flashes in DM pairs, leading to a depletion of the cage's calcium available for release, evoked postsynaptic membrane potentials much reduced in magnitude from the initial flash (Fig. 2E and 2F). UV-photolysis of the caged calcium did not generate changes in presynaptic membrane potential or, in CM pairs, changes in postsynaptic potential (Fig. 2C).

To determine if photolytic release of caged-calcium had effectively elevated intracellular calcium concentrations in both CM and DM groups, we monitored presynaptic calcium using the ratiometric fluorescent indicator, Fura-2 AM (Fig. 3A-D). In neuronal pairs cultured in CM, an average resting calcium concentration of 150 ± 25 nM was elevated to 460 ± 100 nM following UV photolysis (Fig. 3F). Similarly, the

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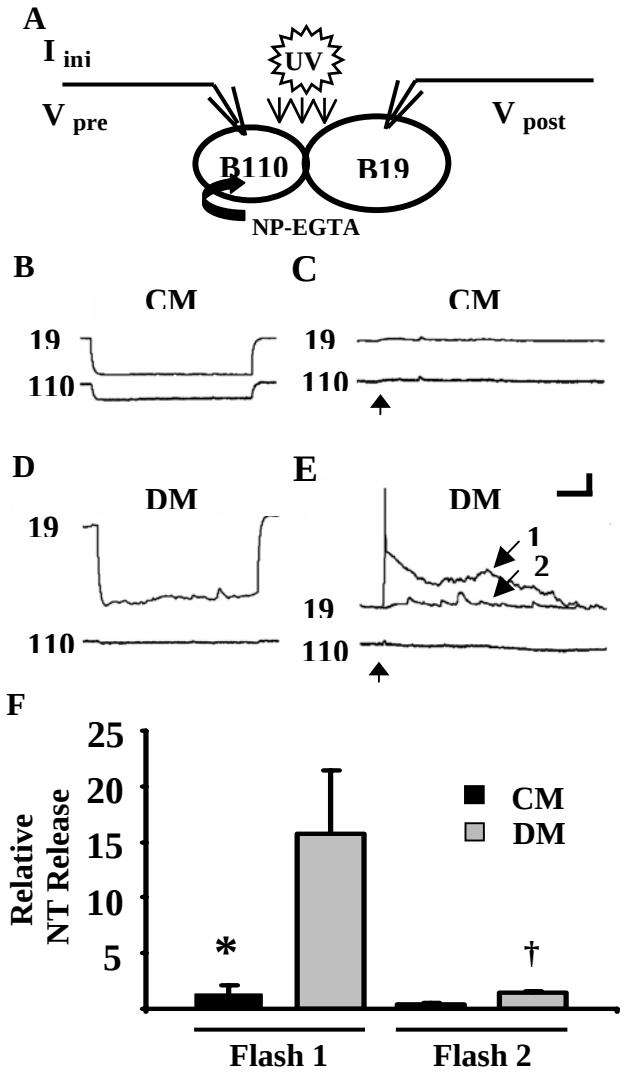

Figure 2.
Photolytic release of calcium induced neurotransmitter secretion at uncoupled, but not electrically coupled, synapses. (A) This schematic diagram of neuronal somata demonstrates the arrangement for intracellular recordings and NP-EGTA loading (black arrow) in cell pairs. (B-E) An inverse relationship existed between electrotonic coupling and NP-EGTA/UV-evoked neurotransmitter release. Electrophysiological recordings of electrical coupling in cell pairs cultured in CM (B and C) and DM (D and E) are paired with respective recordings of neurotransmitter release in these same cell pairs. The membrane potential recording of neuron B19 is shown at the top and that of neuron B110 is represented in the middle for each set of recordings. Horizontal bar equals 1.0 sec; vertical bar equals 20 mV. (C) Following the initiation of the UV pulse (indicated by the arrow), no change was detected in membrane potential in either neuron of this CM pair. (E) In a DM cell pair, the first UV pulse (trace #1) generated a significant change in the membrane potential of B19 (postsynaptic cell). A second UV pulse (trace #2) elicited less of a postsynaptic response. There was no change in membrane potential of B110 (presynaptic cell). (F) This histogram quantifies the average levels of chemical neurotransmission as measured by relative magnitude of PSPs. Following the first UV pulse, CM cell pairs ($n = 7$) exhibited significantly less neurotransmission than DM pairs (*, $p < 0.02$). After a second UV pulse, neurotransmitter secretion was greatly reduced in DM pairs (†, $p < 0.01$).

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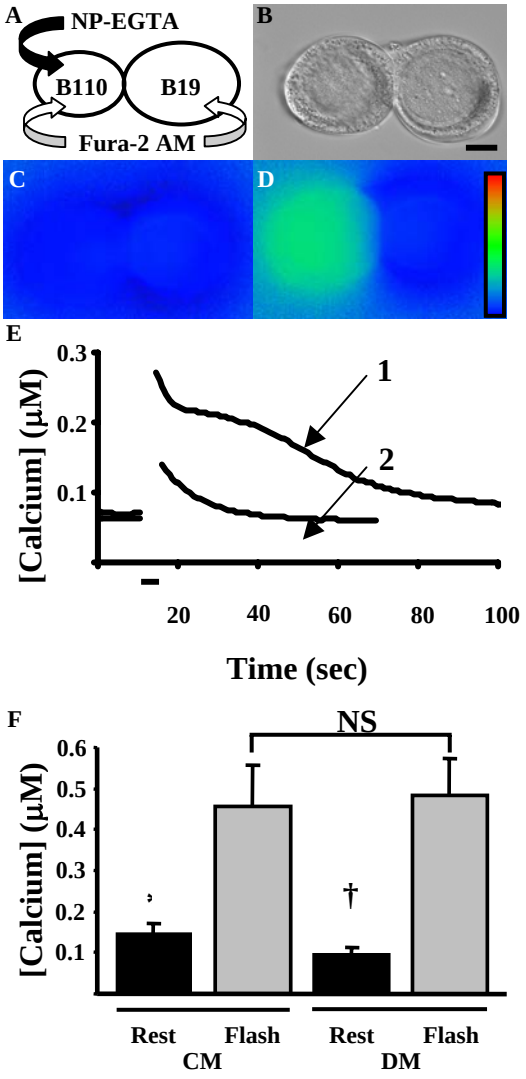

Figure 3.
NP-EGTA photolysis elevated presynaptic calcium levels equally in CM and DM cultured somatic synapses. (A) This schematic diagram illustrates the loading procedure utilized to manipulate (Ca^{2+}-loaded NP-EGTA; closed arrow) and visualize (Fura-2; open arrow) intracellular calcium. (B) The Nomarski image of the giant somatic synapse shows a typical B110-B19 neuronal pair cultured in DM (B110 is on the left). The scale bar equals 10 μm. (C, D) The representative fluorescent images indicate resting calcium levels before UV photolysis (C) and the elevated calcium concentration in B110 after photolysis (D). On the scale bar, the transitions between blue-green and green-orange are approximately 100 nM and 1000 nM, respectively. (E) This graph represents the intracellular calcium concentration of a DM pair after an initial (trace 1) and a subsequent (trace 2) flash of ultraviolet light. The first pulse elevated the calcium concentration to at least 300 nM, which was twice the level generated from the second pulse. Bar indicates UV flash (2-3 sec). (F) The graph represents the average intracellular calcium concentration in B110 for both CM and DM cell pairs. There was a significant rise in presynaptic calcium concentration after UV photolysis for both CM (n = 9) and DM (n = 8) controls (*, $p < 0.01$; †, $p < 0.02$); however, there was no significant difference in the intracellular calcium concentration between the presynaptic neurons in CM and DM before (rest; $p = 0.2$) or after photolysis (flash; $p = 0.81$).

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average resting calcium level in DM pairs was 100 ± 15 nM and, after photolysis, the calcium concentration increased to 480 ± 95 nM. Subsequent flashes of UV light caused reduced calcium transients as the NP-EGTA cage was depleted of calcium (Fig. 3E). Although photolysis resulted in a significant increase in presynaptic calcium in both CM ($n = 9$; $p < 0.01$) and DM pairs ($n = 8$; $p < 0.02$), there was no difference in the final level of calcium attained between the two groups (Fig. 3F). Therefore, the ability of DM synapses, but not CM synapses, to respond to photolysis with calcium-dependent exocytosis was not due to differential function of NP-EGTA in the two trophic environments. Additionally, synapses formed in these disparate culture conditions did not differ in their capacities to buffer changes in intracellular calcium.

To determine if electrical coupling or trophic stimulation altered postsynaptic receptor sensitivity, and thereby chemical neurotransmission, we examined postsynaptic neuronal responses to acetylcholine (ACh) using a combination of electrophysiological and pharmacological techniques (Fig. 4A). The average ECC in CM pairs ($n = 11$) was 0.27 ± 0.06 compared to 0.06 ± 0.03 in DM pairs ($n = 12$) in this experiment. Following focal application of acetylcholine to the postsynaptic cell of the giant synapse, both CM and DM preparations displayed an increase in postsynaptic membrane voltage (Fig. 4B). No significant difference in postsynaptic membrane potential change was found between CM and DM groups (Fig. 4C). One minute after applying 3.75 μM tubocurarine chloride, a cholinergic receptor antagonist, ACh-evoked responses were abolished in both CM and DM pairs (data not shown). Thus, differences in sensitivity to acetylcholine between DM and CM cultured synapses were not detectable in this study.

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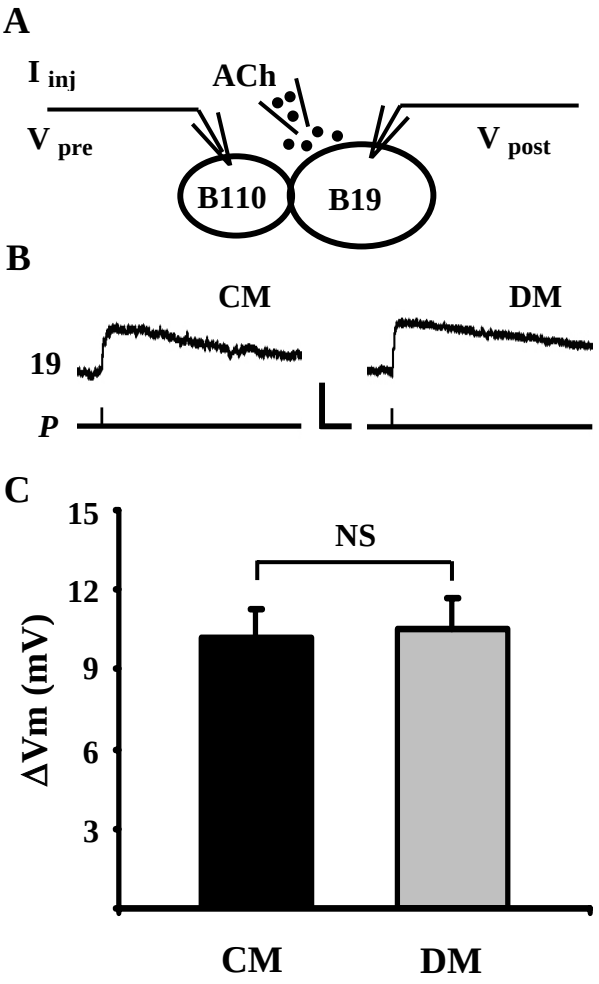

Figure 4.
Suppression of chemical neurotransmission at electrically coupled synapses was not due to changes in postsynaptic cholinergic receptor sensitivity. (A) This schematic diagram illustrates the experimental configuration employed to test for the presence of functional postsynaptic cholinergic receptors. After determining the ECC at a synapse, acetylcholine (ACh) was focally applied to the postsynaptic membrane to examine postsynaptic sensitivity. (B) Representative recordings of ACh-evoked potentials in CM or DM cultured cell pairs showed similar changes in amplitude. The time lines, running below the traces, indicate the moment (5 ms pulse) when ACh was administrated. Horizontal scale bar equals 1 sec; vertical scale bar equals 5 mV. (D) This histogram represents the average change in membrane voltage (at peak amplitude) after exposing neuronal pairs to acetylcholine. There was no significant difference in postsynaptic responses between CM (n = 12) and DM (n = 13) pairs (p = 0.7).

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Since calcium-dependent regulation of neurotransmitter release is a process localized to sites of vesicle fusion at the presynaptic membrane, we hypothesized that gap junctional coupling at these synapses might form a conduit for calcium shunting. This idea suggests that calcium ions would readily move down their concentration gradient from the presynaptic to postsynaptic cytoplasm during action potential- or photolysis-induced elevations. To test this prediction, we monitored postsynaptic calcium levels in these giant somatic synapses using Fura-2 (Fig. 5A-D). After UV photolysis of NP-EGTA in the presynaptic neuron, no significant difference was detected between the average postsynaptic calcium concentrations in CM and DM pairs ($p = 0.67$; Fig. 5E). Furthermore, in CM-cultured synapses that possessed the strongest electrical coupling (i.e., average ECC of 0.35 ± 0.15; $n = 3$), no change in postsynaptic calcium concentrations were detected following photolysis (Fig. 5F). Thus, no evidence of calcium movement from presynaptic to postsynaptic neurons emerged from these studies.

Having determined that differences in presynaptic calcium handling and postsynaptic sensitivity were not detectible between CM and DM cell pairs, we examined the nature of synaptic vesicle mobilization at these synapses. We tested the hypothesis that electrically coupled synapses formed in CM possess a diminished capacity for secretory machinery recruitment, thus imposing a deficiency in functional neurotransmission. To address this idea, the fluorescent synaptic vesicle dye, FM 1-43, was used to stain vesicles in neuronal somata prior to the formation of electrical and chemical connections. Following a stimulation/dye-loading protocol (see methods), there was a significant increase in fluorescence intensity (i.e., staining) of these cells compared to negative-loading controls (i.e., stimulated without dye); ($p < 0.003$; Fig. 6A-G).

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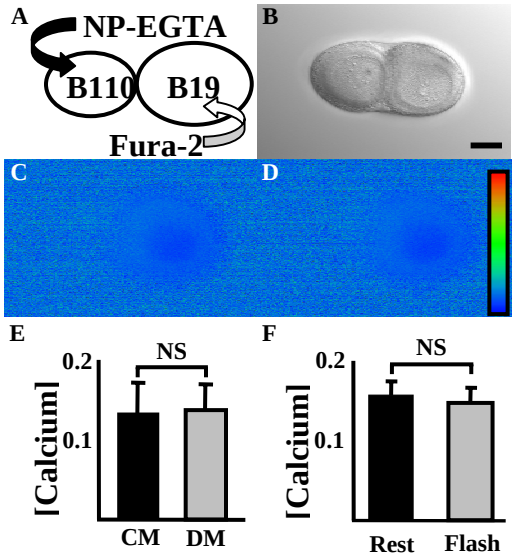

Figure 5.
Experimental elevation of presynaptic calcium did not alter postsynaptic calcium levels. (A) This illustration depicts the conformation employed for manipulation of intracellular presynaptic calcium (Ca^{2+} loaded NP-EGTA; closed arrow), and detection of postsynaptic calcium levels (Fura-2; open arrow). B) This representative DIC/Nomarski image shows a giant somatic synapse characteristic of cell pairs cultured in CM. The scale bar equals 10 μm. (C, D) These representative fluorescence images indicate the calcium levels in B19 before and after UV photolysis for this cell pair. No change in postsynaptic calcium was detected. On the scale bar, the transitions between blue-green and green-orange are approximately 100 nM and 1000 nM, respectively. (E) This histogram shows that no significant difference was found in the average postsynaptic calcium concentration between CM (n = 13) and DM (n = 5) cell pairs after UV photolysis (*, p = 0.67). (F) In electrically coupled CM cell pairs (n = 3; ECC = 0.34), no significant difference in postsynaptic calcium concentration was observed when comparing levels before (rest) and after UV photolysis (flash; *, p = 0.11).

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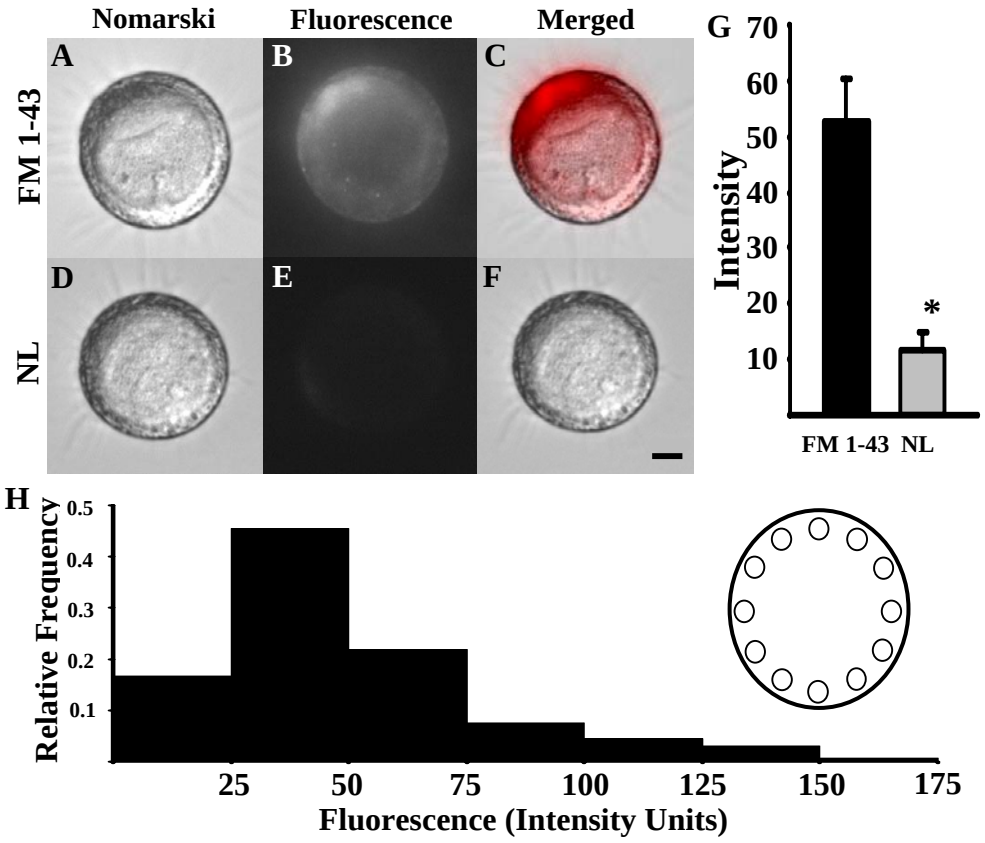

Figure 6.
Nascent presynaptic neurons possessed hotspots of vesicle clustering as indicated by FM 1-43 staining. (A-F) These merged Nomarski and fluorescent images represent single cells stained with FM 1-43 following high K^+ stimulation loading (FM 1-43; A-C) and non-loaded (NL; D-F) cells stimulated in the absence of dye. Images were acquired using Nomarski (A, D) or fluorescence (B, E) optics and then merged (C, F). The scale bar is proportional to 10 μm. In single cell loading, the entire cell contained synaptic vesicles stained with FM 1-43; however, some areas of the cytoplasm exhibited greater uptake of dye (upper left of cytosol in B and C). (G) This graph displays the fluorescence intensity of cells loaded with dye (FM 1-43; n = 11) or lacking the vesicle stain (NL; n = 5). A significant difference in the fluorescence between the two groups existed when analyzing the intensity of the entire somata (*, $p < 0.003$). (H) This graph shows the relative frequency for twelve regions of interest (ROI) with known fluorescence intensity units that ranged from 1-175. The schematic inset in D indicates ROI spaced every 30 degrees both clockwise and counterclockwise from the starting point. ROIs were a constant diameter of 7 μm. Approximately, 67 percent of the ROIs fell into the fluorescence intensity range of 26-75; however, all cells contained hotspots with greater fluorescence.

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Furthermore, cells were not uniformly loaded; that is, some regions of the cytoplasm contained greater fluorescence intensity, suggesting a higher level of vesicle turnover at hotspots prior to the commencement of synaptogenesis. Analyzing regions of interest (ROIs) around the periphery of stained B110 neurons revealed that hotspots constituted approximately 10% of all ROIs prior to the formation of synaptic contact (Fig. 6H).

Synaptic vesicles were localized to sites of contact at newly forming synapses possessing electrical coupling. In fact, ROIs at the site of contact in CM pairs (following 24 hours of synapse formation) possessed high fluorescent intensity; whereas, fluorescence was more homogenously distributed in DM pairs (Fig. 7A-H). In CM pairs, a pronounced band of fluorescent staining was present at the interface of the two synaptic membranes (Fig. 7B and C). In DM pairs, these bands of synaptic staining were not observed. Fluorescent intensity was not significantly greater at presynaptic sites of cell-cell contact after 24 hours in DM as compared to cells after only 30 minutes of contact ($p = 0.052$; Fig. 7G). When comparing synaptic vesicle distribution for each of the three groups, only neuronal pairs cultured in CM exhibited a significant increase in fluorescence at ROIs proximal to the synapse compared to the most distal regions ($p < 0.003$; Fig. 7G). Thus, mobilization of synaptic vesicles occurred at CM-cultured synapses. These studies demonstrated that the presence of electrical/biochemical coupling in CM-cultured neuronal pairs enhanced vesicle recruitment and possibly facilitated this synaptogenic process.

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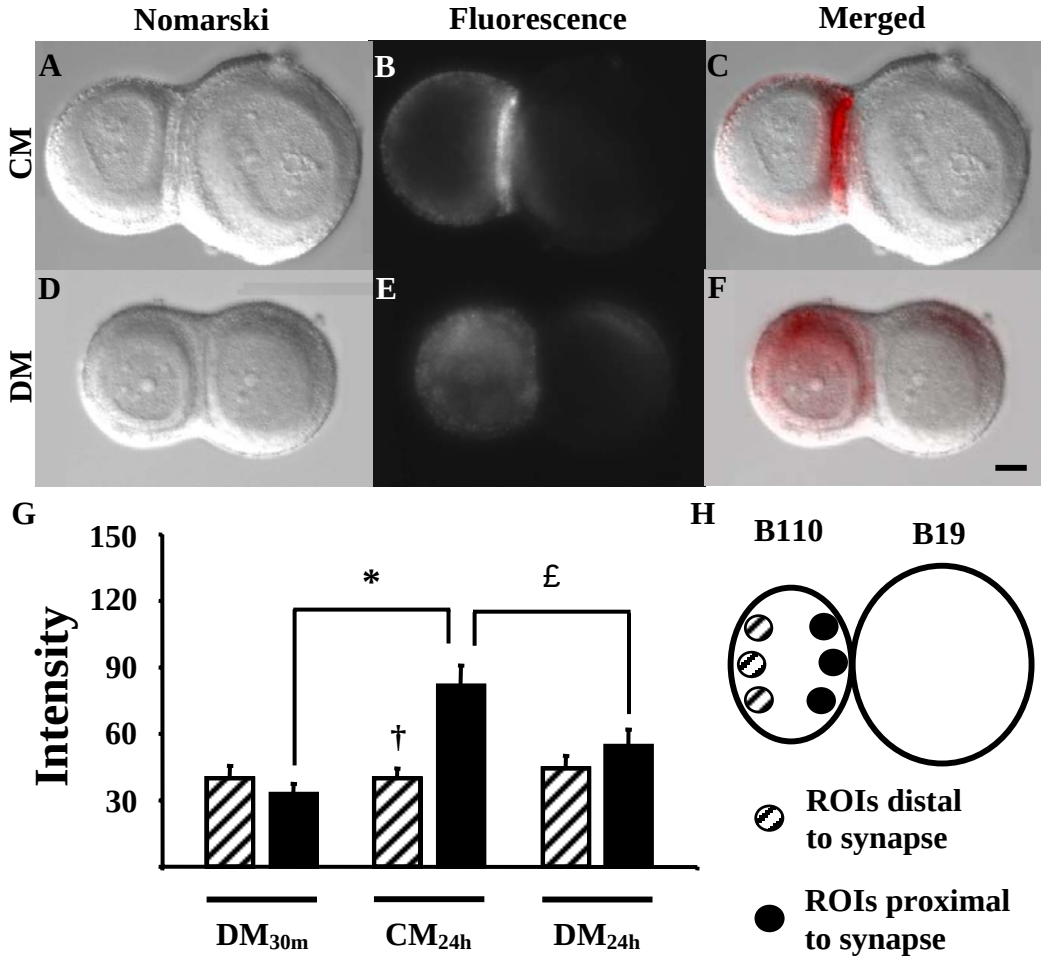

Figure 7.
Trophic factors enhanced vesicle mobilization even in the absence of chemical neurotransmission. (A-F) These representative images indicate cell pairs cultured for 24 hours in either CM or DM. Images were acquired using Nomarski (A, D) or fluorescence (B, E) optics and then merged (C, F). (G) This histograph represents the average FM 1-43 fluorescent intensity for three regions of interest located closest and furthest from the synapse (as indicated in Fig. 7H). (A, B, C, G) These panels illustrate cell pairs cultured in CM for 24 hours, and reveal the presence of stained synaptic vesicles around the peripheral cytoplasm; however, a heavy band of staining is visible at the newly forming synapse. (D, E, F, G) Neuronal pairs cultured in DM for 24 hours exhibit no significant accumulation of staining at the synapses, but more of a comprehensive distribution. The scale bar equals 10 μm. (I) This graph represents the average FM 1-43 fluorescence at both the newly forming synapse (black box) and most distal regions (stripped box) following 30 minutes paired in DM (DM_{30m}; n = 7), 24 hours paired in CM (CM_{24h}; n = 15), and 24 hours paired in DM (DM_{24h}; n = 14). The FM 1-43 intensity near the synapse was significantly different between CM and both other groups (*, $p < 0.002$; £, $p < 0.04$); whereas, no difference between groups was detected distally to cell-cell contact. When comparing regions within a group, neuronal pairs cultured in CM showed an increase in fluorescence at the boundary compared to the section situated farthest away from the synapse (†, $p < 0.003$).

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DISCUSSION

The establishment of physical contact between neurons, in many embryonic and regenerating nervous systems, triggers a developmental progression of synaptic communication involving an initial formation of electrical connectivity that switches to predominantly chemical neurotransmission as electrotonic coupling declines (Allen and Warner, 1991; Penn et al., 1994; Personius et al., 2001; Chang et al., 2000; Szabo et al., 2004). These observations prompt the speculation that gap junctional intercellular communication may coordinate electrical or biochemical neuronal activities during the initial phases of synaptogenesis (Kandler and Katz, 1995). Strength of electrical coupling is inversely correlated with the amplitude of chemically-transmitted synaptic potentials during early synaptogenesis between cultured *Helisoma* neurons (Szabo et al., 2004). This observation indicates that the presence of gap junctional coupling delays the onset of chemical neurotransmission by means of a mechanistic interaction between the two forms of synaptic communication Here, we investigated the mechanism by which electrical coupling delays chemical connectivity at developing synapses and demonstrated that electrical coupling suppresses transmitter release by disrupting the capacity for a presynaptic calcium influx to trigger vesicular exocytosis. Therefore, electrotonic coupling provides a developmental mechanism for modulating the emergence of chemical neurotransmission at newly forming synaptic contacts and for regulating the establishment of a neural network.

Exposure to ganglia-derived trophic factors enhanced electrical synaptic connectivity and suppressed calcium-dependent exocytosis. Fluorescence imaging demonstrated that cells, with and without trophic factor treatment, were capable of

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producing similar elevations in presynaptic calcium. Therefore, differences in ability to handle presynaptic calcium were not the basis for differential synaptic responses between groups. Additionally, calcium accumulated presynaptically was not shunted into the postsynaptic cell through gap junctional conduits connecting neuronal pairs. Therefore, the transient development of electrical synapses disrupted a process down-stream of the calcium accumulation mediating vesicular release of neurotransmitter.

Since gap junctional coupling did not alter calcium accumulation, molecular mechanisms that link, directly or indirectly, influx of calcium with the process of vesicular release must have been affected. Vesicle mobilization appeared normal, or possibly enhanced, despite the fact that transmitter release was compromised during electrical synaptogenesis. Therefore, we conclude that electrical synapses do not disrupt the developmental recruitment of a presynaptic pool of vesicles; however, this pool is not readily-releasable. An essential component of chemical neurotransmission is the movement of vesicles from the reserve pool to a cluster of the readily-releasable vesicles (Kuromi and Kidokoro, 2003). Once mobilized, vesicular release occurs randomly throughout the readily-releasable pool and is possibly based on the ease of vesicle detachment from cytoskeletal elements (Rizzoli and Betz, 2004). Since gap junctional proteins form complexes with nearby cytoskeletal proteins (Toyofuku et al., 1998), the possibility exists that a direct protein-protein interaction could occur between gap junctional proteins and synaptic vesicle proteins, thus altering the availability of vesicles for release.

Alternatively, transient electrotonic coupling could perturb local cellular signaling by altering the levels of presynaptic second messengers or the local cellular events they

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mediate. Chemical neurotransmission depends on a conglomerate of cytosolic, vesicle-associated, and plasma membrane bound proteins. Interaction between these proteins is highly dependent on phosphorylation states (Fujita et al., 1996). For example, synapsins regulate vesicle mobilization by linking the synaptic vesicles to actin utilizing a phosphorylation-dependent mechanism (Pieribone et al., 1995). In addition, many regulators of SNARE (soluble *N*-ethymaleimide-sensitive fusion protein attachment protein receptors) proteins and vesicle fusion, such as Sec1/Munc18, synaptotagmin, and tomosyn, are phosphorylated during the regulation of SNARE function (Gerst, 2003). Therefore, critical elements of the secretion machinery are susceptible to altered function due to gap junctional influences on intracellular signaling (e.g., phosphorylation).

Gap junction formation varies depending on cell type, but electrical synapses generally arise from the insertion of hemichannels into the plasma membrane, and subsequently form plaques as junctional proteins accumulate at the periphery of developing clusters of connexons (Johnson et al. 2002; Gaietta et al. 2002; Lauf et al., 2002). A potential explanation for an inverse relationship between electrical and chemical neurotransmission might involve the physical disruption of the active zone machinery by this process of plaque formation. That is, the spatial requirements of gap junctional plaques might exclude the function or construction of the components necessary for chemical transmission; consequently, impeding vesicle exocytosis. Since mixed synapses exist in some systems where both electrical and chemical neurotransmission function in close proximity (Lin and Faber, 1988; Rash et al., 1996), gap junctional obstruction of synaptic vesicle release is not likely, but nevertheless a possibility.

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The enhancement of electrical transmission, and subsequent reduction in chemical neurotransmission, was accomplished by the exposure of neurons to trophic factors that were released into the culture medium from damaged neural tissue (Wong et al., 1981; Barker et al., 1982). Some of these ganglia-derived factors have been identified and they can modulate synaptic formation (Magoski and Bulloch, 1998; Munno et al., 2000). One potential site of this modulation is on calcium signaling, since voltage-gated ion channels, and hence neuronal excitability and signaling, are affected by trophic factors (Lesser et al., 1999; Yamuy et al., 2000). In this study, the increase in transient electrical coupling and the reduction in neurotransmitter release was not due to changes in voltage-gated currents, since the disruption occurred downstream of calcium influx.

Besides promoting cell survival (Oppenheim et al., 1992; Pinzon-Duarte et al., 2004) and neuronal differentiation (McAllister et al., 1997; Cohen-Cory, 1999; Lom and Cohen-Cory, 1999), trophic factors regulate several aspects of synaptic transmission (Lohof et al., 1993; Kang and Schuman, 1995; Boulanger and Poo, 1999) and alter electrical communication between neurons (Nadarajah et al., 1998; Reuss et al., 1998; Aberg et al., 2000; Szabo et al., 2004). For these reasons, trophic factors and the developmental timing of their presence can profoundly impact neural network formation. During the initial phases of synaptogenesis, the synchrony of neuronal activities is electrotonically governed (Kandler and Katz, 1995). Transient electrical coupling is detected prior to the emergence of chemical connectivity at developing and regenerating synapses, thus regulation of gap junctional communication by trophic factors serve as a functional switch between two forms of synaptic transmission, chemical and electrical. In regenerating *Helisoma* neurons, the duration that electrotonic coupling coincides with

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the length of time that is required for extending axons to establish growth cone contacts with potential targets. The decline of neural network coupling following the downregulation of electrical synaptic transmission is replaced by widespread inhibitory chemical synaptogenesis within the nervous system (Szabo et al., 2004). The question remains to be determined as to whether or not this functional shift in synaptic communication is regulated by trophic factor signaling *in vivo*. Nevertheless, our demonstration that trophic factor-induced changes in electrical synapse formation between cultured neurons suggests that such a mechanism is functional and conceivably alters chemical synapse formation.

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CHAPTER IV

GENERAL CONCLUSION

The nervous system is a dynamic, malleable network of interconnected cells that coordinates the totality of an animal's behavior. The emergence of adult patterns of connectivity requires a regulated sequence of events. The initial step occurs when neural progenitor cells are recruited from an undifferentiated set of ectodermal cells and form the neural plate (reviewed in Morest and Silver, 2003). Depending on the various permutations of inducing factors and inducible signals, cells in the neural plate adopt specific fates (reviewed in Kintner, 2002). After differentiating, neurons then migrate to appropriate regions and extend growth cones until specific targets are innervated. The contact between growth cones and targets starts the synaptogenenic process that last until establishing functional pre- to postsynaptic neuronal communication.

Synapses are not hardwired, but rather they are dynamic sites of intercellular communication that progress through a continuous state of refinement. This plasticity helps shape the developing nervous system and refines previously established connections in such regions as the retina (Schmidt, 2004) and the hippocampus (Morris et al., 2003). The flexibility of any network depends on correlated neural activity among sets of inputs (either spontaneous or sensory-evoked), coupled with the ability of postsynaptic cells to detect such correlations (Katz and Shatz, 1996). This activity-dependent refinement of synapse formation is characteristic of Hebbian synapses, in which some contacts are enhanced while others are eliminated.

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During the initial phases of synaptogenesis and neural network formation, the synchronization of electrical or biochemical neuronal activities is electrotonically conducted (Kandler and Katz, 1995). In embryonic chick spinal cord neurons, coupling is generally undetectable among adjoining cells; however, this form of neural communication is occasionally observed, suggesting that coupling is, somehow related to chemical synapse formation (Fischbach, 1972). Additionally, transient electrical coupling is detected prior to the emergence of chemical connectivity at developing spinal cord synapses (Penn et al., 1994), neuromuscular junctions (Allen and Warner, 1991), and retinal synapses (Personius et al., 2001). These, and similar observations in other models used to examine regeneration (Chang et al., 2000; Szabo et al., 2004), prompt the speculation that gap junctional intercellular communication significantly contribute to the development of synapses.

Damaged *Helisoma* neurons regenerate axons that must innervate the correct targets to reestablish a functional network. During regeneration *in vivo* and cell culture, neurons B19 and B110 form novel, transient electrical connections that are later replaced with an inhibitory chemical synapse (Szabo et al., 2004). Following twenty-four hours of synaptogenesis between cultured *Helisoma* neurons, the strength of electrical coupling was inversely correlated with the amplitude of chemical synaptic potentials, indicating that the presence of gap junctional coupling delays the onset of chemical neurotransmission (Szabo et al., 2004). Furthermore, the duration that electrotonic coupling coincides with the length of time that is required for extending axons to establish contact with peripheral targets. These data, together with my current studies,

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suggest a mechanistic, developmentally-relevant interaction between these two forms of synaptic communication.

During this period of synaptogenesis, electrotonic coupling precludes transmitter release by disrupting the capacity for a presynaptic calcium influx to trigger vesicular exocytosis (Chapter III; Figure 8). Furthermore, electrical synapses inhibited chemical neurotransmission despite enhancing the quantity of synaptic vesicles mobilized to the region of cell to cell contact (Chapter III; Figure 8). Therefore, electrotonic coupling provides a developmental mechanism for modulating the emergence of chemical neurotransmission at newly forming synaptic contacts, may contribute to the plasticity required to establish neural networks, and may represent a fundamental mechanism employed in neural development and regeneration.

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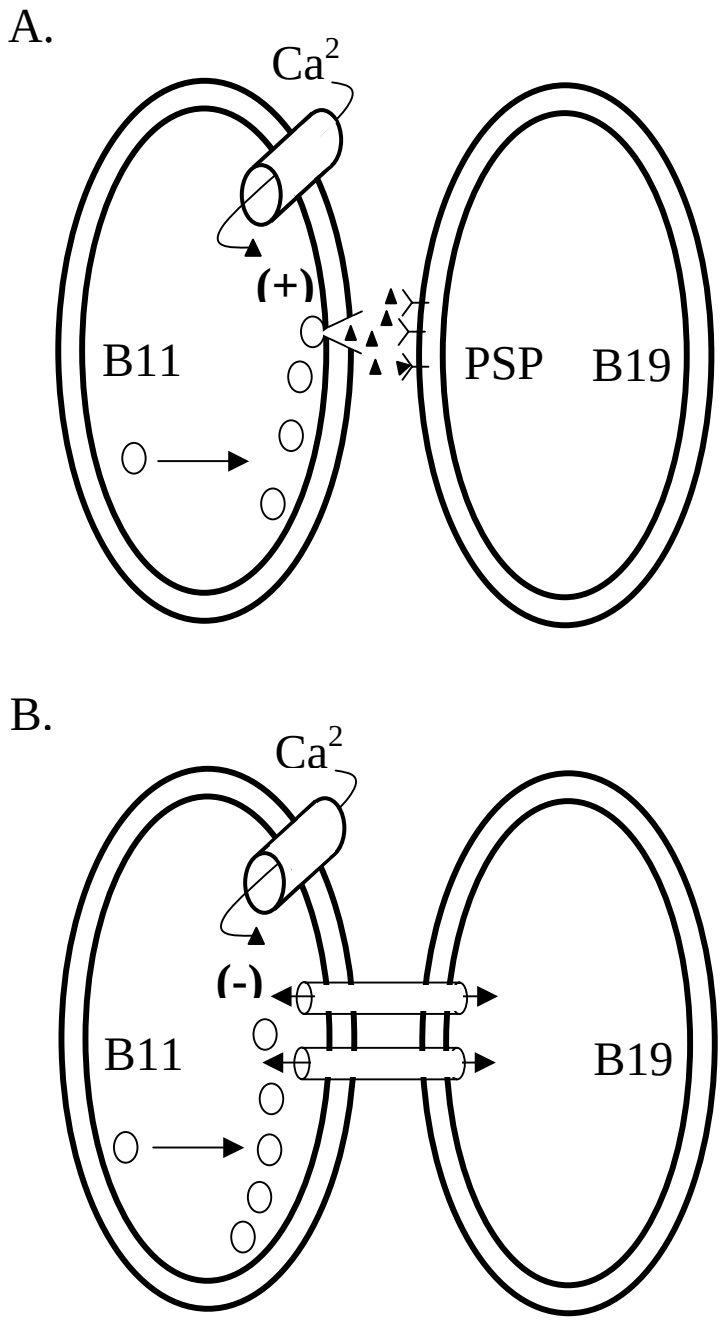

Figure 8.
Electrotonic transmission at developing synapses disrupts calcium-dependent exocytosis despite enhancing vesicle mobilization. (A) This schematic diagram of neuronal somata pairs, B110 and B19, demonstrates the cellular process occurring during the deprivation of trophic factors (DM). In uncoupled cell pairs, the presynaptic B110 responds to an influx of calcium with vesicular exocytosis. (B) This model illustrates the intracellular processes occurring in electrically coupled cell pairs cultured in CM. Electrotonic synapses delay chemical synaptic transmission by imposing a functional block between the accumulation of presynaptic calcium and the synchronized, vesicular release of neurotransmitter. Additionally, electrical coupling augments the recruitment of synaptic vesicles to the newly forming synapse despite impeding the formation of chemical connections.

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Texas A&M University, Department of Biology, College Station, Texas

Spring 2003 & 2004 - Physiology Lab Instructor
Fall 2002, & 2003 - Honors Introductory Biology Lab Instructor
Spring 2002 - Honors Introductory Biology Lab Instructor
Fall 2001 - Introductory Biology Lab Instructor
Summer 2002 and 2003 - Introductory Biology Lab Instructor

FELLOWSHIPS & AWARDS

Texas A&M University, Department of Biology, College Station, Texas

September 2001 - College of Science Biology Fellowship
September 2002 & 2003 - Biology Mini Travel Grant
September 2002 & 2003 - Faculty of Neuroscience Mini Travel Grant
November 2002 - Instrument Certification for TEM
July 2003 - Biology Research Grant
March 2004 - 2nd Place for Texas A&M Neuroscience Poster Presentation

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