

**TRANSIENT MIXED SYNAPSES REGULATE EMERGING
CONNECTIVITY IN SIMPLE NEURONAL NETWORKS**

A Dissertation

by

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

DOCTOR OF PHILOSOPHY

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August 2013

Major Subject: Zoology

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ABSTRACT

The electrical synapse was first described over 50 years ago. Since that time appreciation of its complexity and importance has grown, including the hypothesis that early transient formation of these synapses is important to adult patterns of connectivity in neural networks. Presented in this dissertation are studies utilizing identified neurons in cell culture from the snail *Helisoma trivolvis* to examine discrete periods of electrical synapse formation during regeneration with sustained or transient expression. Extensive knowledge of connectivity patterns of the buccal neurons of *Helisoma* in cell culture and the ganglia, provide a useful framework for looking at modulation and manipulation of electrical synapses and their impact and emerging connectivity in a simple neuronal network.

Two types of electrical connections were observed those that were transient, between a B19 and a B110 and those that were sustained, between a B19 and another B19. Dopamine (DA) modulation of forming electrical synapses (FES) produces a synapse specific effect at those either destined to be transient (TES) or sustained (SES) and may be a direct effect on the gap junctions at the synapses, as is the case at TES, or an indirect effect on other membrane currents, as seen in SES. DA modulation produces different outcomes at SES-centered networks and TES-centered networks with respect to new chemical synapse formation, demonstrating network-dependent effects of electrical synapse modulation.

Pharmacological blockade of chemical and electrical components at forming mixed synapses in some cases alters subsequent synapse formation although due to the variable nature does not appear to be a direct interaction between chemical and electrical synapses. Three-cell networks appear to display a balancing mechanism for overall electrical coupling when electrical synapses are blocked suggesting a competition for some resource in the construction or trafficking of gap junctions. In addition to electrophysiological examinations, network coupling can be assessed utilizing fluorescent calcium imaging to look at coincidence of calcium changes as an output for coupling between cells. This technique provides a useful tool for less invasive studies of neuronal networks and the impact of coupling at mixed synapses.

DEDICATION

*To my dearest wife, without whose love, support and understanding this journey
would not have been possible.*

ACKNOWLEDGEMENTS

I would like to thank my committee members, Dr. Louise Abbott, Dr. Rene Garcia, and Dr. Mike Smotherman for guidance on not only project-specific material, but advise about science and career development in general.

I would also like to thank the various friends (there are too many of you to list here, but you know who you are) for providing an empathetic ear and supporting me as well as my sanity throughout my time at Texas A&M. In addition, my lab peers, Dr. Jeff Burkeen, Dr. Zane Lybrand and Dr. Alisa Womac, also provided immeasurable support and camaraderie the likes of which I was incredibly lucky to have. Thank you all for invaluable friendship and I wish you the best in your future endeavors.

Many people made this work possible. I would like to acknowledge Dr. Theresa Szabo-Maas for help on manuscripts as well as advise on experiments. I would also like to acknowledge Dr. Bryan Luikart, Meredith Bunkers, and Dr. May Boggess for aide in work presented in this dissertation.

There have also been those that were with me from the literal beginning, my parents Don and Darla Richardson that I would also like to thank. I cannot begin to tell you how much your love, support and encouragement have meant over the years and for always being willing to listen when I needed something. My wife, Allison, I would also like to thank for all the support and for sticking with me these years. I know they were tough at times but you always stayed the course with me and for that I can never thank you enough.

Finally, I would like to thank my advisor, Dr. Mark Zoran. My thanks feel woefully inadequate as you have been a mentor in the truest sense of the word. Your support and insight kept me from getting hopelessly frustrated when things would go awry (as I felt happened frequently) and allowed me to complete this journey of my life. Thank you sincerely for all the time you spent in both my professional as well as my personal development.

NOMENCLATURE

5HT	Serotonin
ACh	Acetylcholine
BSA	Bovine serum albumin
CBX	Carbenoxolone
CUR	Curare
DA	Dopamine
DMeth	Deltamethrin
DMSO	Dimethyl sulfoxide
Dyna	Dynasore
FES	Forming electrical synapse
HEX	Hexamethonium
mPSP	Miniature postsynaptic potential
NES	New electrical synapse
nAChR	Nicotinic acetylcholine receptor
nCCS	New chemical cholinergic synapse
nTES	New transient electrical synapse
PLL	Poly-l-lysine
PSP	Postsynaptic potential
SES	Sustained electrical synapse
TES	Transient electrical synapse

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CHAPTER I

GENERAL INTRODUCTION

The general acceptance of the neuronal doctrine as defined by Santiago Ramon y Cajal and others, that the nervous system was comprised of individual cells and not a continuous web-like entity, led necessarily to the question of how signals traveled between the many neural entities (reviewed in Bennett 1999). One hypothesis was that nervous stimulation produced a chemical to which the target effector cells responded with a corresponding signal. Perhaps the most famous experiments in this regard were those of Otto Loewi showing that stimulation of the vagus nerve, and its associated inhibition of heart rate, was due to a factor secreted by the vagus nerve (Loewi, 1921). A second hypothesis, long championed by John Eccles, proposed that there was a direct passage or coupling of electrical current between connected neurons. The first clear demonstration of such electrically-transmitting synapses was not to come until intracellular electrophysiology performed by Furshpan and Potter (1959), showed direct electrical current flow at giant motor synapses involved in crayfish escape behaviors. Today it well documented that both forms of neural transmission, chemical and electrical, exist in most nervous systems, if not all. Furthermore, in many instances synapses actually exhibit mixed connectivity where both forms of synaptic transmission co-exist. Perhaps the best understood and most rigorously studied mixed synapses are those of the Mauthner cell of the goldfish (Lin and Faber, 1988).

Chemical-mediated connections (known as chemical synapses) differ fundamentally in their structure and function from electrical synapses. Functionally, chemical synapses require that action potentials in the signal-sending (presynaptic) cell be coupled to the release of a diffusible chemical signal at the site of contact before action potentials can be generated in the signal-receiving (postsynaptic) cell. This requires very specific specializations in both the presynaptic and postsynaptic cells, particularly machinery in the presynaptic cell for production and release of chemical transmitter and in the postsynaptic cell for the receipt and transduction of those chemical signals into changes in membrane potential. In contrast, electrical synapses form large intercellular channels, known as gap junctions, that allow for the direct passage of electrical current between cells. These gap junctions are formed when extracellular domains of the channel proteins from each cell (known as hemichannels) align to form a large pore providing cytoplasmic continuity.

In contrast to chemical synapses, many electrical synapses are bi-directional, conducting current equally well in either direction. Given the synaptic specializations necessary for chemical synaptic transmission, such bi-directionality would be a difficult proposition. However, in numerous instances gap-junctional conductance displays rectification in which the channel will preferentially conduct current in one direction (Furshpan and Potter, 1959; Hall et al., 1985). As might be expected, chemical synapses contain a longer synaptic delay when compared to the near instantaneous transmission of electrical signals through gap junctions. It is generally considered that chemical synapses have higher metabolic demands and provide a higher degree of modulation than their

electrical counterparts. Recently, however, electrical synapses have been demonstrated to be highly dynamic in expression and degree of plasticity (Condorelli et al., 1998; Pereda et al., 2012).

Electrical synapses are well studied with regard to their mediation of rapid motor escape behaviors (Antonsen and Edwards, 2003), however, the appreciation for their role in the regulation of synaptic function throughout the vertebrate brain is continually growing (Bennett et al., 1959; Connors and Long, 2004). The expression of electrical synapses can be highly diverse. In some instances gap junction-mediated intercellular communication is sustained throughout development and incorporated into the adult neural connectivity of the animal, such as in the retina (Bloomfield and Volgy, 2009). These types of synapses will hereafter be referred to as sustained electrical synapses (SES). However, in numerous instances electrical synapses, present early in nervous system development, undergo a programmed uncoupling (Spitzer, 1982; Connors et al., 1983) often coincident with the emergence of chemical synapses. This brief, but distinct period of expression, led these developmental forms gap junction-mediated coupling to be referred to as transient electrical synapses (TES). Although the function of these short-lived electrical connections is not well understood, TES have long been hypothesized to be important in the formation of appropriate adult connections amongst neural networks (Peinado et al., 1993; Yuste et al., 1995).

As mentioned earlier, gap junctions are known to be important sites of neuromodulation with an increasingly large number of modulatory agents known to affect changes in junctional conductance. One such agent is cytosolic calcium (Rose et

al., 1977), which can act to gate electrical synapses by affecting alterations in their voltage sensitivities. There are also well-studied neuromodulators in the vertebrate retina including dopamine (DA) and nitric oxide, which alter junctional conductance (Witkovsky and Dearry, 1991; Lu and McMahon, 1997). In both instances these gap junction modulators alter kinase activity leading to changes in the relative phosphorylation state of the gap junction proteins and, subsequently, alterations in their conductance (Lasater, 1987; Patel et al., 2006).

Given that gap junction expression is widespread throughout the adult vertebrate brain and that in certain instances its expression is transient, gap junction-mediated synapses have been implicated in the regulation of developing and emerging adult connectivity. Therefore, this dissertation and my doctoral research is based on the central hypothesis that modulation of developing electrical synapses will lead to predictable changes in the formation of synaptic connections within known neural networks. Furthermore, I hypothesize that the persistence, or not, of electrical connections, SES and TES, respectively, within neural networks will have different modulatory effects (i.e., generate different synaptic outcomes) on the networks within which they exist. In order to address these hypotheses, I have used a biological system, *Helisoma trivolvis*, where neurons are visually identifiable and easily manipulated into cell culture. This system allows for the controlled construction of networks of known neuronal identity and for the spatial and temporal manipulation of synaptic contacts and the ready administration of pharmacological and neuromodulatory agents. Using these tools, I

have assessed how modulation of both sustained and transient forms of electrical synapses alters the emergence of neural network connectivity.

Electrical Synapses

Electrical synapses allow for the direct passage of intercellular communication between coupled cells. Although evidence of this mode of communication was functionally described over fifty years ago at crayfish giant synapses (Furshpan and Potter, 1957; Furshpan and Potter, 1959) cardiac ganglia of mantid shrimp (Watanabe, 1958) and supramedullary neurons of pufferfish (Bennett et al., 1959), it has since been demonstrated that electrical coupling occurs at large membrane-spanning channels referred to as gap junctions (Revel and Karnovsky, 1967; Sotelo et al., 1974). Gap junctions were so named because they bring opposing cell membranes into much closer juxtaposition (2-4nm) with one another than is typical for an intermembranous space (around 20nm). It is important to note that, although much of the discussion here of gap junctions will be limited to their function and presence in neural systems, these channels are widely found throughout the body to couple many cell types within most tissues. For example, the expression of connexin (Cx) 43 is widespread in cardiac muscles (Reaume et al., 1995), alveolar capillaries (Parthasarathi et al., 2006), liver epithelial cells (de Feijter et al., 1996), osteocytes (Cherian et al., 2005), myometria (Ou et al., 1997) and in the brain, where gap junctions couple massive networks of both neurons and astrocytes (Yamamoto et al., 1992).

Structure and composition

Gap-junction proteins are contained in two multi-gene families; connexins, which are unique to chordates, and innexins and pannexins, which constitute a second family with representatives extant across both protostome and deuterostome lineages (Phelan, 2005; White et al., 2004). Currently there are 20 connexin genes that have been identified in the mouse genome, 19 of which are orthologs of those found in the human genome (Sohl et al., 2005). Out of these 20 genes only a relatively small number are expressed within the mouse nervous system including Cx26, Cx36, Cx45 and Cx57 (Dere and Zlomuzica, 2012). In comparison, 25 innexins have been identified in *C. elegans* (Starich et al., 2001) and a recent paper reports the identification of 21 innexin genes encoded in the leech genome (Kandarian et al., 2012).

Although initially thought to be distinct to invertebrate lineages, innexin homologs were later found in vertebrates demonstrating a more widespread distribution among animal phyla. It is important to note that the use of the nomenclature, innexin and pannexin, is often considered dubious due to the fact that they are homologs (Baranova et al., 2004). Therefore, it has been suggested that all members be referred to simply as pannexins (pan being Latin for throughout). This name, pannexin, is used very distinctly when found in the chordate genome, however, the innexin identifier is still very prominently used to identify most, but not all, invertebrate gap junction proteins. Currently, there are three identified pannexin genes in mammals (Panchin, 2005). Interestingly, unlike connexins that form fully functional intercellular channels, pannexins have only been shown to function as hemichannels in mammals (MacVicar

and Thompson et al., 2010) though they were capable of forming intercellular channels when expressed in oocytes (Bruzzone et al., 2003).

Structurally gap junction proteins consist of 4 transmembrane domains, 2 extracellular loops, 1 intracellular loop, and cytosolic carboxy and amino termini (Panchin, 2005). Gap junctions are formed when hexameric units of connexins or innexins, termed connexons or innexons respectively, link between two adjacent cells and create a large junction allowing for cytoplasmic continuity between the two cells following channel opening. Docking of the connexons between adjacent cells is thought to occur by interaction of the extracellular loops contained in the connexins comprising each hemichannel (Yeager and Nicholson, 1996). Mechanistically this is suggested to occur by the formation of disulfide bridges among conserved adjacent cysteine amino acids that act to link and stabilize juxtaposed hemichannels (Kumar and Gilula, 1996).

Gap junctions and their constituent hemichannels are highly diverse in their composition (White and Paul, 1999). Connexons can be homomeric, with all six subunits the same connexin proteins, or heteromeric, where connexins of multiple identities form the hemichannel. Additionally, hemichannels contributed by each of the two cells constituting a gap junction are not obligated to have the same composition (Dedek et al., 2006). Resulting gap junctions can therefore be homotypic, identical hemichannels, or heterotypic, different hemichannels. Taken even a step further, gap-junctional plaques that constitute electrical synapses may be either homo- or heterogeneous and, therefore, could contain myriad types of gap junctions each with different signaling properties. All of these different components create a large degree of diversity in the composition of the

intercellular junction, each providing potential variability in gating mechanisms, selectivity and modulation (Veenstra, 1996), which will be addressed later in this section. This diversity is evident in their wide array of functions and expression throughout the developing and adult animal body, of particular interest here- the nervous system.

The channel itself has a large pore diameter that can pass molecules up to 1 kDa in molecular size (Dermietzel and Spray, 1993), allowing the junction to not only passage ions (and more importantly current) for electrical transmission but also small second messenger molecules that mediate a wide range of cellular processes (Harris, 2007; Kandler and Katz, 1998; Kumar and Gilula, 1996; Leitch, 1992). The pore directly links the cytoplasm between cells creating an electrical synapse whose communication is very rapid. For this reason electrical and biochemical coupling can occur in very tight temporal sequences. In fact, throughout early phases of nervous system development, large groups of neurons are connected via electrical synapses (Nadarajah et al., 1997). Thus, the expression of gap junction proteins, the electrical and biochemical coupling they mediate and the modulation of their physiological function in the nervous system is considered tantamount to a regulatory switch during synaptic development and signal transmission within adult neural networks (Kandler and Thiels, 2005). In the following sections I will discuss processes by which these intercellular pores affect changes in neural function.

Gating gap junctions

Electrical synapses are generally thought to be far less dynamic in their neural signaling capabilities than their chemical synapse counterparts. It is becoming evident, however, that gap junctions have a far wider range of synaptic capabilities and degree of modulatory impact in neural regulation than initially credited in early studies of electrical synapses (Furshpan and Potter, 1959). Gap junctions by their mere presence do not dictate an open pore for direct cellular communication. Electrical synapses can be gated into an open or closed conductance state and there are several well-known mechanisms by which they can be activated. Gating is thought to occur through one of the two termini with some clear evidence involving the amino terminus and voltage-gating, as determined by studies of Cx26- and Cx32-based channels (Purnick et al., 2000, Oh et al., 2004). Phosphorylation-based gating occurs at the carboxyl terminus (Lampe and Lau, 2000) and levels of cytosolic calcium are known to influence gating (Rose and Loewenstein, 1976). Therefore, the key players in a host of biological regulatory pathways, including membrane potential, phosphorylation state and calcium signaling, are also critical participants in the gating of gap junctions.

It has been oft demonstrated that gap junctions are gated by the voltage across the junctional membrane (Spray et al., 1979, Srinivas et al., 1999), though sensitivities to voltage is largely dependent on the hemichannel in question and its connexin makeup (Revilla et al., 2000). Notably many junctions exhibit both voltage-dependent and independent currents, demonstrating that electrical synapses have multiple conductance states rather than simple on-off mechanisms (Chen and DeHaan, 1992; Moreno et al.,

1994). These multiple gating states show that junctional conductance varies and hence displays a degree of gradation that allows for fine control of electrical synaptic communication. Voltage-dependent junctional currents also provide understanding of the rectification properties that some gap junctions possess, while others produce bi-directional conductance (Bennett, 1997). Most arguments suggest that these rectifying electrical synapses involve heterotypic gap junctions, where one hemichannel has a distinct voltage-dependent conductance while the other exhibits either slow-voltage dependent or voltage-independent gating, allowing differential resistance to current flow in one direction through the channel than the other. A final and interesting note on voltage sensitivity at electrical synapses is that it offers a mechanism for activity-dependent regulation of gap junction physiology. In this regard, the activity within neural networks containing electrical synapses or mixed electrical/chemical synapses can modulate information processing of that network over time, depending on activity states. This idea is central to several aspects of this dissertation.

Changes in cytosolic calcium (Ca^{2+}) levels, as mentioned earlier, also gate intercellular channels, often with in an increase in calcium resulting in channel closure (Rose et al., 1977; Spray and Bennett, 1985). It might seem odd that increased calcium closes gap junctions when they are known to effectively propagate calcium waves among neural cells, particularly astroglia (Scemes and Giaume, 2006; Verkhratsky et al., 2012). However, experiments demonstrating closure of intercellular channels were performed in excess of physiological levels of Ca^{2+} (Spray et al., 1994). Calcium modulation of junctional conductance could occur through changes in transjunctional

voltage, as discussed earlier, due to its nature as a divalent cation. Additionally, Ca^{2+} could act through acidification mediated by calcium entry into the neuron, due to gap-junctional sensitivity to proton levels. However, some evidence suggests that the latter is an unlikely mechanism due to a lack of synergy when calcium and hydrogen ions are co-applied (Peracchia, 2004). In addition to its effects on membrane voltage, and potentially pH, cytosolic calcium can also alter the conductance of gap junctions through calmodulin-dependent interactions, which can directly bind with certain connexins to alter channel conductance (Peracchia et al., 1996; Peracchia et al., 2000) or activate kinases such as Ca^{2+} -calmodulin-dependent kinase II (CaMKII) to phosphorylate gap junctions (Pereda et al., 1998). All three of these mechanisms represent potential sites for activity-dependent modulation of electrical synapses. Furthermore, it should be noted that Ca^{2+} entering a neuron contributes to further depolarization of the cell membrane, particularly at synapses, where changes in gap junction permeability will depend on the dual sensitivities of those channels to calcium ions and membrane voltage.

Adding to the story on channel phosphorylation, many second messengers including cyclic adenosine monophosphate (cAMP), cyclic guanosine monophosphate (cGMP) and the aforementioned Ca^{2+} act to alter junctional conductance by modulation of phosphorylation state of intercellular channels (Saez et al., 1986; Kwak et al., 1995). Studies of Cx43 suggest that there are roughly 21 serines in addition to 2 tyrosine amino acids that are likely the sites of kinase activity and phosphorylation involved in gap junction protein conformational changes that underly phosphorylation-dependent gating (Solan and Lampe, 2005). The retina has provided arguably the most well studied model

of this type of channel gating. Horizontal cells of teleosts undergo a reduction of coupling when injected with cAMP, thereby activating cAMP-dependent protein kinase A, PKA (Lasater, 1987). The same phenomenon happens in horizontal cells of the turtle retina when adenylate cyclase is activated by the addition of forskolin (Piccolino et al., 1982). However, the exact gating induced by phosphorylation is, again, often dependent on the connexin and hemichannel composition, as demonstrated by cAMP increasing, rather the decreasing, junctional conductance among mouse tumor cells (Atkinson et al., 1995).

Gap junction expression and trafficking

Aside from direct gating of individual or populations of gap-junctional channels, another mechanism affecting overall electrical synaptic strength is alteration in plaque size. Membrane bound connexin half-lives in some cell membranes are known to be only a few hours long (Laird, 2006), meaning that regulation of gap junction numbers can be highly dynamic and an important site for potential modulation. This can be achieved through multiple methods including: increased expression of the genes encoding gap junction proteins, specific trafficking of those proteins to desired sites on the cell membrane, or regulation of gap junction turnover within the plaque itself. In the first instance, changes in connexin or innexin gene expression would lead to a potential increase or decrease in the number of proteins and hence potential channels expressed at a plaque. However, mere changes in protein levels do not guarantee their inclusion at synaptic sites. Therefore, trafficking to appropriate regions of the cell membrane is also

required to effect changes in intercellular conductance. Additionally, a given channel's inclusion into, or removal from, a given plaque dictates how strong the coupling at that site can be and can provide potential regulatory control of electrical coupling.

In mammalian reproduction, Cx43 is upregulated in the myometrium of pregnant rats in preparation for parturition (Lye et al., 1993). The increase in transcription of connexin genes during pregnancy in the myometrium acts to increase cell-to-cell coupling, where synchronization of muscle contractions is necessary for the birthing process (Risek et al., 1990, Lefebvre et al., 1995). Likewise, though some connexin expression remains, overall gap junction protein levels are reduced in rat motor neurons during the chemical synapse formation stage of spinal development (Chang et al., 1999), going from a period where the spinal motor networks are largely electrically coupled into the adult state when motor neurons are rarely connected to interneurons by anything other than chemical synapses.

A connexin's pathway from gene transcription to synaptic membrane localization follows the general mechanism of other synaptic proteins. These included protein synthesis occurring at the endoplasmic reticulum (ER), where connexins ultimately are integrated into the ER membrane and their protein topology is finalized. From there, through repeated vesicle budding and fusion cycles, the proteins (perhaps in multimers) are transported from the ER membrane and hemichannels are formed through the various Golgi stacks, trafficked along microtubule-based transport mechanisms and finally inserted into the cell membrane (Yeager et al., 1998). Since Cx43 is best studied in this regard I will briefly summarize the 'birth' portion of its lifecycle. As suggested

above following its synthesis in the ER, Cx43 polypeptide simultaneously gets incorporated into the ER membrane. Although inconsistencies exist on where oligomerization of Cx43 occurs (Falk et al., 1997; Musil and Goodenough, 1993), most recent data suggest that hexamer formation into connexons does not occur until the trans Golgi network (Vanslyke et al., 2009).

Obviously, an important linkage between changes in gene expression and the ultimate incorporation of the finished protein into the cell membrane involves trafficking of the protein to appropriate sites on the cell membrane. As knowledge of the gap junction and gap junction assembly increases, sometimes referred to as the gap junction proteome, many of the players in directing and targeting of its assembly and incorporation are being defined. Phosphorylation of connexins and hemichannels is one such proposed mechanism. Rab20, a member of the Rab family of GTPases known to be involved in intracellular trafficking, directs Cx43 through its many steps of vesicle budding and fusion (Das Sarma et al., 2008). Another mechanism mediated by ERp29, a protein found in the endoplasmic reticulum and known to process ER proteins, has been demonstrated to bind Cx43 suggesting it may act to chaperone connexins through the secretory pathway (Das et al., 2009). Although targeting of gap junctions to appropriate sites on the cell membrane would logically be an important step in the process of plaque assembly there is currently some debate about exactly how specific individual cells are at directing hemichannel assemblies to the plaque site.

Gap junction plaques grow by accretion, where new channels get incorporated into the cell membrane, reportedly at non-junctional sites, and can move laterally to join

with the periphery of a given plaque (Lauf et al., 2002). Also given the knowledge that older channels are removed from the central portions of the plaque (Gaietta et al., 2002), it is now clear that gap-junctional plaque size is regulated by addition of new channels in the periphery and removal of old channels medially. Incorporation of connexons into the gap-junctional plaque occurs through various interactions with the scaffolding proteins or linkers with the cytoskeleton. Zonula occludens-1 (ZO-1) has been well studied in its interactions with members of the connexin family and can regulate distribution of junctions as well as plaque size through interactions with spectrin and the cytoskeleton (Toyofuku et al., 1998; Hunter et al., 2005). In addition to spectrin, other actin-binding proteins, drebrin and cortactin (Butkevich et al., 2004; Vitale et al., 2009), function to link connexins with the actin cytoskeleton.

Turnover of gap junctions from the center of plaques occurs often by internalization of the intact junction, or at least segments of the junction. This internalization results in the formation of a double-membrane vesicle, often referred to as annular gap junctions or connexosomes (Jordan et al., 2001). Interestingly, but not surprisingly, this double-membrane vesicle contains both plasma membrane from the internalizing cell as well as the juxtaposed cell. Internalization of this connexosome is thought to occur via clathrin-mediated endocytosis, as shown in Gumpert et al, 2008 where use of RNA interference of clathrin or associated machinery, AP-2, Dab2, or dynamin resulted in a reduction of internalized gap junctions. After undergoing endocytosis, connexosomes can then be targeted for degradation. There have been suggested roles for a sequential degradation of gap junctions by both lysosomal and

proteasomal degradation pathways. A report by Qin et al. (2003) demonstrated that in tumor cells proteasomal pathways act to promote endocytosis of Cx43 while lysosomes are more integral to the actual degradation of the internalized junctions. It is important to note that although the mechanism described here is the most widely studied, many tissues appear to lack connexosomes (Laird, 2010), suggesting that either other mechanisms for internalization may be present, or that data on connexosomes is incomplete.

Several aspects of these house-keeping mechanisms are central to my research approaches, results and interpretations to follow in this dissertation. First, the fact that gap junctions and the plaques and synapses that they underlie are highly labile is important. The idea that electrical synapses can be constructed and deconstructed in hours will become a resonating theme here. Our understanding that the building of hemichannels is largely an ER-golgi process and that the construction of an electrical synapse is dependent on directional trafficking to that synapse will be central to my arguments. Furthermore, the ongoing endocytotic cycle at synaptic sites and its role in the maintenance of synapse communication will be exploited by pharmacological disruption of the dynamin-mediated aspects of this process. Finally, these processes and more are very likely important sites of the regulation of early and ongoing electrical synapse formation with neural networks. Therefore, modulatory mechanisms that alter the construction and maintenance of gap junction plaques are as important as those that affect changes in junctional gating properties. The next section will expand upon these issues.

Gap junction modulation

A final principle of gap junction physiology important here is that they are readily modulated by a number of factors exogenous to the cells. For example, many neurotransmitters, such as dopamine (DA) and glutamate, affect changes in junctional coupling. A well-known mechanism involves DA activation of a D1 receptor in the horizontal cells of the retina to reduce junctional conductance (Lasater, 1987; McMahon et al., 1989; Piccolino et al., 1982; Teranishi et al., 1983). This effect is mediated via D1 activation of adenylate cyclase to stimulate an increase in cAMP levels leading to changes in PKA-mediated phosphorylation. Another cell type in the retina, the AII amacrine cells, also undergo modulation of gap-junctional coupling in response to DA. As suggested in Kothmann et al. (2009), DA-induced PKA activity in amacrine cells activates a phosphatase, causing dephosphorylation of the gap junction leading to reduced coupling. In the retina DA may also activate D2-like receptors in the rods and cones of the photoreceptor layer to increase junctional conductance at certain times of the day (Ribelayga et al., 2008). Aside from the retina, DA modulation of junctional conductance has been demonstrated in the nucleus accumbens (O'Donnell and Grace, 1993), the mixed synapse of the Mauthner cell of fishes (Pereda et al., 1994), and the lobster stomatogastric ganglion (Johnson et al., 1993).

Nitric oxide (NO), a gaseous neurotransmitter, can also modulate junctional conductance. Patel et al. (2006) demonstrated that HeLa cells transfected with Cx35 underwent a reduction in junction permeability due to NO application that involved both cGMP-dependent protein kinase (PKG) and PKA. NO also stimulates guanylate cyclase

to increase cGMP levels in horizontal cells, amacrine cells and bipolar cells of the retina, where it alters phosphorylation of connexin proteins through activation of PKG (Mills and Massey, 1995; Xin et al., 2000). The neurotransmitter serotonin (5HT) can also modulate coupling in the nervous system. Rurig and Sutor (1996) showed that 5HT reduced cell coupling in the somatosensory cortex through its actions on the 5HT₂ receptor. Reduction of coupling in this serotonin-mediated mechanism involved release of calcium from intracellular stores. Invertebrate neuronal gap junctions are also modulated by 5HT, with some these effects caused by non-junctional actions (Szabo et al., 2010), a topic addressed in the next section of this introduction.

Aside from modulation via neurotransmitters, junctional conductance can also be altered through interactions with cell adhesion molecules. Connexin proteins directly interact with various cadherins, a class of Ca²⁺-dependent adhesion molecules. E-cadherin, for instance, is proposed to be a key player in the mechanism underlying Ca²⁺-dependent modulation of junctional signaling (Jongen et al., 1991). Similarly, antibody treatments that block N-cadherin function lead to the failure of Cx43 to form junctional channels (Meyer et al., 1992). Additionally, laminin 5 through its interactions with integrin proteins produces modulatory effects on intercellular communication through gap junctions and this mechanism is thought to involve Rho-GTPases (Lampe et al., 1998).

Non-junctional regulation of electrical synapses

When discussing changes in the coupling strength of electrical synapses it may be tempting to think of these in terms of direct effects on junctional conductance through modifications in the functional properties of gap junctions themselves. However, non-junctional factors including both membrane resistance and capacitance of the cells harboring the plaques also impact coupling (Bennett, 1966). Coupling strength is vitally dependent not only on the resistance through the junction itself, but also the resistance of the cell to current flow across its own plasma membrane or across the electrical synapse with its other targets. Therefore, anything that alters membrane resistance of the neuron will functionally alter its coupling, where an increase in membrane resistance will promote stronger coupling and vice versa. A simple and common example of a non-junctional modulatory effect is the decrease in membrane permeability to K⁺ ions induced by many neuromodulators. Closure of K⁺ channels leads to an increase in membrane resistance, thereby shunting a greater percentage of current through the gap junction into the neighboring cell.

Interaction of chemical synapses in close proximity to electrical synapses is also likely to affect the degree of electrical coupling. Though some chemical synapses undoubtedly impact electrical coupling through steps that involve changes in intracellular calcium and relative phosphorylation levels of gap junctions, they also possess the means to regulate coupling by non-junctional mechanisms. Inhibitory chemical synapses proximal to gap junctions can counteract the effects of depolarizing ionic currents, and consequently gap-junctional currents, by shunting away some of that

signal through the ligand-gated channels. As recently reviewed in Pereda et al. (2012), there is an interesting example of this phenomenon where chemical inhibitory synapses seemingly serve this specific purpose at mixed electrical/chemical connections. Electrically coupled networks of inferior olive neurons form gap junction plaques at their dendritic spines and communicate exclusively through these electrical synapses. However, these spines also interface with afferent inhibitory inputs from neurons of cerebellar nuclei (De Zeeuw et al., 1998). The cerebellar neurons release the inhibitory neurotransmitter GABA onto the spine and shunt away depolarizing signal that might otherwise flow between the inferior olive neurons. Thus, the purpose of this inhibitory synapse is to indirectly regulate gap junction coupling and, consequently, the synchrony of firing between inferior olive neurons.

Several aspects of the mechanisms of gap junction modulation, direct or indirect, are central to this dissertation. First, I will show that dopamine is a potent regulator of gap junction coupling within motoneuronal networks and found that the mechanisms underlying this DA-mediated modulation are both direct and indirect. Second, a central theme of my research involves the interaction of inhibitory chemical neurotransmission and electrical transmission at emerging mixed synapses within neural networks. The significance of these inhibitory synaptic components as mechanisms of current shunting is addressed.

Electrical Synapses in Development

It is now evident that electrical synapses are much more widespread than initially thought (Meier and Dermietzel, 2006) and are also incredibly dynamic (Cachope and Pereda, 2012; Pereda et al., 2012). Electrical synapses can be found in neurons of the adult vertebrate brain (Bennett et al., 1959; Connors and Long, 2004; Korn et al., 1973), as well as among glia (Pannasch et al., 2011; Sanderson et al., 1994; Sneyd et al., 1995), whose importance in regulating neuronal signaling has now been well established (Araque et al., 1999). Electrical synapses are discernible even at early developmental states of the nervous system (Connors et al., 1983; Spitzer 1982; Walton and Navarette, 1991). The coordination of neuronal differentiation is thought to partly involve mediation of biochemical signaling through gap junctions. NT2-D1 progenitors differentiate into NT2-N neurons through a retinoic acid pathway. Gap junction blockers reduce differentiation into NT2-N neurons without directly affecting retinoic acid signaling (Bani-Yaghoub et al., 1999). Additionally, gap-junctional communication in mice is necessary and sufficient to maintain cells in their neural progenitor state, while disruption of Cx43 gap junctional assembly causes embryonic cells to exit progenitor states and differentiate (Cheng et al., 2004).

An early discovered and now canonical role for electrical synapses in circuits is their ability to coordinate synchrony of activity within coupled neurons (Deans et al., 2001; De-Miguel et al., 2001; Kiehn and Tresh, 2002; Mann-Metzer and Yarom, 1999), a mechanism that can influence the firing activities of large neuronal and muscle

ensembles. One of the most well examined illustrations of electrical signaling was in the mediation of motor behavioral outputs associated with rapid escape reflex circuits (Edwards et al., 1999; Phelan, et al., 1996). This synchrony can also be involved in a number of important processes including synapse stabilization or removal during development (Cohen-Cory, 2002; Personius et al., 2007), coordination of rhythmic oscillations critical to pattern generation, and coupling of firing rhythms associated with aspects of cognition (Hormuzdi et al., 2001; Draguhn et al., 1998). Further, electrical synapses have been implicated in neural circuit adaptation mechanisms where discrete outputs depend on the synchronous integration of multiple incoming signals (Edwards et al., 1998). A straightforward example of the latter is found in the rat retina where amacrine cells, receiving separate inputs, use electrical coupling as a coincident detector to produce different electrical outputs depending on the temporal sequence of the incoming signals (Veruki and Hartveit, 2002). Coincident subthreshold inputs can summate due to electrical coupling among amacrine cells allowing these neurons to produce suprathreshold outputs only when incoming stimuli are coincident. In this manner even a relatively simple setup like neurons coupled with a bi-directional electrical synapse can allow neural networks to be modified based on integration of multiple inputs. Though there are numerous points of evidence showing electrical synapses act to support synchrony in neural networks, it is also important to note that there are some networks where electrical synapses function to counter synchrony such as in the golgi cells of the mouse cerebellum (Vervaeke, et al., 2010).

As discussed in Relá and Szczupak (2004), electrical synapses act to functionally delineate signaling compartments via creation of cytosolic continuity between the cells. Compartmentalization of neural networks within the nervous system can be useful in augmenting the outputs of those circuits. In this instance, coupled neurons that receive separate inputs, but have the same target, can both stimulate the target provided one cell receives a suprathreshold potential from its input. The spatial expression of the electrical synapse is important though, as this synchronous effect is most reliably produced when the coupling occurs in close proximity to the neuron's spike initiation zone. Additionally it is often convenient when discussing electrical synapses to think of 'coupling' as occurring throughout the entire cell. However, branch-coupled cells such as dendritic spines or axon terminals can function as discrete coupling compartments.

Certain systems demonstrate sustained use of gap junctions, such that after development the electrical connections are maintained in the adult connectivity. This can occur in certain motor behaviors, as mentioned earlier, as well as sensory inputs, including those from the retina, cochlea, and olfactory bulb. In the retina, gap junction coupling is found at numerous cell contact sites, including the synapses of rods and cones (Tsukamoto et al., 2001). AII amacrine cells mediate signaling between rod bipolar cells and ganglion cells and this signaling pathway is largely through gap junctions (Strettoi et al., 1990). In the cochlea, Cx26 and Cx30 constitute gap junctions of mice and the mutation of these connexin proteins is linked to genetic deafness (Ahmad et al., 2003). Finally, synchrony in external tufted cells, proposed to be

important for encoding olfactory stimuli, occurs through gap-junctional coupling (Hayar et al., 2005).

Many electrical synapses do not persist in neural networks and instead are transient in nature. As suggested by earlier statements regarding neural progenitor differentiation, many processes require tightly sequenced coupling and uncoupling of cells in order to fulfill their physiological needs in a precise manner. During the maturation of developing neural networks, electrical synapses are initially expressed and later undergo a programmed uncoupling from their synaptic partners (Bittman et al., 1997; Chang and Balice-Gordon, 2000a; Dupont et al., 2006; Spitzer 1982). This electrical coupling and uncoupling during development led to the identification of a temporary expression pattern for some electrical connections, called transient electrical synapses (TES). The presence of TES has been found in a diverse number of nervous systems. These systems include the vertebrate visual cortex (Kandler and Katz 1998), other neocortical regions (Connors et al., 1983; Dupont et al., 2006; Peinado et al., 1993), neuromuscular junctions (Personius et al., 2001), the hypothalamus (Arumagam et al, 2005), the retina (Penn et al., 1994) and the spinal cord (Chang, et al., 1999; Mentis et al., 2002). Furthermore, electrical synapses are wide spread among invertebrate nervous systems, but transient electrical synapses also are found in these phyla. For example, the visual system of flies (Curtin et al., 2002) and the motor systems of molluscs (Szabo et al., 2004) and annelids (Marin-Burgin et al, 2006) express these short lived connections. An important largely untapped gap in our knowledge about neural networks and their development involves temporal changes in the type of synaptic

signaling present during their formation. The fact that TES formation and subsequent uncoupling coincides with discrete events during development, gives rise to the possibility that the pattern of electrical coupling/uncoupling may have an important role in the formation of neural networks (Bittman et al., 2002; Peinado et al., 1993; Yuste et al., 1995) and that the regulation of these intercellular synapses is likely vital to how the nervous system functions.

Aside from this observable phenomenon in the developing nervous system, some injured nervous systems reproduce TES, further supporting its fundamental role in proper networking between cells. After axotomy, dye-coupling across electrical synapses is observable in mammalian spinal motor neurons similar to that in development (Chang et al., 2000B), leading to the conclusion that electrical coupling may be important even in the reestablishment of neuronal connections following injury. Likewise the pulmonate snail *Helisoma* exhibits patterns of transient electrical coupling in both injured buccal ganglia as well as in networks regenerating in vitro (Haydon et al., 1987; Haydon and Kater; 1988; Szabo et al., 2004).

An important aspect of signaling in neural networks is the interplay between electrical and chemical synapses and their regulation of the opposing form of communication. This can occur when one synaptic type modulates signaling in the other at mixed synaptic connections, such as in the Mauthner cell, where electrical synapses are maintained in response to synaptic activity that is dependent upon co-localized NMDA receptor activation (Pereda et al., 1998). In the glomerulus of the olfactory bulb, Cx36 deficient mice fail to develop appropriate chemical signaling between mitral cells

and thus suggest that gap-junctional coupling is necessary for glomerular circuit maturation (Maher et al., 2009). In invertebrate development electrical synapse-dependent formation of adult chemical synapses is also seen in the *Drosophila* eye (Curtin et al., 2002) and in the leech ventral nerve cord (Todd et al., 2010). In the mammalian neuromuscular junction (NMJ) where mice have Cx40 mutations, synapse elimination occurs at earlier developmental time points than in mice normally expressing Cx40, suggesting that correlation of activity via gap junctions is responsible for appropriate timing of NMJ maturation.

The functions of transiently expressed gap junctions are starting to be elucidated, but still remain largely mysterious and this is particularly true with respect to the regulation of neural network development. These transient synapses have long been known to coordinate electrical activity among synaptic partners and coordinate synchrony in neural circuits, but it is also evident that they are coordinating biochemical events among synaptic partners (Kandler and Katz, 1998). The biochemical coupling of cells can coordinate intracellular signaling cascades among neural networks and regulate refinement of those circuits. Transient gap junctions behave as cell adhesion substrates at choice points during development and in proper neuronal migration (Kristan et al., 2000; Elias et al., 2007). Therefore, it is not unreasonable to hypothesize that electrical synapses mediated by gap junctions are critical components in many of the processes underlying finding and connecting of neurons with their appropriate network constituents during development. This idea is one of several key foundations of the hypothesis I have tested in this dissertation.

It is known that gap-junctional coupling inhibits chemical neurotransmission within several neural systems, including those of neonatal rats (Mentis et al., 2002) and regenerating *Helisoma* motor systems (Bulloch et al., 1984; Szabo et al., 2004; Szabo et al., 2007). These data suggest that neuronal coupling may be responsible for delaying chemical transmission until an undetermined time when chemical neurotransmission is efficacious. Interesting, data from leech using injection of RNAi against Hm-inx1 show that formation of TES between certain neurons in the midbody ganglia is a required precursor to the formation of more mature chemical neurotransmission (Todd et al., 2010). This suggests that TES could not only be important for the timing of developing mature chemical synapses, but their ability to form during a critical window of time. Recently, it has been shown that inhibitory chemical synaptic transmission of thalamic relay neurons in the mammalian brain emerged as electrical coupling decreased during the first and second postnatal weeks. It remains unclear whether interactions between electrical and chemical synapses during early postnatal development play a mechanistic role in the loss of electrical coupling as chemically-mediated inhibitory circuit mature.

Coincidence of synaptic signaling is critical to the analysis and interpretation of my dissertation research. Coincidence detection of Ca^{2+} signals is used in aspects of my work to predict electrical coupling within neural networks of mixed connectivity. Furthermore, most of the synapses studied here are, in fact, mixed to varying degrees, but usually expressing both electrical and chemical components. The interactions between these two types of synaptic connections and the impact that those interactions have on the synaptic outcomes of neural network formation constitute the central

premise of my work. *Helisoma* motor neurons, during network formation in vivo and in vitro, form both electrical synapses and inhibitory chemical synapses at their sites of cell-to-cell contact. In some instances electrical coupling is short lived, a form of connection described earlier as transient electrical coupling or TES. In other instances, the electrical coupling is strengthened over time and maintained. These forms of electrical connection I have called sustained electrical synapses or SES. Finally, to differentiate newly forming electrical synapses from those being diminished or enhanced, I will use the term forming electrical synapse or FES for clarity.

Synaptic Connectivity and Formation in *Helisoma*

Invertebrate model systems, since early investigations of the nervous system around the mid 20th century, have been used for the study of neural physiology at the cellular level. Key examples of this include a series of papers by Hodgkin and Huxley (1952a; 1952b) in the 1950's fundamentally describing the ionic nature of the resting membrane potential and the action potential in the squid giant axon as well as the aforementioned initial demonstration of electrical synapses in the crayfish by Furshpan and Potter in 1957. Many of these systems became popular experimental models and successful due to their large neuronal size, identifiable nature, and ease of access, at the time, by new techniques like intracellular electrophysiology. Invertebrate systems were also attractive because of their relatively simple behaviors and the reduced neural circuits eliciting those behaviors, probably best exemplified in studies by Eric Kandel and colleagues on

the nervous system of *Aplysia* and its regulation of the gill withdrawal reflex (Pinsker et al., 1970).

Neurons of the *Helisoma* buccal ganglia are quite large (in some cases with a diameter greater than 30 μ m) and mediate feeding behavior of this freshwater snail. Due to the large neuron size and contrast of pigmentation beneath the ganglion sheath these neurons are easily identifiable and are readily accessible via electrophysiology to assess neural connectivity and physiology. Studies culminating in Stanley Kater's 1974 overview, thoroughly described the simple *Helisoma* feeding behavior and the underlying neurons and buccal muscles involved in mediating movement of the radular feeding structure. These observations provided the basis for future studies on neural networks both in vivo and in vitro by providing the necessary framework of both individual neurons involved in feeding and the functioning of the broader feeding circuit. Groups of interneurons, collectively known as central pattern generators (CPG), are electrically coupled to one another and mediate the rhythmicity of motor neurons involved in snail feeding (Granzow and Kater, 1977; Kaneko et al., 1978). The motor circuit involved in feeding was initially described to generate a two-phase rhythmic pattern (Kater, 1974), however it was later determined to be tri-phasic in nature as reviewed by Don Murphy (2001) and as summarized below.

Helisoma feeding circuit

During phase 1 (S1), S1 interneurons, which include N1a and BCN1, respond to inputs from sensory afferents by stimulating a subset of buccal motor neurons, of which

B6 and B8 are best described. N1a and BCN1 are thought to mediate their effects on motor neurons via different mechanisms, with the former using dopaminergic signaling, while the latter is thought to use a GABAergic system. In response, S1 motor neurons that innervate the posterior jugalis muscle, cause a protraction of the odontophore and extension of the radula.

S1 interneurons in addition to stimulating S1 motoneurons also elicit activity in phase 2 (S2) interneurons to initiate the next phase of feeding. Initiating this phase are neurons B2 and B29, referred to as influential neurons given that they have both interneuronal connections and motor outputs to muscles. These motor neurons stimulated S2 interneurons include B110, B26, B27. B2 is thought to work primarily through a glutamatergic pathway. S2 motor neurons innervate portions of the anterior jugalis (AJ) as well as the supralateral radular tensor (SLrT) to initiate retraction of the odontophore back into the oral cavity. S2 CPG neurons additionally inhibit S1 production as well as phase 3 (S3) initiation until phase 2 is complete.

S3 interneurons, which include N3a, start to fire due to postinhibitory rebound from the S2 portion of the CPG. In response these interneurons activate S3 motoneurons like B17, B18 and B19. The motor neurons also innervate segments of the AJ and SLrT muscles to produce a hyper-retraction of the odontophore and radula, bringing them in close proximity to the esophagus. During the interphase, before another bout of feeding, S1 interneurons are still recovering from S2 inhibition and will eventually fire again if sufficient stimulation exists.

Regeneration and cell culture

Aside from its relative simplicity, large sized neurons and ease of manipulation, another attractive feature of *Helisoma* is its potent regenerative capabilities. Axotomy of existing connections in certain neurons demonstrated the capability of identifying appropriate axonal pathways and synaptic targets. Although the process of neuronal regeneration is more discerning with regard to peripheral connections (Murphy and Kater, 1978), central connections like those of neuron B5 exhibit novel coupling following injury (Bullock and Kater, 1982). Potent regeneration of this molluscan system makes it an appealing option for observing how synaptic connections are formed. A recent publication on neuromuscular contact regeneration proposes that in *Helisoma* this occurs via a three step processes (Turner et al., 2011). Upon initial contact with potential muscle targets, miniature NMJ potentials are robust with a virtual absence of action potential-evoked release of neurotransmitter. This was subsequently followed by a strong reduction in miniature potentials while evoked potentials became weakly efficacious. In the last phase, miniature potentials were virtually absent and neuromuscular contacts regained fully functional adult synapses with robust evoked release of neurotransmitter.

The *Helisoma* system benefitted greatly following the demonstration that neurons could easily be dissociated from their ganglia into cell culture. The system was further aided by the revelation that isolated neurons cultured in media supplemented with trophic factors from *Helisoma* central ring ganglia were capable of neurite extension (Wong et al., 1981). Utilizing this cell culture system, many neuronal properties

fundamental to the feeding neural system were determined. In Haydon et al. (1987), it was demonstrated that growth cone extension and motility is differentially regulated and that extension of neural processes was intimately involved in the regulation of synapse formation in vitro (Haydon and Kater, 1988). In 1989, Haydon and Zoran demonstrated that neurons have different synaptogenic strategies. Neuron B19 has constrained synaptogenesis and requires an activity-dependent contact with appropriate SLrT muscle fibers in order to gain action potential-coupled evoked chemical transmission (Zoran et al., 1991; Poyer and Zoran, 1996). Meanwhile neuron B5 is promiscuous in its synaptogenesis and will indiscriminately secrete neurotransmitter on novel targets in culture (Haydon and Zoran, 1994). More recently the dynamics of synapse formation have been demonstrated. Existing synaptic connectivity or its inability to form results in changes in the sequence and efficacy of synaptic plasticity (Neunuebel and Zoran, 2005; Szabo et al., 2004), as well as the formation of connectivity within small neuronal networks (Szabo and Zoran, 2007).

Electrical synapses in Helisoma

One of the first demonstrations of an electrical synapse in *Helisoma* came when Bulloch and Kater (1981) observed electrical connections between bilateral neuron B5s following axon crush in reduced buccal ganglia preparations. This study also demonstrated a short-lived coupling between ipsilateral neuron B4 and B5. Thus, the first evidence of transient electrical synapses following nerve injury were reported. Hadley and Kater (1983) used electrophysiology on intact buccal ganglia preparations to

show that bilateral B19s possessed electrical synapses and demonstrated that junctional channels aide in synchronizing left and right side behaviors during feeding. Electrical coupling between B5s, however, does not exist unless regeneration is induced and transient forms of coupling emerge. This novel connection is seen both in vivo as well as in vitro and requires coincident neurite growth, such that neurons that were not actively growing before cell-cell contact failed to couple (Hadley and Kater, 1983; Hadley et al., 1983).

Work done by Szabo (2004) demonstrated that in addition to B19, fellow S3 motor neuron B18, was extensively coupled to the opposing B18 in intact ganglia preparations. B19, B18 and B110, an S2 motor neuron, were shown to form robust and sustained electrical coupling in cell culture when paired with homotypic partners. In the same study, short-lived, transient electrical synapses formed between B110 and B19 both in vivo and in vitro. Furthermore, inhibitory chemical synapses that propagated only from B110 to B19 were also formed at these heterotypic connections. B110 bears similarities to B5 in that it has unconstrained synaptogenic capabilities and will therefore be promiscuous in developing evoked neurotransmitter release onto any neuronal target that is sensitive to acetylcholine, the primary neurotransmitter of molluscan motor neurons. These studies provided a foundational framework for the formation and function of electrical synapses during regeneration of the *Helisoma* feeding network.

Electrical synapses in the regulation of connectivity and network formation

As previously discussed, ongoing electrical coupling in the adult feeding circuit occurs between neurons B19 and B18 and is likely to aid in the coordination of bilateral motor outputs during a round of feeding, in this case hyper-retraction of the odontophore. Although coordination of motor behavior likely occurs in part due to this method, it is redundant due to the fact that these neurons possess both ipsi- and contralateral projections to the muscles. B19 is strongly dye-coupled to other S3 motor neurons, B17 and B18 (Szabo, unpublished), suggesting that the entire output of phase 3 motor activity is synchronized. As occurs in the mammalian retina (Veruki et al., 2002), coupling of these neurons could act to ensure that the behavioral output only occurs when there is coincident stimulation of S3 motor neurons, ensuring that erroneous hyper-retraction doesn't occur.

Neuron B5 shows no evidence that it projects contralaterally to the esophagus and does not connect with its homologous B5 (Bulloch and Kater, 1981), suggesting that its target organ, the esophagus, uses an alternative mechanism to synchronize its activity. However, upon nerve injury B5 sends a contralateral projection and forms a novel synapse with the other B5. This synapse appears to be maintained for at least a week, and then is likely eliminated. B110 neuron, as indicated earlier, bears certain physiological similarities to B5 and has not been shown to connect with its contralateral counterpart, although it does indeed have contralateral projections (Murphy, 2001). B110 neurons develop electrical synapses that are maintained at least throughout the 5 days in cell culture (Szabo et al., 2004). Hypothetically, these neurons might benefit

from the synchrony provided by electrical coupling yet in normal development achieve this through a separate pathway that has yet to be demonstrated. The formation and persistence of these novel connections provide an intriguing and potentially exploitable facet of synapse and network formation.

Aside from SES in the buccal ganglia, there are also a few examples of TES amongst buccal neurons. The first discovered transient coupling was between B5s mentioned earlier, but also at time B4-B5 and B5-B19 short-lived connections were found to exist following nerve injury (Hadley and Kater, 1983). TES peaked in coupling at about 2 days of contact and by day 7 were uncoupled. Interestingly, the transient nature of the B4-B5 synapse was dependent on the presence of coupling between the two B5s (Bulloch and Kater, 1981). In the absence of that coupling, B4-B5 electrical connections were maintained roughly twice as long. Szabo et al. (2004) also describe a transient connection between B110 and B19 following commissural crush. Similar to TES formed by neuron B5, B110-B19 coupling peaked at day 2 and underwent significant reduction by day 7. Perhaps most interesting about this synapse was that by day 3 a pronounced inhibitory chemical synapse had formed from B110 onto B19. It was subsequently found in vitro that reduction of electrical coupling via deprivation of trophic factors caused an earlier formation of the chemical synapse, leading to the hypothesis that TES might regulate the timing of subsequent chemical synapse formation. This idea of an inverse relationship between chemical and electrical synapse formation has subsequently been demonstrated between hypothalamic (Arumagam et al, 2005) and thalamic neurons (Lee et al., 2010) of the developing mammalian brain. This

transition from electrical to chemical synapses has been documented for many species and many brain regions in mammals, but the mechanisms that mediate this switch and the possible interactions between these forms of connectivity during neural network development have remained elusive (Kandler and Thiels, 2005).

Neunuebel and Zoran (2005) followed up this idea by looking at how TES might regulate chemical synapse formation. In the absence of transient electrical coupling, neuron B110 was capable of evoked transmitter secretion in response to release of Ca^{2+} from an intracellular cage at 24 hours of contact with B19. At the same synapse B110 was incapable of evoked secretion when TES was present. This effect was not due to changes in cytosolic calcium accumulation or cholinergic receptor sensitivity. In fact, cells that were incubated in conditioned medium (CM), and capable of forming TES, exhibited better recruitment of vesicles proximal to the site of contact than those lacking TES following incubation in trophic factor-deprived media. Thus, a functional disruption of the ability of neuron 110 to couple calcium influx to vesicle release may occur when TES is present.

Further work on the role of transient coupling on synaptic connectivity was demonstrated using a simple three-cell network in cell culture as shown in Fig. 1B, where cells were dissociated and placed into novel configurations. Szabo and Zoran (2007) used a protocol where two neurons were allowed to undergo synaptogenesis over a 4-day span of time after which an additional neuron was placed in contact with the existing 2-cell network. An exhaustive study was performed where all permutations of 3-cell connectivity were assessed for electrical and chemical connectivity. The culmination

of those studies demonstrated that new synapse formation was dependent on the synaptic connectivity already present within the network, and suggested networking outcomes were due at least in part to connectivity-dependent junctional coupling. This study in particular laid the framework for the basic neural network/cell culture protocol I have used in the present studies.

Modulation of electrical synapses

Modulation of synaptic connectivity is a key component in the regulation of many physiological processes and is a fundamental part of the neural regulation of most animal behaviors. Changes in synaptic connectivity are known to occur in response to neural (neurotransmitter) and humoral (hormone) signals. In the *Helisoma* feeding circuit, two major modulatory inputs are known to exist: C1, a serotonergic neuron from the cerebral ganglion, and P11, the FMRFamideergic neuron from the pleural ganglion (Murphy, 2001). Application of these two transmitters modulates motor function of the buccal feeding circuit (Zoran et al., 1989). Serotonin is known to depolarize buccal neurons (Achee and Zoran, 1997; Szabo et al., 2010) and, either through application or C1 stimulation, is known to act to phase-lock retraction and hyper-retraction of the radular movements (Price and Godlberg, 1993; Quinlan and Murphy, 1996).

To date there has been no demonstrated role for FMRFamide in electrical synapse modulation, though it has also not been ruled out. Meanwhile, 5HT selectively inhibits neurite elongation, a process proposed to be necessary for electrical coupling, between buccal neurons (Haydon et al., 1987). Szabo et al. (2010) showed that serotonin

had variable effects on electrical coupling between buccal neurons, where cells with weaker coupling exhibited stronger reduction in coupling. However, 5HT's effects on the gap-junctional coupling were not direct, but instead were extra-junctional in nature through the modulation of a hyperpolarization-gated inward current.

DA, another important neuromodulator, through exogenous application is capable of initiating the feeding behavior in its full and distinct three phases (Quinlan and Murphy, 1996). Feeding stimulants that induced the full feeding behavior activate neuron N1a, a phase 1 interneuron, which subsequently initiates feeding (Quinlan et al., 1997). DA also suppresses neuronal activity of some buccal neurons via activation of D2-like receptors (Zhong et al., 2012). These receptors, in turn, activate phospholipase C (PLC), which leads to K^+ channel activation and membrane hyperpolarization. Thus, DA is a key component in the modulation of the *Helisoma* feeding circuit. The various neuromodulators found in the *Helisoma* feeding circuit suggest that multiple pathways interact to produce the various patterns of feeding seen in the snail. These modulatory effects are certain to impact synaptic connectivity amongst neurons involved in feeding and the formation and regeneration of the neural circuits that mediate it.

Summary

Gap junctions are found throughout the tissues of animal bodies in most taxa studied. The intercellular coupling they provide impact many aspects of the lives of cells, from differentiation and apoptosis to heart contraction and brain function. Unsurprisingly,

mutation of some of these proteins can lead to a variety of disorders including X-linked Charcot-Marie-Tooth syndrome (Bergoffen et al., 1993) and some forms of hereditary hearing-loss (Martinez et al., 2009). These junctions are not mere static entities, but rather are highly dynamic in the degree in which they communicate and are regulated. Notably electrical synapses in numerous nervous system preparations undergo programmed uncoupling during important windows of synapse formation. I have hypothesized that transient electrical synapses play a vital role in how neural networks are developed, subsequently impacting how behavior is regulated by those networks. Here, I use *Helisoma* neuronal cell culture for its ease of manipulation and its direct access to assessment of electrical connectivity within networks of identifiable neurons. *Helisoma* buccal neurons reach a choice point between 1 and 2 days of contact where coupling either is downregulated, and therefore transient or TES, or strengthens and persists, therefore sustained or SES, as summarized in Figure 1C. Ultimately the temporal expression is such that as TES declines cholinergic chemical synapses (CCS) are upregulated. The question however, remains how these two temporal sequences of expression regulate emerging connectivity. Using either soma-soma giant synapses (Fig. 1A) or 3-cell networks (Fig. 1B), the chapters that follow attempt to address how electrical synapses regulate the formation of connectivity within these neuronal networks. In particular they address how emerging connections, chemical and electrical, respond to manipulation of extant connectivity of the network within which they are emerging.

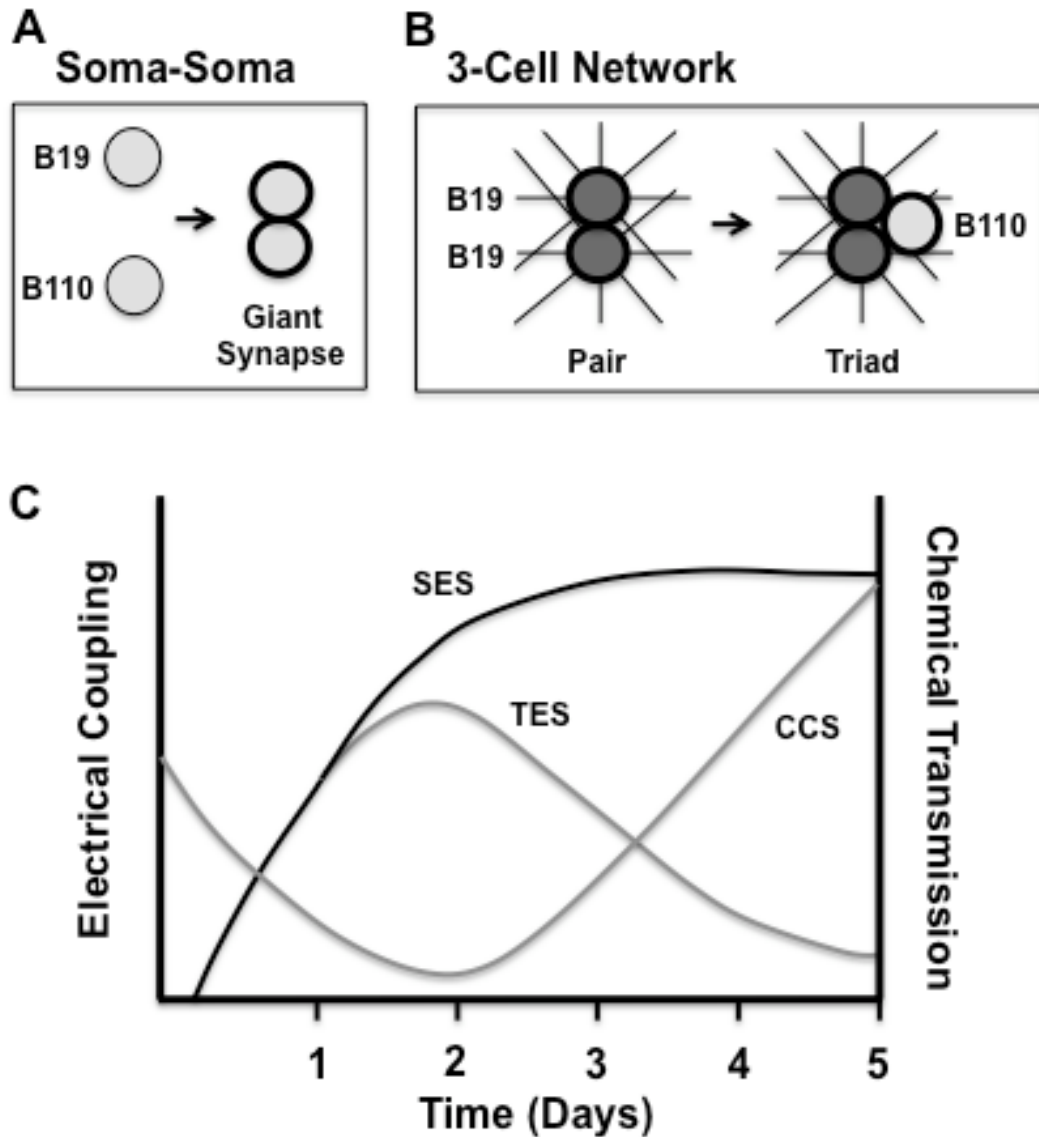


Figure 1. Electrical synapse formation in *Helisoma* pairs and 3-cell networks. (A) Diagram representing two neurons extracted from the buccal ganglia of *Helisoma* and plated into cell culture. After 3 days of culture in isolation, neurons completely reabsorb their axons, becoming spherical, and are stuck together as giant somatic synapses. (B) In 3-cell networks, neurons are plated as shown in A except they are cultured in adhesive conditions allowing neurite extension. At 4 days in culture a third cell is brought into contact with the pair creating the 3-cell network with extant and emerging connectivity. (C) Represents a general timeline for electrical and chemical synapse formation. In the first 24h chemical components are known to exist before the formation of electrical synapses. Shortly after day 1, connections either express transient electrical synapses (TES; gray line) or sustained electrical synapses (SES; black line). The downregulation of TES is known to occur at times when cholinergic chemical synapses (CCS; gray line) strengthen, suggesting a regulatory interaction.

Chapter II addresses how extant electrical synapses can be modulated via dopamine, as well as how modulation of electrical synapse formation effects subsequent synapse formation. In Chapter III, I assess the use of a calcium-imaging protocol to predict network connectivity and neural activity as they relate to TES and SES formation and their modulation. In Chapter IV direct pharmacological manipulation of synapses was used to assess how emergent connectivity in simple three-cell networks affect changes in electrical and chemical connectivity with the goal of determining the mechanism by which these two forms of synaptic transmission interact to impact the others formation.

CHAPTER II

NEURON-SPECIFIC MODULATION OF ELECTRICAL SYNAPSE FORMATION BY DOPAMINE

Introduction

Electrical synapses have historically been shown to couple neurons in many regions of adult mammalian brain (Korn et al., 1973; Connors et al., 1983; Parker et al., 2009), and have recently have been ascribed functions far beyond their classically known role in rapid transmission of electrical signals (for review, see Pereda et al., 2012). For example, they are thought to play a crucial role in mixed electrical-chemical transmission between hippocampal mossy fibers and pyramidal cells (Vivar et al., 2012) and in an extreme case in the inferior olive, they have been shown to constitute the only form of interneuronal communication (de Zeeuw et al., 1998).

During development, electrical synapses are expressed transiently (Peinado et al., 1993; Kandler and Katz 1995, 1998; Dupont et al., 2006); for example in the hypothalamus (Arumugam et al., 2005), spinal cord (Mentis et al., 2002) and neuromuscular junction (Personius et al., 2001). In this capacity, they are thought to be involved in local circuit formation (Szabo et al., 2007) and affect the subsequent formation of chemical synapses (Mentis et al., 2002; Szabo et al., 2004). Thus, the role of electrical synapses in neuronal function and their impact on neural processing is only beginning to be understood (Bennett and Pereda, 2006).

Along with an emerging understanding of the role and prevalence of electrical synapses in neural processing, it is becoming clear that electrical synapses are modifiable and can be important sites of neural plasticity (Pereda et al., 2012; Cachope and Pereda, 2012). In particular, DA has long been known to impact electrical connectivity via its modulation of cAMP-dependent pathways (Piccolino et al., 1982; Lasater and Dowling, 1985; Pereda et al., 1992; Rorig et al., 1995; Rorig and Sutor, 1996).

DA's role as a modulator of neuronal function is central to numerous neurologic deficits, such as Parkinson's disease, schizophrenia, attention deficit and hyperactivity disorders (Fasano et al., 2010). Activation of D2 autoreceptors has been shown to inhibit synaptogenesis by mesencephalic dopamine neurons through translational mechanisms (Fasano et al., 2008). DA receptor activation has also been shown to decrease GABAergic synaptogenesis with medium spiny neurons (Goffin et al., 2010). Thus, while the impact of dopamine signaling on synapse formation and neural network development is far-reaching, the underlying cellular mechanisms are not well understood. We have previously demonstrated that the differential presence of transient electrical synapse (TES) or sustained electrical synapses (SES) alters the outcome of simple neural network formation in identified cell cultures (Szabo et al., 2007). In this study, we have examined the mechanisms governing the modulatory effects of DA at existing electrical synapses and during synaptogenesis using identified TES and SES neural networks. These studies demonstrate that DA induced modulation of existing connections differentially affect TES versus SES coupling and that these effects may

have different mechanisms. In addition, modulation of electrical synapse formation alters new emerging synapses at a simple 3-cell network.

Materials and Methods

Animals

Experiments were conducted on laboratory stocks of albino (red) pond snails, *Helisoma trivolvis*, which were maintained in 20 gallon aquaria at 26°C. Aquaria were kept on a controlled photoperiod of 12 hour light/12 hour dark and snails were fed lettuce and trout chow daily.

Reduced ganglia preparations

Snails were deshelled and pinned to a Sylgard-coated dissecting dish. For studies of semi-intact ganglia preparations, a midline incision was made in the dorsal body wall. Removal of the buccal ganglia consisted of severing the cerebrobuccal connectives, as well as the heterobuccal, ventrobuccal, and posterior buccal nerves. In addition, the esophagus was cut from its site of connection to the buccal musculature. Electrical connections between paired *Helisoma* buccal motoneurons B19 and B19-B110 were studied. These neurons innervate radular tensor muscle groups (Kater 1974; Zoran et al. 1989). When ganglia were pinned in appropriate configurations, neuronal cell bodies of B19 and B110 were readily identified. The right buccal neuron B110 might have a symmetrical partner in the left buccal ganglia but it has been difficult to consistently

identify, possibly due to the slightly asymmetric arrangement of the posterior nerves at this site; therefore all experiments have utilized neuron B110 from the right ganglia (when the rostral side is up and neuron B110 is visible). Prior to recording, dissected buccal ganglia were stored briefly in defined medium (DM). DM consisted of Leibowitz-15 (L-15, Formula No. 82-5154EC Gibco Laboratories) containing *Helisoma* salts (40.0 mM NaCl, 1.7 mM KCl, 4.1 mM CaCl₂, 1.5 mM MgCl₂, and 10.0 mM HEPES) at pH 7.5.

Neuronal cultures

For studies of neurons isolated into cell culture, excised buccal ganglia were placed into 0.2% trypsin (Sigma) in DM for 20 minutes to partially digest the neural sheath. Ganglia were pinned to a Sylgard dish containing 3 ml of high osmolarity DM (56.0 mM NaCl, 2.4 mM KCl, 5.7 mM CaCl₂, 2.1 mM MgCl₂, and 14.0 mM HEPES). The buccal commissure and the relevant nerve trunks, containing the axons of 19 and 110 neurons, were crushed with fine forceps. The sheath of each ganglion was cut along the dorsal surface, next to a neuronal soma, using an electrolytically sharpened microknife. Pressure applied to the ganglion forced the neuronal cell body through the incision and the neuron was collected into a fire-polished, non-adhesive (hemolymph-coated) micropipette using negative pressure produced by a microsyringe (Gilmont). Neurons were then transferred into specific culture conditions, as described below for each experiment.

For studies of neurons exhibiting neuritic growth, cells were transferred directly into adhesive 35 mm culture dishes (No. 3001 Falcon) containing 2 ml of conditioned medium (CM). The dishes were made adhesive by pretreatment with 0.1% poly-L-lysine (PLL) in a 0.15M Tris buffer. CM was generated by incubating 2 central ring ganglia per 1 ml of DM in these PLL-coated culture dishes for 3 days. Brain-derived factors in CM are required for neurite outgrowth in these cultures (Wong et al. 1981). Neurons were maintained in these culture conditions while neurites extended and established contacts.

For studies of neurons lacking neurite outgrowth, cells were transferred into non-adhesive, 35 mm culture dishes (No. 1008 Falcon) containing 2 ml of CM. CM was bulk-cultured in silicone-treated (Sigmacote) glass petri dishes before being transferred to culture dishes that had been made non-adhesive by pretreatment with a 0.5% solution of bovine serum albumin (BSA). Neurons were maintained in these culture conditions as single, spherical cells for 24 h before being transferred into fresh CM dishes and paired into contact. Cell pairs were cultured for an additional 24 h and then transferred to recording chambers (PLL-treated culture dishes containing 2 ml DM) for electrophysiological study.

General electrophysiology

Electrophysiological properties of neurons were examined using intracellular recording techniques. Glass microelectrodes (borosilicate; FHC), possessing tip resistances ranging from 10-20 M Ω , were filled with 1.5 M KCl. Current-clamp

recordings of neuronal membrane potentials were amplified using a bridge-balanced electrometer (Getting Instrumental Inc.) and records were viewed on a storage oscilloscope (Tektronix). In most experiments unless otherwise stated, neuronal membrane potential was maintained with base current injection (BCI) at approximately -70 mV. Electrical coupling was measured by injecting constant amplitude, hyperpolarizing current pulses (3 s in duration) into one neuron (0.2 - 3nA) while simultaneously recording membrane voltage changes in the presynaptic (injected) neuron (approximately 30-50 mV) and its synaptic partner. Coupling coefficients were determined as the ratio of postsynaptic to presynaptic voltage changes (Bennett 1977). Data analyses for coupling ratios and input resistance measurements were taken at the peak of the membrane hyperpolarization. Electrophysiological recordings were digitized by a MacLab A/D data acquisition system linked to a Macintosh Quadra 950 computer using Chart software. Records were archived onto a magneto-optical disk for later analysis and printing.

Analysis of reduced ganglia preparations

Dual recordings were made from electrically coupled cell pairs. Preparations were pinned onto a Sylgard-coated glass petri dish containing 2 ml of 10x calcium saline (40.0 mM NaCl, 1.7 mM KCl, 41.0 mM CaCl₂, 1.5 mM MgCl₂, 10.0 mM HEPES). Increased levels of Ca²⁺ were used to reduce general motor activity of the preparation. Neuronal pairs were then penetrated with glass microelectrodes and hyperpolarizing current injections were applied sequentially to each cell (0.3-1 nA for 3 s). Constant

perfusion of the recording chamber (3 ml/min) was maintained throughout the experiment using a peristaltic pump (Pharmacia). Solutions of 0.5, 5.0 or 50 μ M serotonin creatine sulfate (5HT; Sigma) in 10x calcium saline were perfused through the recording chamber for 2-3 minutes. Treatment was followed by a 20-30 min wash with 10x calcium saline. The modulatory effects of DA on electrical coupling were determined by injecting multiple hyperpolarizing current pulses into each neuron of the pair before, during, and after perfusion. Data measurements were taken at peak membrane potential changes associated with hyperpolarizing current injections unless otherwise indicated. Analysis of the data determined that these electrical connections were non-rectifying. Thus, bi-directional analyses were averaged and a single mean coupling coefficient was calculated for cell pairs at each phase of the experiment.

Analysis of neuronal cell cultures

The effects of exogenous DA on electrical coupling between neurons in cell culture were examined as described above. Cells were penetrated with microelectrodes, hyperpolarizing current pulses were injected, and coupling coefficients were calculated. 50 μ M DA in DM was perfused over each cell pair for 30 seconds, followed by 5-10 min DM wash.

Triplet synapse formation

To achieve triplet synaptic networks two neurons were placed into contact for 4 days, and receive either the treatment (DA) or the vehicle. After 4 days of contact the

central pair was washed to remove treatment and the third cell was brought into contact with one of the original two, as an emerging or new contact.

Pressure injection

Sharp glass pipettes, with a tip diameter ranging from 0.5–1.0 μm , were created from capillary tubes (Borosilicate, 1.5 mm; FHC) and filled with internal solutions containing the neural tracer, Neurobiotin (NB; Vector Laboratories, Burlingame, CA). Solutions were pressure-injected with a Picospritzer II (General Valve, Marietta, GA). The duration of the injection pulses was 5–10 msec at 20–30 psi. Solutions were injected until the diameter of the cell detectably increased by 5–10% and then allowed 1hr to diffuse between coupled partners before being fixed for 5 min in 4% paraformaldehyde in PBS.

Cellular imaging

Soma-soma pairs were transferred to DM-containing adhesive (PLL-coated) glass-bottom dishes and visualized using phase-contrast and epifluorescence optics. Images were captured and analyzed using a Olympus inverted microscope, Hamamatsu (C5810) CCD camera, SimplePCI image capture and Adobe Photoshop software (Adobe Systems, San Jose, CA). A portion of the image devoid of cells was sampled for dark background (DB) subtraction.

Mean fluorescence intensity values were obtained from images, and dye coupling coefficients (DCCs) were determined as the ratio fluorescence intensity of the non-injected (postsynaptic) neuron to that of the injected (presynaptic) neuron.

Data analysis

Two-tailed Student's t tests, ANOVA (post-hoc LSD), or Chi-square were used for statistical analysis. Statistical significance was determined at $P < 0.05$. Data are presented as mean plus or minus SEM, unless otherwise indicated.

Results

DA modulation of electrical synapses

Identified neurons from the snail, *Helisoma trivolvis*, were examined. Cellular properties, synaptic connectivity, and synaptogenic capabilities of these neurons have been extensively described (for review, see Murphy 2001). The neuromodulator dopamine (DA) inhibited electrical communication between neuron B19 and its bilateral counterpart in isolated buccal ganglia (Fig. 2A and B). Bath perfusion with DA (50 μM) induced a significant reduction in electrical coupling coefficient (ECC; $n=5$; $p < 0.001$), an effect that was completely reversed upon saline washout (Fig. 2C). To determine the site of DA action on electrical coupling, identified neurons were isolated into cell culture and synaptic connections were allowed to reform over 24 h of soma-to-soma contact (Fig. 3A and B). A giant somatic synapse configuration (Haydon, 1988) was used to

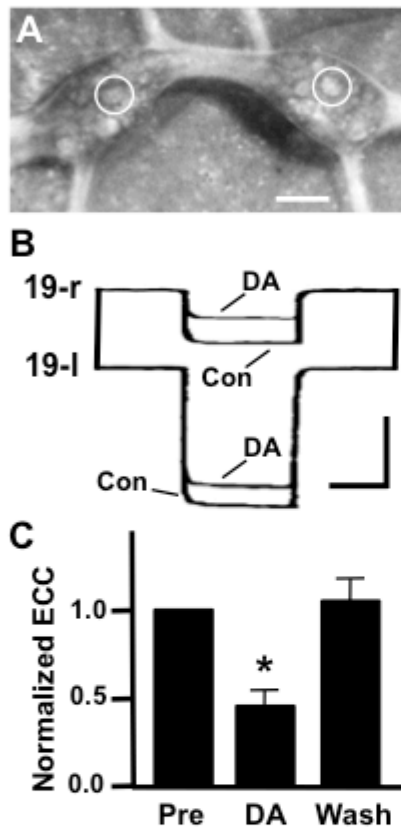


Figure 2. Dopamine decreased electrical coupling between buccal neurons in reduced ganglionic preparations. (A) Micrograph shows a caudal view of the paired *Helisoma* buccal ganglia. Specific neurons were identified by cell size and position, based on pigmentation of the ganglia (dark background) and neuronal cell bodies (light spheres), such as neuron 19 (circles). Scale bar equals 100 μm . (B) Representative traces from electrically coupled neurons 19. Hyperpolarizing current was injected into neuron 19 in the left ganglion (19-l), and membrane potential was monitored in both this neuron (lower traces) and the postsynaptic target neuron in the right ganglion (19-r; upper traces). Superimposed recordings demonstrated initial control responses to current injection in saline alone (Con) and responses obtained during bath perfusion with 50 μM dopamine (DA). Vertical scale bar equals 20 mV; horizontal scale bar equals 0.5 s. (C) Electrical coupling coefficients (EEC) were calculated before DA application (Pre), during exposure (DA), and following saline exchange (Wash). DA treatment caused a 50% reduction in normalized ECC and this relative ECC was significantly different from the recovered ECC values following washout (*, $P < 0.001$; Student's *t*-test; $n = 5$).

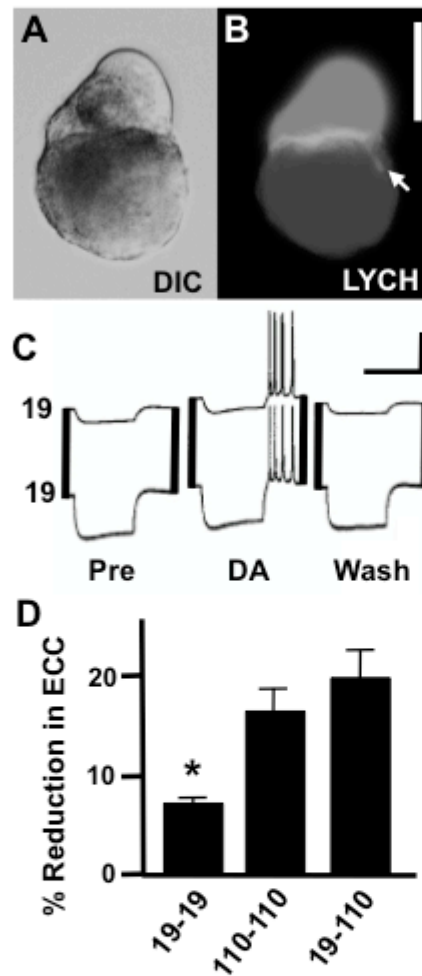


Figure 3. Synapse-specific modulation of electrical coupling by acute dopamine treatment *in vitro*. (A) DIC image of giant somatic synapse formed between a neuron 19 (bottom cell) and neuron 110 (top cell) cultured in outgrowth-restrictive conditions. (B) Lucifer yellow injection into 110 revealed extensive cell-cell contact between somata and minimal neurite outgrowth over the surface of the neuron 19 (arrow). Scale bar equals 20 μm . (C) Membrane potential recordings demonstrate the modulatory effects of 50 μM dopamine (DA) on a 19-19 somatic synapse. Bath perfusion with DA caused a membrane depolarization, an increase in post-inhibitory rebound spiking and a slight reduction in electrical coupling. Vertical scale bar equals 30 mV, horizontal scale bar equals 3 s. (D) DA-induced reductions in ECC is synapse-specific. Dopamine caused significantly greater reduction ($P < 0.04$; ANOVA; post-hoc LSD) at 110-110 ($n=4$) and 19-110 ($n=6$) cell pairs, as compared to 19-19 pairs ($n=25$).

maximize the area of synaptic contact and the strength of electrical coupling. Treatment with DA (50 μ M) again caused a reduction in electrical coupling between two B19 neurons (Fig. 3C and D); however, the extent of the DA-dependent reduction in ECC was synapse-specific, as demonstrated by the fact that suppression of electrical coupling at B19-B19 synapses was markedly less than at B110-B110 and B19-B110 synapses ($P < 0.04$; Fig. 3D). Along with suppression of electrical coupling, DA also caused a membrane depolarization and increased excitability in neurons B19 and B110. Increased neuronal excitability was particularly evident following hyperpolarizing voltage changes, where bursts of action potentials were activated by post-inhibitory rebound (Fig. 3C). Thus, DA's inhibitory effects on electrical synaptic transmission were neuron-specific and not an indirect product of a polysynaptic network modulation.

DA modulation of electrical synapse formation

Having determined that both neurons B19 and B110 respond to acute DA application, I next sought to determine the effects of chronic DA treatment on electrical synapse formation. To achieve this chronic treatment, homotypic B19-B19 pairs or heterotypic B19-B110 pairs were cultured in the giant somatic synapse configuration. Synaptogenesis between these neurons has been characterized previously (Szabo et al., 2004). Within 24 hours, sustained electrical synapses (SES) were formed between B19-B19 neuron pairs (Fig. 4A). SES are long-lasting connections maintained beyond initial synapse formation. In contrast, transient electrical synapses (TES), short-lived

connections that exist during the initial stages of synapse formation and are then lost, formed between B19-B110 neuron pairs.

Somatic synapses were treated chronically with 50 μ M DA throughout the first 24 h of synapse formation and, following their transfer into DA-free medium, electrical coupling was assessed. Chronic DA treatment caused a significant reduction in TES formation at B19-B110 contacts ($n=10$; $p<0.007$), where coupling was less than 25% of untreated control levels ($n=10$, Fig. 4B). In contrast, the establishment of SES at B19-B19 contacts was not reduced by DA treatment. Rather, chronic DA exposure during initial stages of synaptogenesis caused a small but significant increase in ECC at homotypic B19-B19 synapses ($n=12$; $p<0.0005$), as compared to untreated controls ($n=12$; Fig. 4C). Thus, TES formation was markedly diminished by DA treatment, whereas SES formation was strengthened.

Cellular mechanisms of DA-induced modulation of electrical synapses

To determine whether DA was acting directly on gap junction channels or extrajunctional sites, we used the tracer molecule neurobiotin (NB), which has been shown to diffuse through gap junction channels formed between these neurons (Szabo et al., 2004). A reduction in the number of gap junction channels present should result in a reduction in the passage of dye between cells. To determine if DCC values were representative of electrical coupling, prior to dye injection, ECCs were determined for each cell pair, and these values ranged from 0.0 to 0.8 (Fig. 5A). Dye coupling coefficients (DCC) were then determined, and at B19-B19 somatic synapses these DCC

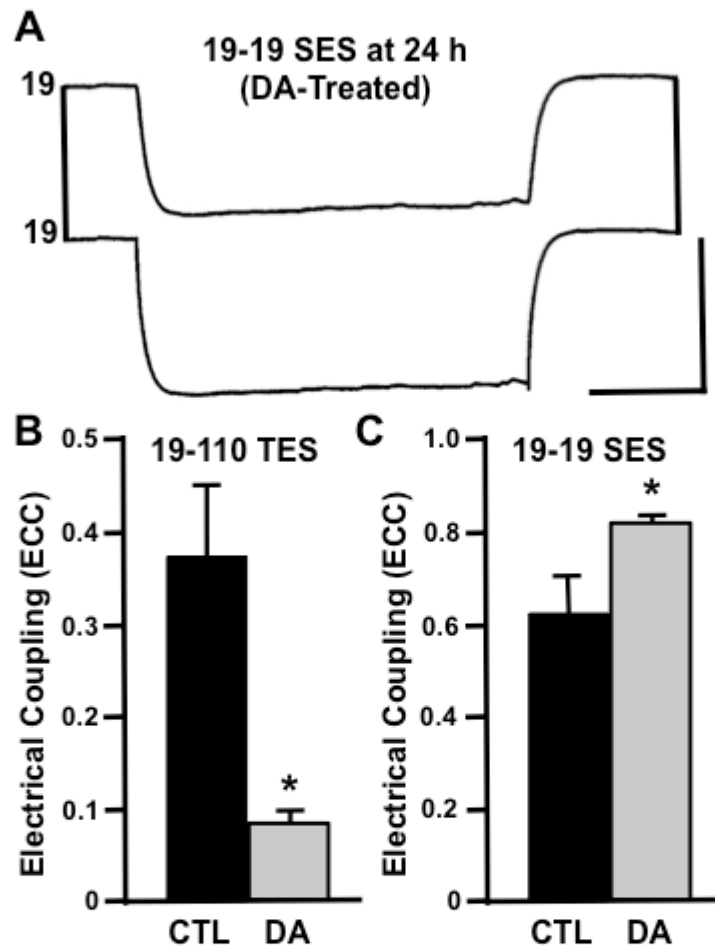


Figure 4. Formation of electrical synapses is modulated by dopamine. (A) Representative traces of from a 19-19 somatic pair exposed to 50 μ M DA during 24 h of initial contact. Hyperpolarizing current was injected into one 19 (bottom trace) and membrane voltage was recorded in both this cell and its partner 19 (top trace). Vertical scale bar equals 40 mV, horizontal scale bar equals 1 s. (B) Synapse formation at 19-110 transient electrical synapses (TES) was significantly suppressed by DA (n=10) compared to untreated controls (CTL, n=12; *, p<0.007, Student's *t*-test). (C) Synapse formation at 19-19 sustained electrical synapses (SES) was significantly enhanced by DA (n=12) compared to untreated controls (CTL, n=12; *, p<0.001, Student's T-test).

values strongly correlated with the EEC values determined for the same somatic synapses ($R^2=0.57$; $n=15$).

Since we determined that the NB dye-coupling procedure was a reliable indicator of electrical coupling at these somatic synapses, NB injection and fluorescence imaging were used to assess dye coupling in both untreated control (CTL; Fig. 5B) and DA-treated somatic synapses (DA; Fig. 5C). No effect of DA treatment on dye coupling was detected at B19-B19 electrical synapses ($p=0.72$; Fig. 5D). However, similar to the pronounced DA-induced reduction in electrical coupling previously seen at B19-B110 synapses, DCC was significantly suppressed at these connections when treated with DA ($p<0.0002$; Fig. 5E). Thus, the reduction in electrical coupling caused by DA was at least partially due to a decrease in gap-junctional conductance at B19-B110 transient electrical synapses. In contrast, the absence of DA-induced changes in dye coupling at B19-B19 sustained electrical synapses suggested that modulation of non-junctional channels indirectly mediated the reduction in electrical coupling at these contacts.

DA modulation of chemical synapse formation

The differential presence of TES or SES alters the outcome of simple neural network formation, both in terms of electrical and chemical synaptogenesis (Szabo et al., 2007). Therefore, we tested whether DA-induced diminution of TES formation altered subsequent synapse formation in defined three-neuron networks (Fig. 6A). In three-cell (B110-B19-B110) TES networks, an initial heterotypic pair (B110-B19; TES expressing) was treated with 50 μ M DA for 4 days (Fig. 6B, gray cells) prior to

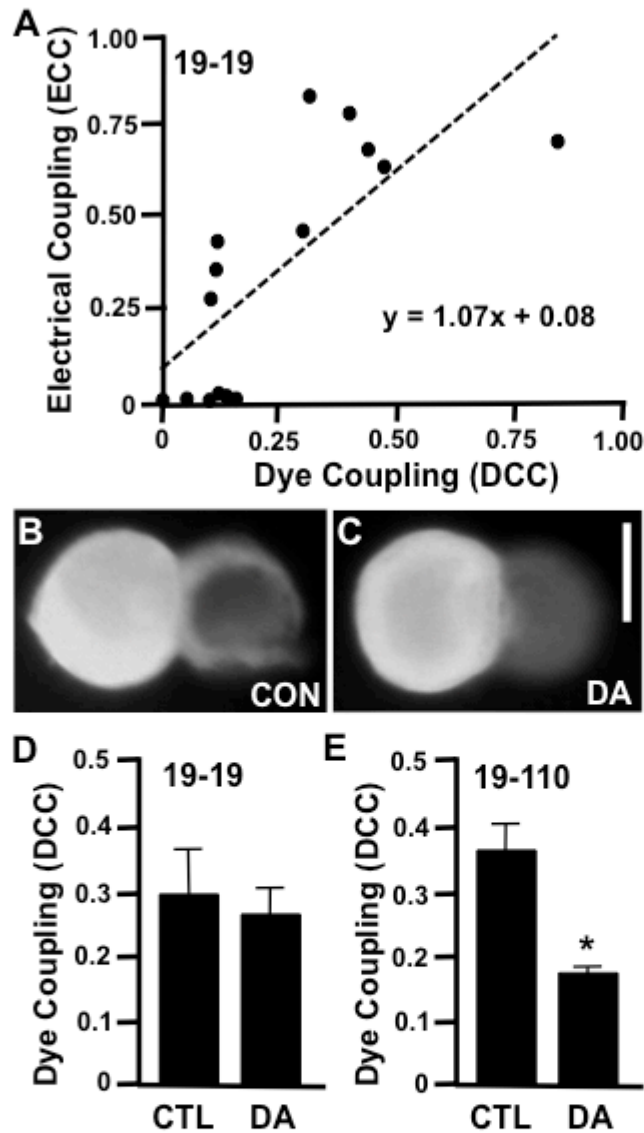


Figure 5. Mechanism of DA modulation of electrical synapse formation. (A) Electrical coupling coefficients (ECC) and dye coupling coefficients (DCC) were obtained for 19-19 somatic synapses between 12 and 24 h of contact and a linear relationship between the two was demonstrated ($n=15$; $R^2 = 0.57$). (B and C) 19-110 somatic synapses were treated with conditioned medium alone (B; CTL) or conditioned medium containing 50 μM DA (C; DA) and the passage of neurobiotin from the injected cell (larger left cell) to its uninjected partner (right cell) was examined. Higher levels of fluorescence were detected in the cytoplasm of neuron 110 in control somatic synapses. Note the resolution of the nucleus in B due to the cytosolic accumulation of neurobiotin fluorescence. (B,C) Scale bar shown in C equals 20 μm . (D) DCC values were used to quantify dye coupling between 19-19 somatic synapses in the presence (DA) and absence (CTL) of dopamine. No significant differences in dye coupling were detected between treated and untreated cell pairs. (E) DCC values were significantly lower in DA-treated 19-110 somatic synapses, as compared to untreated controls (*, $P < 0.05$, Student's *t*-test).

establishment of contact with a third untreated neuron (B110; Fig. 6B, white cell), thus generating a network containing synapses of varying age and DA-treatment status. Since older neurons B19 do not readily form strong electrical synapses, electrical coupling was weak (mean ECC <0.15) at the newly formed B110-B19 contacts in untreated control (CTL; n=7; Fig. 6C and 7A). Also, as predicted, DA-treated networks (DA; n=6; Fig. 6D and 7A) possessed weak coupling that was not significantly different than control levels. Thus, a temporal limit or permissive window exists following initial synaptic contact for DA-induced reduction in electrical coupling.

Since an inverse relationship exists between electrical and chemical synaptic transmission at transient electrical connections (Szabo et al., 2004), we also examined chemical neurotransmission in these three-neuron networks. Approximately 30% of both CTL and DA TES networks possessed chemical coupling at these newly formed synapses (Fig. 7B), and the percentage of preparations with chemical coupling, the waveforms of the evoked postsynaptic potential (PSP) and PSP amplitudes were not different between treated (DA; Fig. 6F) and control groups (CTL; Fig. 6E).

As shown above, in contrast to DA-induced TES diminution, DA enhances SES formation (Fig. 4B). We therefore examined the effect of DA on synapse formation in three-cell SES networks, with two older neurons B19 and one younger neuron B110. In these three-cell (B19-B19-B110) networks, an initial homotypic pair (B19-B19; SES expressing) was treated with 50 μ M DA for 4 days prior to establishment of contact with a third untreated neuron B110. Electrical coupling was weak (mean ECC < 0.15) at the

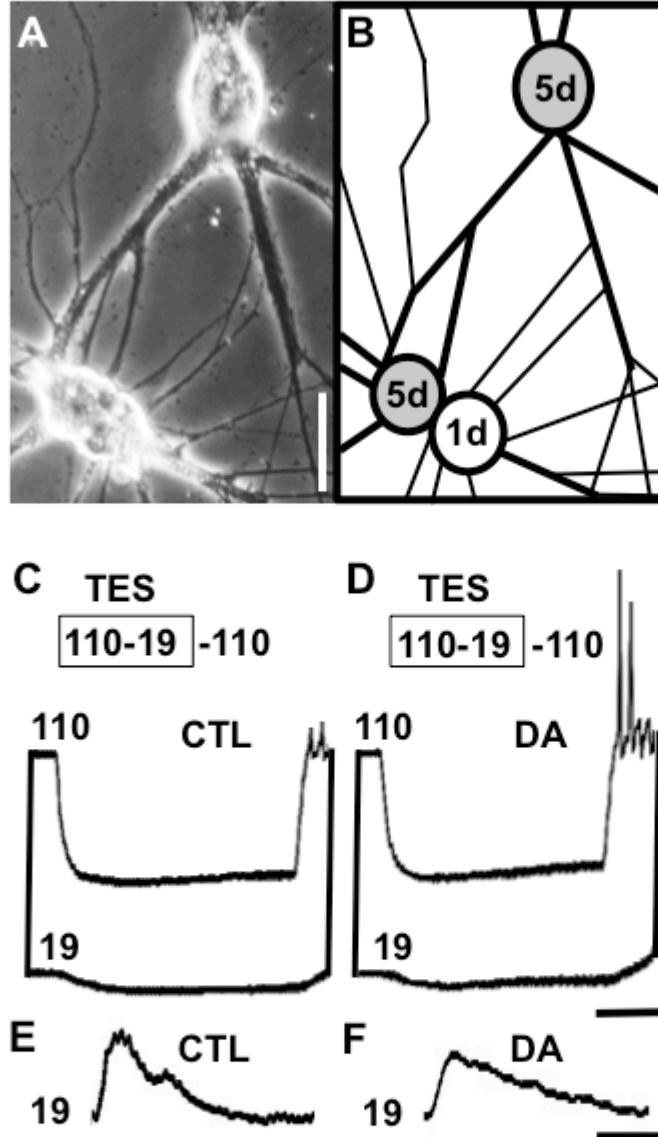


Figure 6. Electrical and chemical synapses in three-cell networks. (A) Phase contrast micrograph showing a representative 3-neuron network. Neurons (light iridescent spheres) were plated into adhesive cell culture conditions for 4 days of neurite outgrowth. A third cell was plated into culture on day 4 in close apposition to one of the older neurons, thereby forming a three-cell network. Scale bar in equals 30 μm . (B) Schematic of the three-cell network in (A). The two 5d neurons (shaded) were connected by large neurites, while finer processes extended from the third, younger (white) neuron. 5d cells were exposed to either untreated medium (CTL) or 50 μM DA for 4 days, prior to plating the third 1d cell. Cells were then cultured in medium only (no DA) for an additional day. (C and D) Representative traces from two cells in a 3-cell 110-19-110 network showing electrical coupling between the 5 day old neuron 19 (top, enclosed in the light black box) and the 1 day old neuron 110 (top, not enclosed). Simultaneous recordings of membrane potential were made from the injected (presynaptic) neuron 110 (top trace) and the 5d (postsynaptic) neuron 19 (bottom trace). Weak electrical coupling was detected at this 1d synapse, in both control (CTL, panel C) and dopamine-treated networks (DA, panel D). Vertical scale bar in C and D equals 20 mV, horizontal scale bar equals 1 s. (E and F) Depolarizing current injection into the 1d (presynaptic) neuron 110 evoked postsynaptic potentials (PSPs) in the 5d (postsynaptic) neuron 19. The amplitude of PSPs in control (E, CTL) or dopamine-treated networks (F, DA) was similar. Vertical scale bar in E and F equals 5 mV, horizontal scale bar equals 0.4 s.

newly formed B110-B19 synapses in both DA-treated (n=7) and untreated connections (n=6; Fig. 7C). Newly formed (1d) connections in untreated control networks did not possess chemical coupling (CTL; Fig. 7D). In contrast, DA-treated B19-B19-B110 SES networks were significantly more likely to possess chemical coupling at their 1d connections than untreated control networks ($P < 0.05$; Chi squared test; Fig. 7D), even though electrical coupling was not different between treated and untreated groups (Fig. 7C). Thus, treatment of neural networks with DA, although enhancing SES and diminishing TES, had no impact on electrical synapses within those networks that emerged following DA. However, newly forming chemical synapses were more prevalent at DA-treated networks with sustained electrical synapses (SES), rather than those undergoing transient electrical coupling.

Cellular mechanisms of DA-induced modulation of chemical synapse formation

While the enhancement in chemical synapse formation observed in DA-treated neural networks might have been the result of altered electrical coupling in these networks (Szabo et al., 2007), alternatively, chronic DA treatment might also have caused a long-term alteration in acetylcholine receptor (AChR) expression and acetylcholine (ACh) sensitivity. To test this possibility, neuron B19s were cultured for 4 days in 50 μ M DA, in a protocol similar to that used for network modulation studies. Following transfer into DA-free medium, neurons were assessed electrophysiologically for ACh-induced membrane depolarization following pulsed application of 10 μ M ACh.

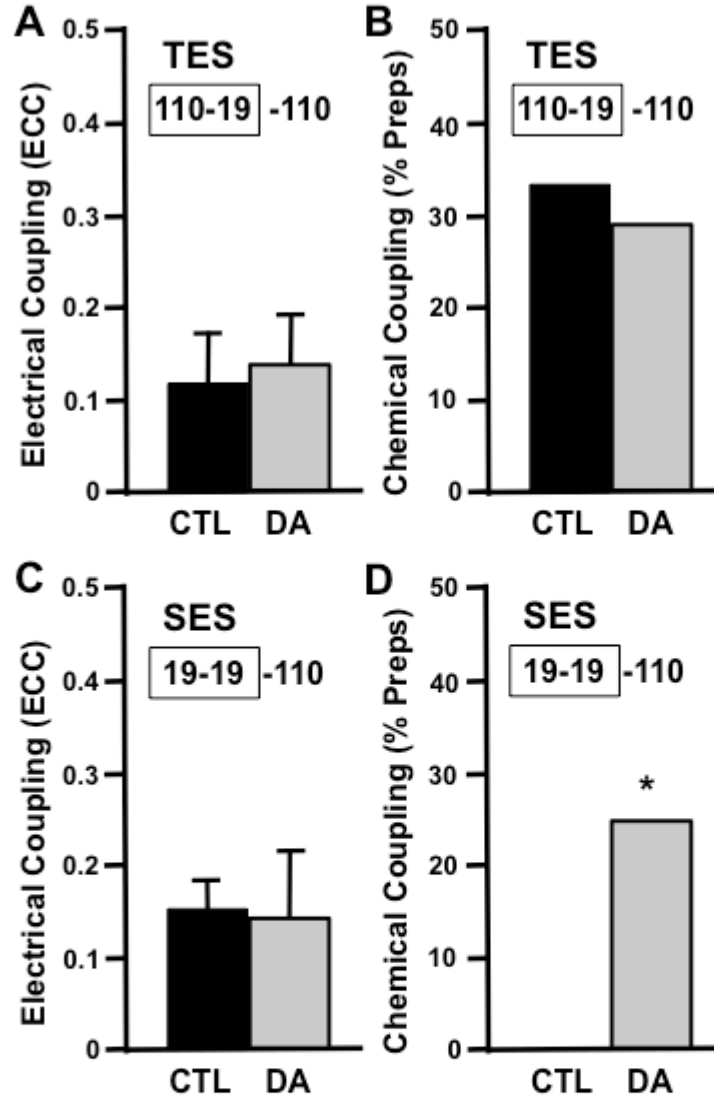


Figure 7. Synapse-specific alterations in chemical synapse formation following exposure to dopamine . (A) ECC values for 5d-1d 19-110 connections in TES (110-19-110) networks cultured in conditioned medium (CTL, solid bars; n=6) were not different from those determined for identical connections in dopamine-treated networks (DA, open bars; n=7). (B) The percentage of preparations with chemical coupling at the same 19-110 connections (in A) was not significantly different from identical connections in DA-treated networks. (C) ECC values for 19-110 connections in SES (19-19-110) networks cultured in condition medium (CTL, solid bars; n=4) were not different from identical connections in dopamine-treated networks (DA, open bars; n=8). (D) Chemical removal. However, coupling was not present in control B19-B19-B110 networks, but was detected in 25% of DA-treated 1d B19-B110 connections (*, $P < 0.05$; Chi-square test; n=8).

pretreatment with DA did not change the amplitude of ACh-evoked depolarization (DA; $n=6$; $p=0.75$), as compared to untreated control neurons (CTL; $n=6$; Fig. 8A and B). In support of this finding, the amplitude of PSPs in DA-treated TES networks (3.2 ± 0.8 mV; $n=6$) and DA-treated SES networks (2.8 ± 1.1 mV; $n=7$) were not different ($p=0.66$). Therefore, enhancement of chemical synaptogenesis, in networks possessing DA-enhanced SES formation, was not a byproduct of long-term changes in the sensitivity of the postsynaptic neurons to ACh.

Discussion

It is becoming increasingly clear that electrical synapses are important regulatory sites of neural communication, and that they are dynamic and modifiable (Bennett, 1997; Pereda et al., 2012). Neuromodulation of electrical coupling has been shown in mollusks (Carrow and Levitan 1989), annelids (Colombaioni and Brunelli 1988), arthropods (Kepler et al., 1990), fish (Harsanyi and Mangel 1992; McMahon 1994; Curti and Pereda 2004) and mammals (Maher et al., 2009; Goffin et al., 2010). Potential modulators of electrical coupling include peptides (Wolinsky et al. 1985), lipids (Guan et al. 1997) and neurotransmitters, such as norepinephrine (Blue and Parnavelis, 1982).

Dopamine has long been known as a modulator of electrical coupling (Piccolino et al., 1982; Rorig et al., 1995; Rorig and Sutor, 1996; Fasano et al., 2008). It is also a potent regulator of neuronal activity in the circuit controlling the feeding behavior of

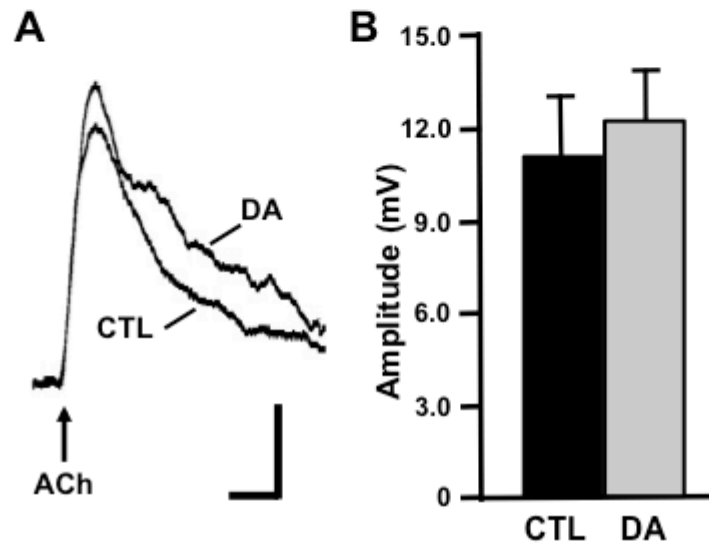


Figure 8. Chronic dopamine treatment did not alter neuronal sensitivity to ACh. (A) Neuron 19 membrane depolarization evoked by pulsatile application of 10 μ M ACh from a micropipette was similar in amplitude, rise time and decay both in neurons treated for 4 days with 50 μ M dopamine (DA) as well as in untreated controls (CTL). (B) Mean amplitude of ACh-evoked membrane depolarization was not different ($P=0.74$) between untreated control (CTL; $n=6$) and dopamine-treated (DA; $n=6$) neurons.

Helisoma (Trimble and Barker 1984; Quinlan et al., 1997; Murphy 2001; Fig. 1). We therefore investigated the role of DA in the formation of identified *Helisoma* neural networks, where electrical coupling formed during synaptogenesis can be either sustained or transient (Szabo et al., 2004). We found that chronic DA treatment reduced a transient form of electrical coupling, but strengthened sustained electrical synapses in a neural network-specific fashion. This result demonstrates that DA can produce opposing effects on electrical synapses that are dependent on the identity of the neurons within a developing network.

Modulation of electrical coupling can be due to direct effects on gap-junctional channels; although additional extrajunctional mechanisms abound (Pereda et al., 2004, 2012). In this study, we demonstrated that the suppression of transient electrical coupling caused by DA was due to a decreased conductance at gap junctions formed between neurons of differing identity. However, modulation of gap junctions was not involved in changes induced by DA at sustained electrical synapses. Since changes in non-junctional conductance can indirectly modulate electrical coupling (Szabo et al., 2010), DA-dependent enhancement in SES is likely due to changes in extrajunctional channels. Although much is known regarding the diversity of dopamine receptors in vertebrates (Callier et al., 2003), less is understood with regard to invertebrates, particularly within molluscan neural networks. Furthermore, much information is emerging regarding the diversity of innexins, the proteins that form the majority of gap junctions in invertebrates (Kandarian et al., 2012). The specific innexins underlying *Helisoma* transient and sustained electrical coupling remain unknown, and further

studies characterizing these proteins should help elucidate the synapse-specific effects of dopamine on synaptogenesis in these simple networks.

The regeneration of *Helisoma* buccal neurons in vivo involves the formation of new TES connections (Bulloch and Kater, 1981; Hadley et al., 1982) and the short-term increase in coupling at sustained electrical synapses (Murphy et al., 1983). Similar transient changes in electrical coupling occur in the mammalian nervous system during development and regeneration (Kandler and Katz, 1998; Chang and Balice-Gordon, 2000b), although the specific functions of TES remain largely unknown. It has been hypothesized that transient electrical coupling in *Helisoma* imposes a synchrony of activity upon large populations of neurons, thereby influencing coordination of process outgrowth, the survival of damaged neurons, and activity-dependent mechanisms of synapse formation (Turner et al., 2011). The idea that TES may aid in the construction or maturation of chemical synapses (Kandler and Thiels, 2005; Marin-Burgin et al., 2006; Montoro and Yuste, 2004; Neunuebel and Zoran, 2005) is supported by a switch from transient electrical to chemical signaling seen in mollusks (Szabo et al., 2004; Turner et al., 2011) and mammals (Arumugam et al., 2005). The chemical connection that emerges between neuron 110 and 19 following TES is cholinergic and inhibitory (Szabo et al., 2004). Furthermore, the loss of electrical coupling, and the formation of this inhibitory connection, occurs at precisely the time when synaptic boutons and evoked synaptic potentials appear in the target musculature of these regenerating motoneurons (Turner et al., 2011), thereby providing a potential mechanism for neuromuscular synaptic competition.

Extensive transient coupling has been shown to exist during discrete periods of neural circuit formation (Peinado et al., 1993; Yuste et al., 1995), suggesting that electrical coupling may be an important regulator of neural network formation. We've previously shown that the differential presence of TES or SES alters not only the outcome of electrical synapse formation but chemical synaptogenesis as well (Szabo et al., 2007). In this study, we have shown that although DA had no effect on cholinergic synapse formation within a TES network, chemical synaptogenesis within SES neural networks were significantly enhanced by DA treatment. This increase in chemical synaptogenesis in networks possessing DA-enhanced SES formation was not due to changes in the sensitivity of the postsynaptic neurons to ACh, but rather was a cell specific effect of DA influenced by the presence of this particular form of electrical coupling.

DA has wide-ranging neuromodulatory effects on neural networks involved in learning and memory (Rossato et al., 2009), behavioral reinforcement (Wise, 2004), social displays (Anstrom et al., 2009; Aragona and Wang 2009), vocal communication (Leblois et al., 2010; Sasaki et al., 2006), and feeding (Leininger et al., 2009). The extent to which DA-dependent modulation sculpts aspects of neural network connectivity during development remains unknown. Nonetheless, the current studies demonstrate that both electrical and chemical synapse formation are influenced by DA in complex fashion, such that the identity of the neurons and the strength of existing synapses within an emerging neural network impact the nature of DA-induced synaptogenic modulation.

CHAPTER III
CALCIUM OSCILLATIONS IN *HELISOMA*
NEURONAL NETWORKS

Introduction

The ability to functionally assess how neural networks are constructed is vital to understanding how those neural circuits mediate the physiology in a behaving animal. Expansion and refinement of electrophysiology techniques drove the early growth of fundamental research on the nervous system and continues to drive various aspects of neuronal studies such as ion channel function and their relationship to pain (Bohlen et al., 2011, Samways et al., 2011). Intracellular electrophysiology in particular allows for direct assessment of electrical properties of neurons when they are penetrated with glass microelectrodes. This technique is limited in that recording from large numbers of cells at the same time becomes technically impractical. Furthermore, intracellular electrophysiology can greatly perturb the physiology of the neurons being monitored. Although, no technique is without its drawbacks other methodologies were explored to complement my electrophysiological approaches, with the intent to reduce or eliminate physical damage of neurons.

Two useful examples of relatively noninvasive neural activity include extracellular electrophysiology and cellular imaging. Extracellular electrophysiology, as its name suggests, measures electrical changes at the surface of, but not inside, the

neuron with respect to an extracellular site distant from the neuron. This technique is frequently used to measure field potentials from populations of neurons and is still used today for clinical studies measuring brain activity during sleep to assess sleeping disorders, to record electroencephalography, and to measure heart physiology by way of electrocardiograms. Many studies attempting to address neuronal networking questions have developed extracellular recording techniques in rat hippocampal neurons, including ways to use extracellular recordings as predictors of intracellular physiology (Henze et al., 2000), as well as complex microelectrode arrays that could detect coincident activity among a population of neurons (James et al., 2004). Fluorescent cell imaging techniques likewise have a long history of usefulness in monitoring intracellular physiology in a population of cells, which can include both calcium-sensitive probes (Tsien, 1981; Yuste and Denk; Ikegaya et al., 2005) and voltage-sensitive probes (Yuste et al., 1997; Bradley et al., 2009).

Voltage-sensing probes have frequently used chromatophores bound to the cell membrane whose distribution or orientation changes based on changes in membrane voltage that moves the charged chromatophore and alters fluorescence (Ehrenbert et al., 1988). Other voltage-sensing indicators have used genetically targeted green fluorescent protein to sense domains of ion channels that alter its fluorescence state (Ataka and Pieribone, 2002). Calcium-sensitive probes based on the chelator ethylene glycol tetraacetic acid (EGTA) like Fura-2, were developed by Roger Tsien and colleagues and have found wide use as indicators for cellular physiology (Tsien, 1980; Grynkiewicz et al., 1985). They became useful tools because they had a high sensitivity to calcium and

could be loaded non-invasively into large groups of cell, while maintaining the ability to monitor single cells amongst the larger population (Tsien, 1981; Cosart et al., 2003; Yuste and Katz, 1991). Many of these probes were ratiometric and therefore fluoresced at two emission wavelengths allowing for estimations of calcium concentrations regardless of the strength of loading due to the ratioed output.

Not long after its development, Fura-2 became a molecule frequently used in growth cone physiology studies using *Helisoma* neuronal cultures. These included demonstrations that neuronal depolarization caused local increases in calcium and that these calcium changes were responsible for alteration in growth cone dynamics (Cohan, 1992; Davenport and Kater, 1992, Welnhofner et al., 1999). In addition, neuromodulators that affect neurite outgrowth, like serotonin, were shown to work in part by mediating changes in intracellular calcium (Cohan et al., 1987; Goldberg et al., 1992). Growth cones were also demonstrated to be capable of homeostatically regulating intracellular calcium concentrations (Rehder et al., 1991). Previous work with *Helisoma* giant somatic synapses, using Fura-2 imaging, demonstrated changes in intracellular calcium in response to trophic factor-induced upregulation of electrical coupling (Nueneubel and Zoran, 2005). In addition to being a proxy for voltage-dependent changes in neurons, calcium probes also act as readouts for intercellular coupling of neuronal activity. Monitoring of calcium transients in large neuronal populations have been used previously, such as in Smetters et al. (1999), where single action potentials could be reliably detected using this optical approach and were indicative of the spiking patterns in the population. Stosiek et al. (2003) also demonstrated that ratiometric calcium

imaging could be efficiently used to monitor neuronal populations while still achieving high resolution monitoring of activity in individual cells.

Connectivity of simple 3-cell neuronal networks has demonstrated that ongoing synaptic connectivity can alter emerging electrical and chemical connectivity at these networks (Szabo and Zoran, 2007). This simple neuronal culture system uses identified neurons with known synaptic phenotypes, also addressed previously in Chapter II, and provides a useful tool to look at coincidence changes in calcium during network interactions. As such, I attempted to develop a system whereby I could monitor changes in intracellular signaling amongst cells that would not have the drawback of inducing physical damage, but would complement previous electrophysiological studies and provide pairwise analysis of synaptic interactions a defined neural network.

An important aspect in the formation and maturation of neural networks is neuronal activity, which is typified by large, activity-dependent increases in cytosolic calcium. Cytosolic calcium changes between neurons comprising a network is, therefore, not only indicative of neuronal excitation, but also ongoing synaptogenesis. Fura-2 AM is a cell permeable form of the calcium indicator dye, whose multiple excitation/emission wavelengths allow for determination of cytosolic calcium levels through ratiometric fluorescent imaging. Using coincidence detection on data sets of calcium-dependent fluorescence between neurons, I monitored neural network function with minimal mechanical disturbance of the cell membrane and electrophysiological properties. Given that large gap-junctional pores are capable of rapidly conducting electrical and biochemical signals (including in some cases calcium ions themselves),

stronger electrical coupling between neurons was hypothesized to lead to higher degrees of coincidence in calcium changes. Therefore, coincidence detection of calcium was used as an indicator of electrical coupling. In the following sections, I describe an approach modeling a computational means of assessing network coupling and this approach was assessed using known approaches to modulate coupling and to construct networks with predicted electrical communication. In the following sections, I address the question, can coincidence detection of calcium oscillations between neurons report synaptic interactions with reasonable fidelity?

Materials and Methods

Animals

All experiments performed were conducted on neurons extracted from *Helisoma trivolvis*, an American albino pond snail, as previously described in Chapter II were maintained in laboratory aquaria. Snail culture conditions were maintained at 26°C with a 12-hour light/dark cycle. Snails were fed either lettuce or trout chow each day.

Cell culture

Isolation of neurons from the *Helisoma* buccal ganglia were performed as previously described in Chapter II. Buccal ganglia were treated with trypsin (Sigma) in a 1 to 2 ratio of mass of trypsin per volume of solution. Trypsin-treatment was conducted for 15 minutes and allowed for the softening of the ganglionic sheath before neuron

extraction. Ganglia were first washed in media before being pinned onto Sylgard (Dow Corning) plate in high osmolarity media (in mM: 56 NaCl, 2.4 KCl, 5.7 CaCl₂, 2.1 MgCl₂, and 14 HEPES). Pinned ganglion underwent nerve crush and, using a tungsten microknife; an incision was made near the neuronal cell body desired for extraction. Isolation of the desired neurons was performed using a glass micropipette created from micro-hematocrit capillary tubing (VWR) with an inner diameter of 1.2mm. Capillary tubing was pulled using a vertical pipette puller (Kopf) that was cut so that the tip was slightly larger than the neuronal diameter (approximately 50µm) and was subsequently fire-polished. Pipettes were placed into a micromanipulator with a micrometer syringe attached to plastic tubing, which was filled with media, allowing for negative and positive pressure to be applied to the ganglia. Desired neurons were isolated by visually locating the neuron cell body using a stereoscopic microscope and applying negative pressure to suck the neuron into the pipette before transferring the cell into 35mm culture dishes (Falcon 1008) coated with non-adhesive 0.5% bovine-serum albumin (BSA) obtained from Sigma.

Transferred neurons were cultured in BSA coated dishes for 3 days in 2mLs cultured media (CM) before experimental protocols are started. CM was created by culturing 2 central ring ganglia per mL of defined media (DM) as previously described in Wong et al., 1981. DM consisted of Leibowitz-15 media (L-15 Gibco Formula No. 82-5154) with added glutamine (30mg per 100mL of L-15) and physiological levels of salts to *Helisoma* (40mM NaCl, 1.7mM KCl, 4.1mM CaCl₂, 1.5mM MgCl₂, and 10mM HEPES) adjusted to a pH of 7.5.

General electrophysiology

All electrophysiology performed was done using intracellular recording methods. Glass microelectrodes (FHC, borosilicate 1.5mm OD x 1.12mm ID with omega dot fiber) were pulled to tip resistances of approximately 20M Ω were filled with 1.5M KCl prior to penetration of cells. Synaptic connectivity was assessed using dual current-clamp recordings of identified neurons with Duo-773 electrometers (World Precision Instruments). Recordings were analyzed by digitization of signals using ADInstruments Powerlab data acquisition system in conjunction with Chart software. Electrical coupling was assessed by injection of a hyperpolarizing current of constant amplitude, approximately 1nA, into one cell (presynaptic) while simultaneously recording membrane voltage changes in both the presynaptic cell, usually around 30mV, and the non-injected synaptic partner (postsynaptic) neuron. Analysis of the coupling is assessed by determining the ratio of postsynaptic to presynaptic voltage change at the peak of each change. Analysis of chemical connections between cells was performed by stimulating 10 action potentials in the presynaptic neuron at a rate of 1 action potential (AP) every 2 seconds and observing any resulting postsynaptic potentials (PSP). Since electrical coupling would also result in a depolarization of the postsynaptic cell and many *Helisoma* synapses in culture were mixed in nature, a protocol was developed for assessing when a postsynaptic depolarization was due to chemical connectivity. Since electrical coupling occurs near instantaneously it was determined that any PSP occurring

after 95msec of the peak of a presynaptic AP was due chemical and not direct electrical transmission.

Imaging

Unless otherwise noted all images observed were taken from 20x magnification using an IX70 inverted scope (Olympus). Images were taken using either that of differential interference contrast (DIC) microscopy or Fura-2 AM (Molecular Probes) ratiometric calcium imaging with a pseudocolor applied to better visualize changes in calcium levels. Prior to imaging neurons are plated onto poly-l-lysine coated coverglass slides (Lab-Tek). Fura-2 excitation was performed using light emitted from a system-controlled Lambda DG4 monochromator (Sutter Instruments) where light passes through excitation filters at 340 and 380nm. Neurons imaged were loaded with Fura-2 AM for 1 hour at a final concentration of 4 μ M. Following loading cells undergo 3 washes with DM over the course of 30min. Fura-2 as mentioned above is excited at both 340nm and 380nm and the ratio of the fluorescence (F340/F380) can be directly correlated to intracellular calcium levels. Ratio data was collected using SimplePCI software (Compix). Due to cells often appearing inactive during imaging, 30 minutes prior to imaging 10 μ M of deltamethrin (DMeth, Sigma) in dimethyl sulfoxide (DMSO) was added to the bath to hyper excite cells. Unless otherwise noted the delay between time points where images were taken is 5 sec.

Data analysis

Ratio fluorescence data were analyzed using ImageJ software (National Institute of Health) to create a timescale of change in fluorescence over the entire recording. The determination of calcium oscillation duration and rate was determined using the equation used in Grynkiewicz et al., 1985:

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

where K_d is the dissociation constant of Fura-2AM, F_{min380}/F_{max380} is the ratio of fluorescence intensity measured at zero calcium over the intensity at calcium saturation. R represents the measured ratio intensity versus those at both zero and saturated calcium (R_{min} and R_{max} respectively). Calcium transients were considered to be 5% change in the Fura-2 ratio, R , from resting levels. Raw data from calcium imaging was collected as the mean intensity of F340/F380 ratio from regions of interest (ROIs) within each cell. F340/F380 ratio was then transformed using several steps for analysis of coincident changes between specific neuronal pairs. Data transformations were performed using the numerical computing software MATLAB (MathWorks). The first transformation involved a time series analysis where each data point was converted to the change in ratio between consecutive time points (t) with the difference in ratio (R change between $t-1$ and t). Ratio differences for each time point were correlated to the temporal changes in calcium ratio in one cell with the change in another cell within the neural network at that time point. For each synapse within an imaged neural network, the two-coordinate temporal differences in calcium fluorescence ratio was plotted using a two-dimensional Cartesian coordinate system, with values for one neuron (a) plotted in the y-axis and

values for the other neuron (b) plotted on the x-axis. By convention, a data point with coordinates that fall in quadrant I (+,+) and quadrant III (-,-) are indicate of temporal coincidence in calcium change, whereas paired values with coordinates that fall in quadrant II (-,+) and IV (+,-) indicate change in calcium occurring in inverse directions. For a given time series, the coordinate points within each of the 4 quadrants were counted and a coincidence index was calculated where values close to 1 represent no correlation. The higher the index the stronger the direct correlation in calcium changes and as values fell under 1 the more inversely correlated they became.

Results

Intrinsic activity of Helisoma motor neurons

Helisoma buccal motor neurons B19 and B110 exhibited intrinsic bursting patterns of activity as assessed by intracellular electrophysiology. Individual bursts in isolated single neuronal cultures were found to have durations of 1.94 ± 0.20 s (n=8) and often occurred in rapid succession with inter-burst intervals ranging from 1 to 4 seconds (Fig. 9A). Having determined that buccal neurons were spontaneously active in culture and fired action potentials independent of stimulation, I then determined how calcium transiently changed in these neurons. Unlike the robust level of activity seen with electrophysiology, neurons loaded with Fura-2 AM and imaged with fluorescence microscopy showed only rare observable oscillation in fluorescence intensity with a calcium transient rate of 0.04 ± 0.02 per minute (n=8; Fig. 10A and C). This suggested

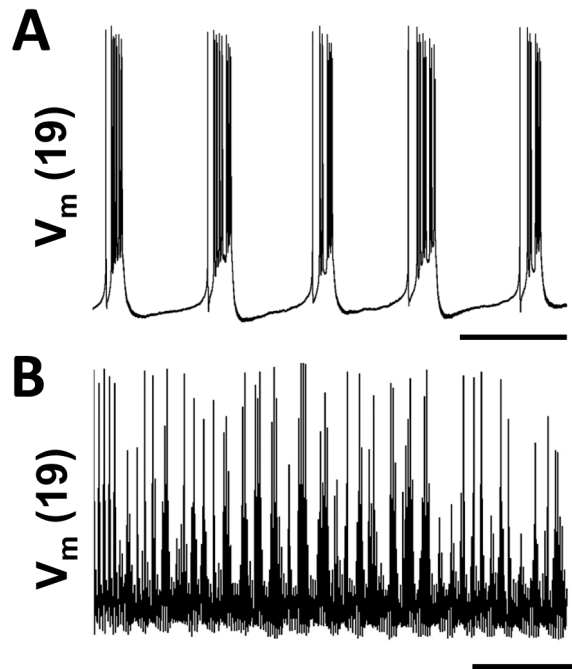


Figure 9. DMeth modulation of intrinsic electrical activity in neuron B19. (A) Representative trace of characteristic B19 bursting activity in cell culture when current-clamped at a resting membrane potential near -65mV . (B) Neuron B19 excitability increased following acute treatment with DMeth, such that prolonged high frequency action potential bursting was produced. Horizontal scale bar in A and B equals 1 s, vertical scale bar in A and B equals 30 mV.

that buccal neurons loaded with this calcium indicator were largely inactive. Although many possibilities exist for why these discrepancies were observed, two of the more obvious ones are: 1) that penetration of cell membranes causes membrane leakage that resulted in a net depolarization of the cells, thus making them abnormally excitable, or 2) that the buffering capacity of Fura-2 calcium binding led to a reduction in observable calcium-dependent response to membrane excitation. To test this we looked at ways to increase membrane excitability in Fura-2 loaded neurons that were otherwise unperturbed.

To make the cells more excitable DMeth, a synthetic pyrethroid similar to natural insecticides used by certain plants, was applied to neuronal cultures to prolong opening of voltage-gated Na⁺ channels. Electrophysiological assessment of DMeth treated neurons demonstrated a pronounced increase in action potential bursting compared to vehicle-treated neurons (Fig. 9A and B). DMeth treatment showed a significant increase in the firing rate ($5.7 \pm 1.5\text{Hz}$; $n=4$; $p<0.05$) of the neuron compared to control cultures ($1.5 \pm 0.19\text{Hz}$; $n=9$). In contrast to the previous calcium imaging studies of untreated neurons, those treated with DMeth for 15 minutes before calcium imaging had much more frequent calcium-dependent changes in Fura-2fluorescence (Fig. 10B and D). As represented in Figure 11A, DMeth increased the rate of calcium oscillations from 2.3 ± 1.1 to 47 ± 21 transients per hour (Fig. 11B, $p<0.05$) as well as the duration of calcium oscillations from 35 ± 5.8 to $53 \pm 11\text{sec}$ (Fig. 11C, $p<0.05$). Additionally, treatment with DMeth increased the percentage of time any cell spent oscillating by 44%. These data

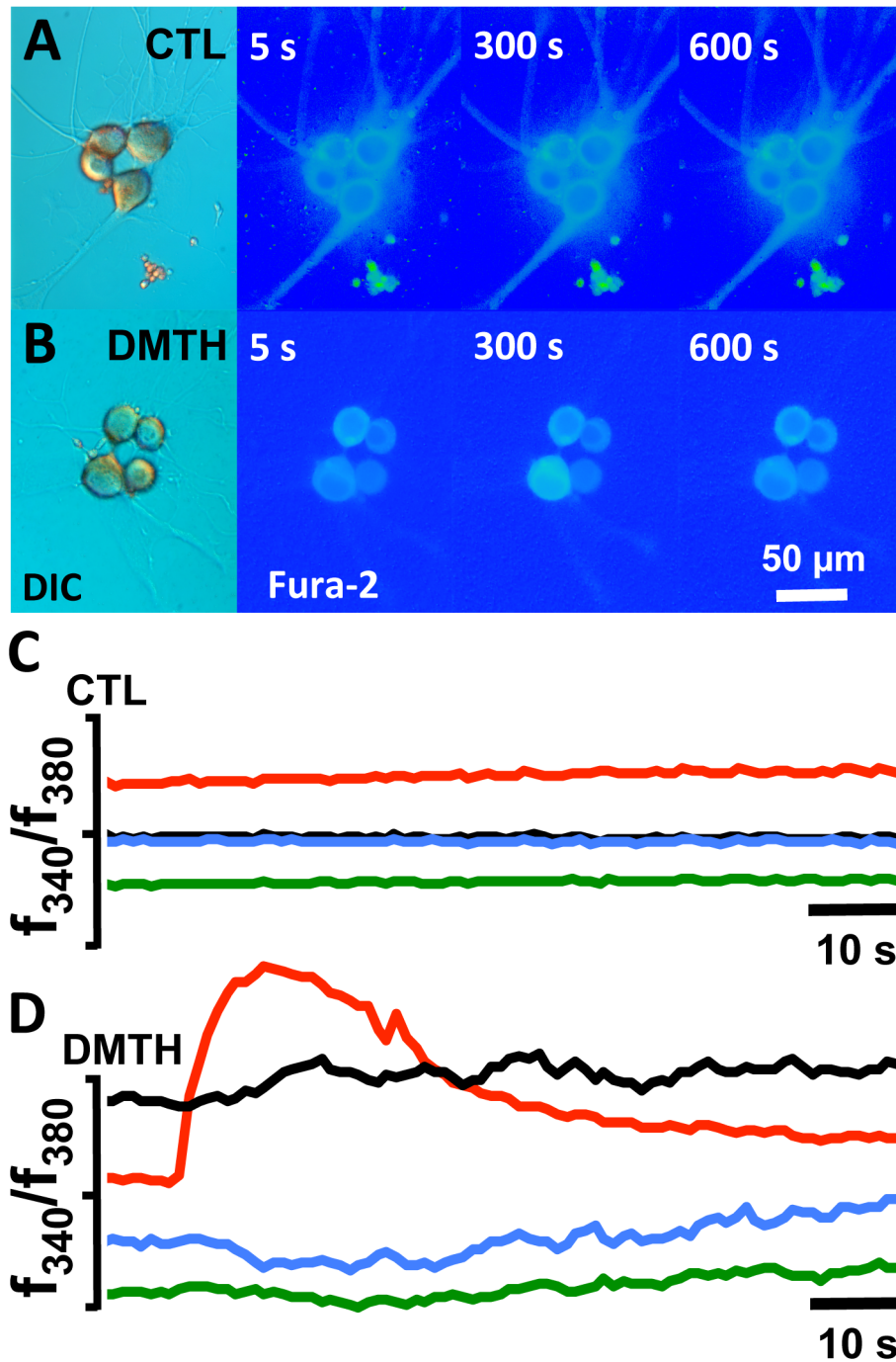


Figure 10. Calcium-dependent changes in Fura-2 fluorescence in response to DMeth. (A and B) Representative images of heterotypic 4-cell networks in either control (A) or DMeth (B) treated cultures. In each the leftmost panels in A and B, a DIC image of the network is shown. The subsequent panels at 5, 300, and 600s show pseudocolor images of Fura-2 fluorescence. (C and D) Representative traces showing simultaneous calcium monitoring (Fura-2 fluorescence ratio; f_{340}/f_{380}) from all 4 cells over time in control (C) and DMeth-treated (D) networks. In C and D the red line represents the bottom left cell of the 4-cell network in A and B respectively, with the blue line representing the bottom right cell, the green representing the top right cell and the black representing the top left cell. A and C show static calcium levels in controls, while B and D illustrate transient changes in calcium-dependent fluorescence in DMeth-stimulated networks.

indicate that increased neuron excitability produced much more robust changes in calcium oscillations in cultured *Helisoma* buccal neurons. As such manipulation with DMeth allows for the detection of calcium oscillations in *Helisoma* neuronal culture and ultimately to test if those oscillations are coincidence amongst neurons in the same network.

A key feature to this study was the ability to detect coincident changes in calcium amongst cells comprising small networks of neurons in culture. As such it was important that I be able to determine if transients of intracellular calcium in one neuron followed temporally in another network neurons. Through the detection of coincident changes in calcium, I hypothesized that synaptic connectivity of the network could be determined.

Temporal coincidence of calcium transients

Having established a method to elicit and monitor network calcium changes, I next developed a method for assessing neuronal connectivity based on the temporal coincidence of calcium transients. First, the ratio of calcium fluorescence F340/F380 was calculated for each combination of cell pairs comprising a simple 3-cell network. A ROI for each cell was selected from Fura-2 calcium images and ratiometric calcium data was plotted over the time course of imaging. Increases and decreases in f340/f380 represented oscillations in intracellular calcium of each neuron comprising the network (Fig. 12A and B). To determine if changes in calcium levels between cells were correlated, I calculated the difference in calcium levels of neurons from one

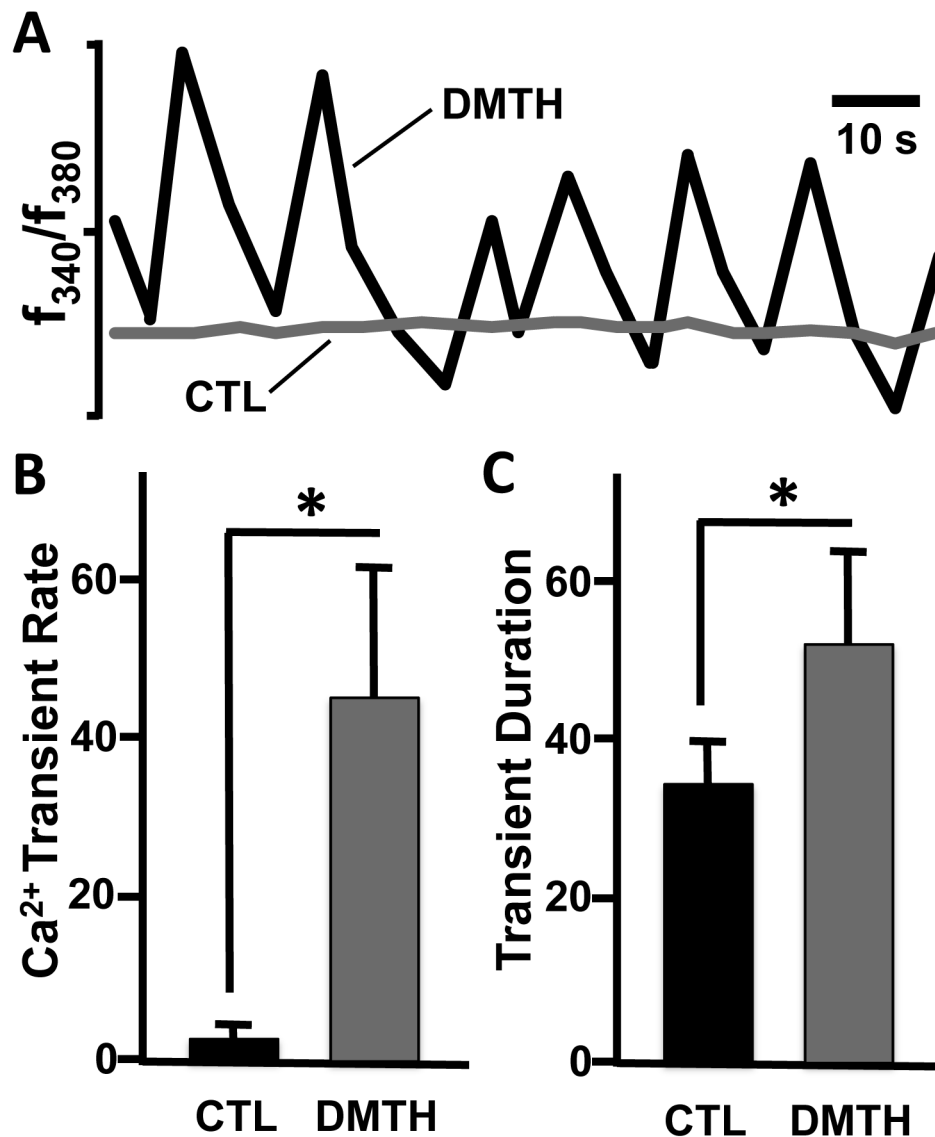


Figure 11. DMeth increases the rate and duration of calcium oscillations. (A) Representatives of both DMeth-induced changes in Fura 2 fluorescence ratio (black line) and inactive, untreated control (gray line) neurons B19. (B) Mean rate of calcium transients was significantly increased (*, $P < 0.05$ Student's t -test) when treated with DMeth (DMTH, gray bars; $n = 13$) versus controls (CTL, black bars; $n = 8$). (C) In the same neurons, calcium transient duration was also significantly increased in drug-treated cells (DMTH; gray bars) compared to vehicle-treated controls (CTL, black bars).

time point to the next. For every time point, the relative change in fluorescence either increased or decreased, from the time point before it. This time series analysis with point-to-point changes in the cytosolic calcium of two cells was analyzed and represented the pairwise activities of a network synapse (Fig. 12C and D). Finally, these temporal differences in calcium for each synaptic pair were assigned X (neuron1) and Y (neuron 2) values and were plotted in a Cartesian coordinate system (Fig. 12E and F).

Points representing correlated calcium transients segregated into quadrants I (+,+) and III (-,-), indicating direct coincidence of calcium changes between neurons, with coordinates in quadrants II (-,+) and IV (+,-) representing inverse correlations in calcium change. Quadrant-specific data points were then tallied and a coincidence index (CI) was calculated, where time points of coincident change were divided by those representing inverse correlation. Therefore, higher coincidence indices were indicative of synapses possessing largely coincident calcium oscillations. Figure 12 illustrates two synapses of different cellular identity, modulatory state, and coincidence of calcium oscillations. A homotypic neuron B19 synapse was characterized by high coincidence of Dmeth-induced calcium oscillations (Fig. 12A and C). This synaptic pair possessed a CI of 5.9 (Fig. 12E), indicative of strongly correlated activity. In contrast, a heterotypic B110-to-B19 synapse had no correlated DMeth-induced calcium transients (Fig. 12B and D), particularly when treated with the neuromodulator DA. This uncoupled synapse possessed a CI of 1.5 (Fig. 12F), indicating very low correlated activity.

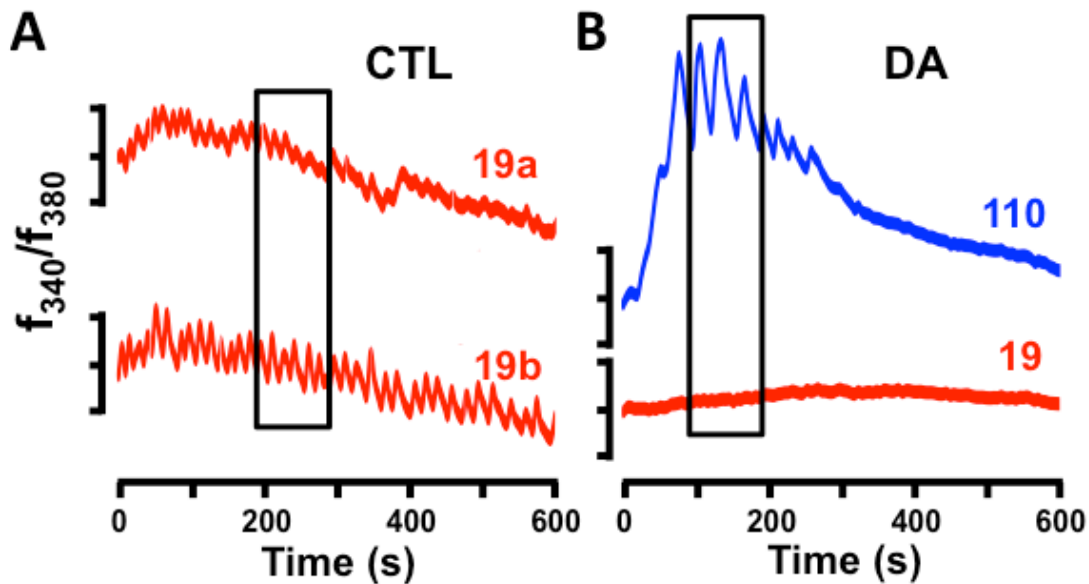


Figure 12. Coincidence of intracellular calcium transients reports synaptic connectivity. (A) Changes in calcium-dependent Fura-2 fluorescence ratio at a strongly-coupled B19-B19 neuronal pair are presented. (B) Fluorescence ration of an uncoupled B110-B19 pair with predicted chemical synaptic transmission following treatment with DA is shown over a 10 min recording period. Red traces indicate B19s, while blue traces represent B110s. (C and D) Time-series analysis of point-to-point changes in Fura-2 fluorescence between two neurons of a 3-cell network. (C) Analysis of point-to-point changes in calcium levels for the synapse in A, while (D) shows the analysis for the synapse in B. (E and F) Cartesian coordinate systems were analyzed for each represented synapse and temporal differences in calcium between the two cells were plotted. E represents the coordinates of temporal changes in calcium at the synapse in A, while F represents the coordinates from the synapse in B. The coincidence index, CI, represents the ratio of those plotted points that are directly correlated (quadrants I and III) versus those that are inversely correlated (quadrants II and IV).

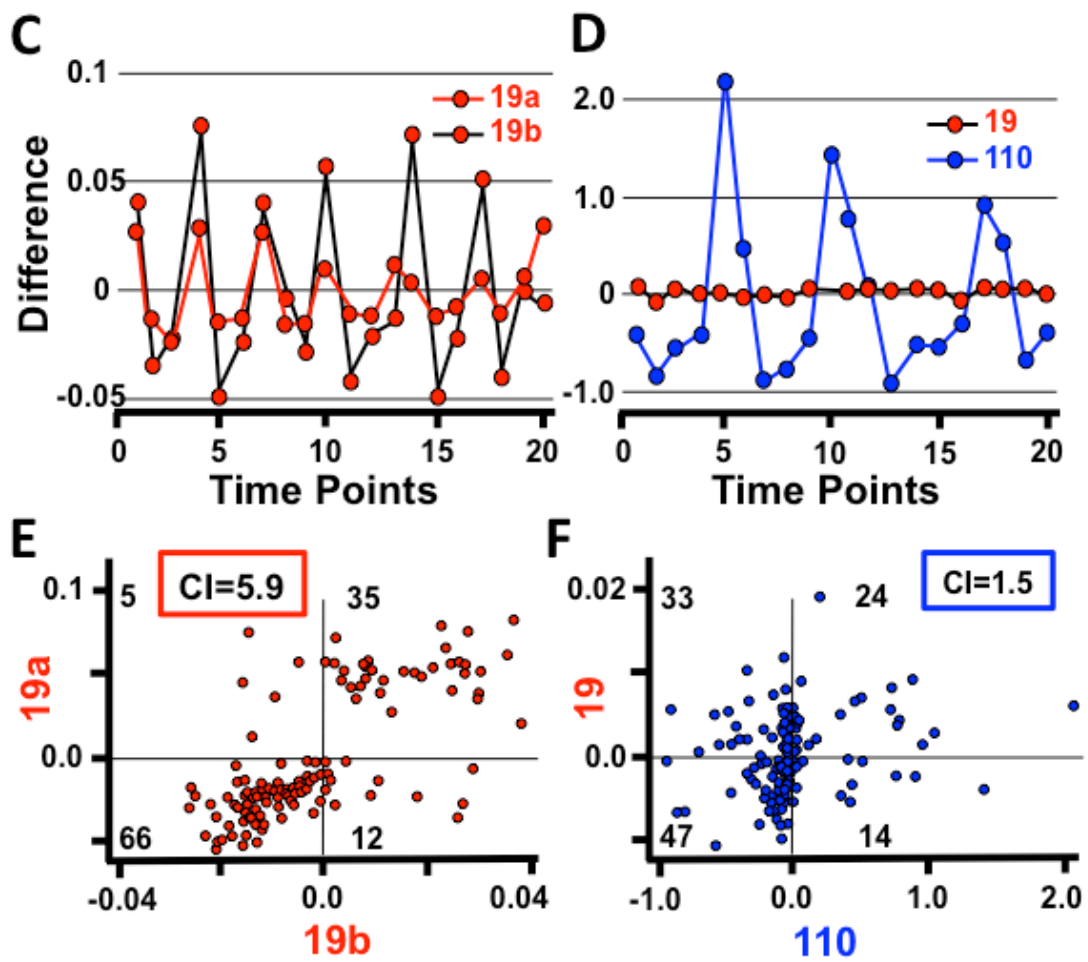


Figure 12 Continued.

Fidelity of coincidence assessment of connectivity

Given the observation that CIs of neural network synapses appear to reflect previous electrophysiological descriptions of these connections, we statistically compared CIs obtained from different types of synapses with known patterns of electrical coupling. To assess the accuracy of calcium-based coincidence detection for the assessment of neural network connectivity, electrical synapses of predicted coupling strength within an identified network were assayed. Synapses with strong electrical coupling were predicted to produce a high degree of coincident calcium transients between cells. In contrast, synapses with either low electrical coupling or chloride channel-mediated inhibitory synaptic transmission were expected to produce low levels of coincident activity.

Network synapses with predictably strong electrical coupling generated calcium oscillations with a significantly greater likelihood of coincidence, as compared to network synapses predicted to lack electrical coupling (Table 1). As suggested above, a highly active B110, predicted to form inhibitory chemical connections with B19, had very little calcium oscillation coincidence between these neurons, indicating a low level of electrical coupling. The use of coincidence detection of calcium transients at identified network synapses appeared to be a highly sensitive approach to predicting electrical coupling. In 100% of synapses (n=18) studied in this way, where the cellular identity and cell culture conditions promoted either strong or weak electrical coupling, coincidence of calcium changes was significantly greater than that predicted by random, uncorrelated activity (Table 1). However, 57% of synapses (n=7), cultured in

Table 1. Statistical analysis of coincidence detection of calcium transients at identified network synapses with predicted electrical coupling strengths.

Prediction	% Coincident	P value range
Strong Coupling	100% (n=8)	0.04 to 0.0001
Weak Coupling	100% (n=11)	0.02 to 0.0001
No Coupling	57% (n=7)	0.01 to 0.001

configurations that reduced electrical coupling, still exhibited significant coincident calcium oscillations. These results suggested that this technique was a sensitive measure of coincident activity, revealing coupling not typically detected by conventional electrophysiological approaches. Alternatively, this approach may inaccurately report a level of non-randomness in the calcium data sets that is not due to correlated biological activity, but rather was a consequence of low amplitude, ubiquitous oscillations in the data caused by culturing, optical measurement, sampling protocols or other procedural issues.

Coincidence indices at sustained and transient electrical synapses

To further analyze whether or not coincidence indices could predict functional classes of electrical coupling (i.e, transient or sustained electrical synapses), we separated the calcium coincidence data set by synapse-specific identity (Fig. 13). Heterotypic connections (e.g., B110-B19 or B5-B18) that were cultured in contact for 1 day or 5 days were predicted to exhibit early and late transient electrical synapses (TES), respectively. Homotypic connections (e.g., B110-B110 or B19-B19) were predicted to exhibit sustained electrical synapses (SES). TES connections when designated as ‘late’ were generally predicted to be uncoupled or very weakly coupled. TES designated ‘early’ were expected to have moderate levels of electrical coupling. The assumption is that SES synapses, because of their high degree of gap junctional connectivity, would likely exhibit the highest CIs. Meanwhile, TES was hypothesized to exhibit lower CIs

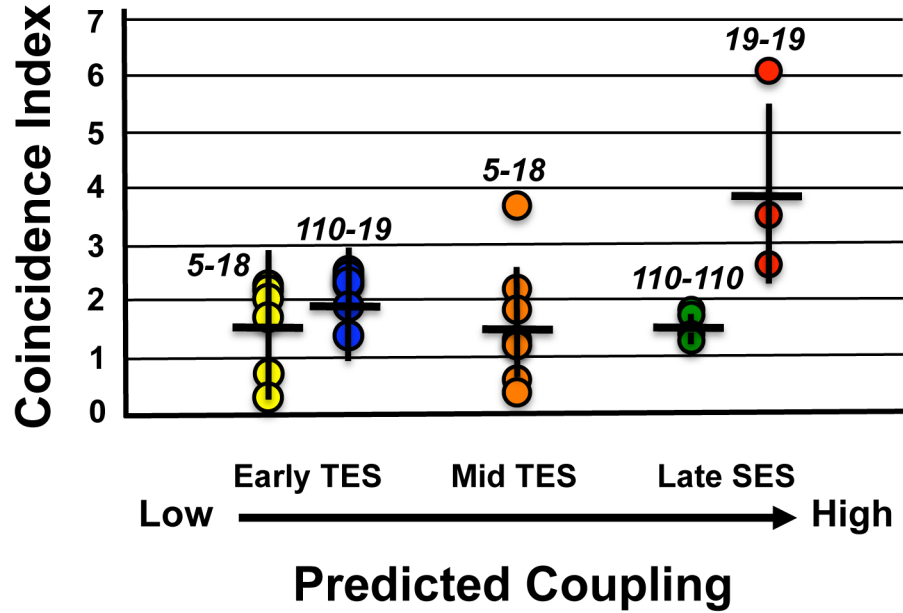


Figure 13. Coincidence indices for synapses predicted to express sustained or transient coupling. Coincidence indices (CI) at B19-B19 synapses, predicted to have high coupling associated with late SES, had high and variable CIs. B110-B110 synapses, also predicted to express late SES, had low and invariable CIs. Both late and early TES, represented by a mix of neuronal pairs, had low CIs, as was predicted. Dots represent an individual synapse's CI, with the horizontal bars representing the group mean and the vertical bar representing the SEM.

due to their generally lower coupling

Late SES connection types had the most striking differences in CI based upon neuronal identity. At B19-B19 SES connections the average CI was 4.1 ± 1.0 (mean \pm s.e.m.; $n=3$; Fig. 13). In contrast, at B110-B110 connections the CI was 1.7 ± 0.1 ($n=4$). Although the sample sizes on these networks did not allow for meaningful statistical comparison, it is worth noting that there was no overlap of the CI range or standard error. Homotypic B110 connections with late SES exhibited weaker electrical coupling in vitro than B19-B19 SES connections. Furthermore, although bilateral B19 pairs are coupled in vivo, the nature of B110-B110 electrical coupling is not known, largely due to asymmetry in the position of cells within the left and right ganglia.

Nonetheless, the degree of dissimilarity detected with calcium coincidence analysis was surprising. In contrast, none of the connections predicted to possess TES synapses, whether early or late in formation, were different in CI indices (Fig. 13). Thus, neurons with similar synaptic phenotypes (i.e., TES connections) but different neuronal identity (i.e., heterotypic B110-B19 and B18-B5), exhibited similar CIs of 2.1 ± 0.2 and 1.8 ± 0.3 , respectively. Late TES synapses, represented entirely by the heterotypic neuronal pairs (B5-B18) had an average CI of 1.8 ± 0.3 .

Discussion

A fundamental and largely unsolved problem of neuroscience is how connections between neurons underlie the global information processing that occurs within larger

neural circuits. Even in the light of well-understood wiring diagrams of a neural network, it is critical to know what kinds of communication are occurring between pairs of neurons within a network and how that communication influences the rest of that circuit (Lee and Reid, 2011). The study of neural network connectivity within large ensembles of neurons is a challenging task. Conventional intracellular (current and voltage clamp electrodes) and extracellular (multi-unit and multi-electrode arrays) each have their limitations in this regard (Liu et al., 2012). Optical analyses of neuronal activities, particularly using fluorescent dyes, have become important tools for probing neuronal ensembles in vivo and in vitro (Grewe and Helmchen, 2009). Here, real-time calcium imaging approaches are combined with thoroughly characterized neural networks in vitro. This system was assessed to determine if computational analyses of that connectivity data generated predictable pairwise synaptic outcomes.

The degree of coincidence of calcium transients produced by identified *Helisoma* neurons was analyzed between contacting neuronal pairs and used as an indicator of the network connectivity. Although multi-electrode intracellular electrophysiology is not impractical in simple 3- or 4-cell networks, monitoring calcium fluxes via fluorescence microscopy provides a high degree of noninvasive access to an individual cell's physiology and provides greater freedom for neuronal manipulation. Fluorescent calcium indicators, such as the Fura 2-AM used here, report intracellular calcium changes evoked by neuronal activity. Calcium probes have proven to be advantageous over voltage-sensitive dyes due to differences in signal-to-noise ratio and phototoxicity (Grewe and Helmchen, 2009). The strong correlation between neuronal spiking activity

and somatic calcium transients has allowed for important discoveries of network properties in vivo, such as those connecting the mammalian visual cortex (Kerr et al., 2007; Greenberg et al., 2008). In excitable neurons, trains of action potentials lead to the summation of individually induced calcium influxes. Calcium fluorophore probes generate fluorescence transients whose temporal dynamics reflect changes in spike frequency underlying neuronal electrical signaling.

An initial obstacle faced in these studies was the unexpected low frequency of calcium transients displayed in the *Helisoma* neuronal networks in cell culture, an observation seemingly in direct contradiction to decades of electrophysiological studies of these motor neurons in vivo (Murphy, 2001) and in cell culture (Turner et al., 2011). A few explanations for these differences are obvious. First, penetrating the neurons with microelectrodes causes excitation through mechanical disturbance of the cell membrane and leakage of Na⁺ and Ca²⁺ ions into the cytoplasm. Second, the use of 1.5M KCl-filled electrodes makes the resting membrane potential slightly more positive and reverses the flow of Cl⁻ ions through acetylcholine (ACh)-activated chloride channels. Together these chloride-based influences may tend to make the neurons, when penetrated with intracellular microelectrodes, slightly more excitable due to a less polarized membrane and inhibitory currents turned excitatory. Third, use of a chelator-derived calcium dye like Fura-2 AM can lead to altered states of cytosolic calcium buffering, which could suppress calcium-dependent excitability. Since the neurons used in these studies are networks of electrically-coupled motor neurons, it is most likely that these cells are weakly active or inactive when isolated from their primary excitatory

interneuronal inputs, a notion likely exacerbated by the calcium buffering capacity of Fura-2.

To circumvent the issue of low action potential firing frequencies *in vitro*, pharmacological treatment was used to hyper-excite neurons in culture to produce robust levels of activity. DMeth, a potent synthetic of pyrethrum that suppresses inactivation of voltage-gated sodium channels (Motomura and Narahashi, 2001), caused hyperactivation and increased frequency of calcium transients in motor neuronal networks. DMeth likely amplified activity already present in low levels in the neuron, instead of stimulating it outright, given that treatment with DMeth often did not immediately induce tonic firing until after a depolarizing current was given. Acute application of DMeth produced a dramatic increase in the rate and duration of calcium transients sufficient to conduct coincident detection analyses in identified neural networks.

Simultaneous calcium imaging from multiple neurons allowed detection of calcium oscillations within each cell over the same time series. Following data collection and ratiometric assessment, cross-correlation analyses were used to detect coincidence of calcium transient activity between neurons. This methodology of analyzing calcium signals allowed indirect inference of neuronal spiking and in doing so allowed deduction of pairwise interactions within the network. Correlated calcium transients were analyzed statistically using a comparison based on a null hypothesis of random interaction. Surprisingly, most neuronal interactions assessed, whether predicted to possess strong, weak or no electrical coupling, had levels of significant correlated calcium signaling.

Furthermore, coincidence indices (CI) calculated from the pairwise interactions were predictive of the type of electrical connectivity present at a given synapse, suggesting that this technique might be useful in diagnostic differentiation between weak transient synapses and strong sustained synapses within a neural network.

Although several synaptic mechanisms exist that might coordinate calcium changes amongst neuronal pairs, electrical coupling via gap junctions seems the most likely contributor as these motoneuronal networks are known to be electrically coupled both *in vivo* and *in vitro* (Szabo et al., 2004). In addition to this electrical communication, chemical neurotransmission in these networks is mostly inhibitory in nature and, therefore, is unlikely to lead to direct correlation of calcium changes. Any depolarization in one cell sufficient to trigger ACh release, would be accompanied by a hyperpolarizing postsynaptic potential in a receptive cell of opposing signal polarity (i.e., one leading to calcium influx and the other not). In the case of an inhibitory potential passing between two electrically coupled neurons, it is unlikely that these small hyperpolarizing membrane potentials would transmit through the low-pass filter of the gap junctions with sufficient fidelity of amplitude to produce coincident resting calcium changes detectible with Fura-2 imaging. Therefore, I conclude that the calculation of pairwise CIs gives reliable assessment of electrical connectivity at these synapses, with higher CIs suggestive of strong electrical coupling and CIs closer to a value of 1 representing low coupling levels. Those CIs under 1, and indicative of inversely correlated calcium changes, would most likely represent synapses with primarily strong inhibitory chemical connectivity, where activity and its associated rise in cytosolic

calcium levels stimulate inhibitory synaptic transmission and hyperpolarization of the postsynaptic cell, which consequently suppresses neuronal activity and leads to a decrease in cytoplasmic calcium levels in the follower cell.

Neuronal pairs with SES, generally the strongest coupling, had higher CIs than pairs predicted to possess either early or late TES. Interestingly, homotypic connections between B110s had much lower CIs compared to those of B19s, even though both of these are predicted to express SES connections. The major difference between these two types of connections is the chemical synaptic strategies employed by the component neurons. Although it does not frequently occur, B110s can form inhibitory connections onto one another, while homotypic B19 synapses are not competent to do so (Poyer et al., 1996; Turner et al., 2011). Therefore, inhibitory chemical synapses may be forming at 110-110 connections and disrupting coincidence of calcium fluxes bringing the CI closer to 1 than in the CI of homotypic B19 SES connections. Although the nature of this discrepancy is not understood, it demonstrates that differences in pairwise evaluations of synaptic interactions with neural network can be achieved using coincident calcium transients rather than membrane potential fluctuations as the basis of the assessment.

Further experimentation is needed to determine if calcium imaging and coincidence computation is sensitive enough to determine complex interactions within large neural networks. Analysis of neuronal firing patterns using computational approaches demonstrate that global firing within an electrically coupled neural network can be predicted by simple pairwise interactions (Shlens et al., 2006; Schneidman et al., 2006). Local interactions at a single synapse can induce long-range correlations through

many cells of the network (Nirenberg and Victor, 2007). That is, at adjacent neuronal pairs connected by gap junctions, a neuron that is only directly coupled to one of the other cells has predictable synchronous efficacies with the non-adjacent neurons of that network. As an example, neurons with an electrical connection strength where roughly one third of the cell's spikes are synchronous would have a predictable spike synchrony in each of the neuron's non-adjacent partners where one-ninth of all spiking events were synchronous. Schneidman et al. (2006) have suggested that pairwise electrical interactions, as described above, present in the firing patterns of the broader neural network in the vertebrate retina serves an error-correcting code function, where signals in the network are correlated so that information can be correctly decoded in the face of background noise levels. Similarly, electrically coupled motor neuronal networks, such as those of *Helisoma*, might benefit from such an error-correcting code, whereby inputs from central pattern generator interneurons are relayed to neuromuscular outputs with high fidelity, particularly when synaptic connections are mixed electrical and chemical synapses. Taken together, these neural network studies using calcium transient coincidence suggest that examinations of relatively small populations of neurons can make global predictions of overall activity within broad networks. What once was a tedious and confounding task of assessing neural connectivity, has today become a manageable undertaking based on the interplay of optical engineering, physiological probes and computational neuroscience.

CHAPTER IV

ELECTRICAL SYNAPTIC REGULATION OF NEW AND FORMING SYNAPTIC CONNECTIVITY

Introduction

Electrical synapses are present in the nervous systems of most animals, being widely found in invertebrates and, to a lesser extent, vertebrates. Often these synapses mediate highly synchronized neural pathways, such as large populations of invertebrate motor neurons. Due to their rapid transduction of electrical signals, these synapses are vital components of escape reflexes, such as the giant fiber escape circuit of crayfish (Antonsen and Edwards, 2003) and the goldfish Mauthner cell-mediated escape network (Pereda et al., 2004). The electrical synapse is not limited to mediating only rapid escape or highly synchronous motor behaviors. In fact, the vertebrate brain is much more widely coupled by gap junction-mediated synapses than once appreciated (Pereda et al., 2012). In adult mammals, astroglial networks are connected through gap junctions and play pivotal roles in regulating ongoing synaptic efficacy in the brain (Nagy and Rash, 2000; Oliet et al., 2001). Electrical synapses functionally couple cells and coordinate their activity patterns through creation of contiguous intercellular compartments that are often bidirectional in nature (Yuste et al., 1992). In addition to synchronization of ionic conductance, electrical synapses underlie biochemical coupling between cells, where

small second messengers, such as cAMP and IP₃, pass through gap junctions (Kandler and Katz, 1998).

During nervous system development, synchronization of neural activity through gap junction-mediated intercellular connections is thought to be important in the construction and development of neural networks (Kandler and Katz, 1995; Personius et al., 2001). Electrical synapses are highly expressed in the developing central nervous system, where they are transient and diminish dramatically prior to the formation of adult chemical connections (Peinado et al., 1993). Additionally, gap junctions are upregulated in the mammalian central nervous system during the regeneration of neural connections following injury (Chang et al., 2000). A common pattern seen in the expression of these transient electrical synapses (TES) is a sequential progression from largely electrical coupling to predominantly chemical synaptic connectivity (Kandler and Katz, 1998; Marin-Burgin et al., 2005; Dupont et al., 2006). In many circumstances, this period of electrical coupling is thought to be crucial to the formation of adult chemical synapses, such as in the optic lamina of *Drosophila* (Curtin et al., 2002), mechanosensory neurons of leech (Todd et al., 2010) and olfactory systems of mice (Maher et al., 2009). An interesting feature of TES is not only that it regulates the formation of adult chemical synaptogenesis, but there appears to also be a mutual regulation of electrical connections by chemical synapses, such that delay or inhibition of chemical neurotransmission appears to prolong the temporal expression of TES (Szabo et al., 2004). In rats, electrical coupling is present between CNS neurons early in development. Later, these synapses are uncoupled by NMDA-mediated glutamatergic

signaling. This sequence of coupling and uncoupling occurs in the hypothalamus (Arumugam et al., 2005), spinal cord (Mentis et al., 2002), and neocortex (Dupont et al., 2006). Blocking of NMDA-mediated signaling leads to a delay in the electrical synapse uncoupling. Thus, a complex co-regulatory mechanism exists between electrical and chemical synapses during neural network formation. Still, the cellular mechanisms involved in mediating these synaptic interactions remain unknown.

In rat thalamic relay neural circuits, transient electrical synapses are replaced by inhibitory chemical synapses during the second postnatal week (Lee et al., 2010). Similarly, regenerating motor neural circuits of the *Helisoma* buccal ganglia express transient electrical synapse that, at specific connections, are replaced by inhibitory chemical synapses in a matter of days. Synapses formed between neurons B19 and B110 both *in vivo* and *in vitro* are strongly coupled at 2 days of contact. Over the next 3 days, the decline of electrical coupling is inversely proportional to the emergence of a unidirectional and inhibitory cholinergic synapse (Szabo et al., 2004). In contrast, B19-B19 homotypic synapses develop sustained electrical synapses (SES) that do not diminish in coupling strength and do not form chemical synapses. When TES are deprived of trophic factors, chemical synapses are formed earlier, suggesting that electrical synapses regulate formation of chemical neurotransmission. Furthermore, block of acetylcholine receptors led to increased electrical coupling (Szabo et al., 2004). Surprisingly, in simple neural networks, the state of electrical coupling influenced the formation of new electrical and chemical synapses with that network (Szabo and Zoran, 2007). In the present study, I have utilized these well-defined *Helisoma* synapses, within

paired connections or simple 3-cell networks, to investigate the contributions of electrical and chemical synaptic transmission to the formation of neural network connectivity. Specifically, I have tested the hypothesis that emerging electrical synapses compete with other electrical synapse and with inhibitory chemical synapses during the formation of connections within motoneuronal networks.

Materials and Methods

Animals

Experiments were conducted on laboratory stocks of albino (red) pond snails, *Helisoma trivolvis*, which were maintained in 20 gallon aquaria at 26°C. Aquaria were kept on a controlled photoperiod of 12 hour light/12 hour dark and snails were fed lettuce and trout chow daily.

Cell cultures

Cultures were maintained to the specifications referenced in Chapter 2. For studies of neurons isolated into cell culture, excised buccal ganglia were placed into 0.2% trypsin (Sigma) in defined media (DM) for 15 to 20 minutes to partially digest the neural sheath. Ganglia were pinned to a Sylgard dish containing 3 ml of high osmolarity DM (56.0 mM NaCl, 2.4 mM KCl, 5.7 mM CaCl₂, 2.1 mM MgCl₂, and 14.0 mM HEPES). The buccal commissure and the relevant nerve trunks, containing the axons of 19 and 110 neurons, were crushed with fine forceps. The sheath of each ganglion was

cut along the dorsal surface, next to a neuronal soma, using an electrolytically sharpened microknife. Pressure applied to the ganglion forced the neuronal cell body through the incision and the neuron was collected into a fire-polished, non-adhesive (bovine serum albumin-coated) micropipette using negative pressure produced by a microsyringe (Gilmont). Neurons were then transferred into specific culture conditions, as described below for each experiment. For studies of neurons exhibiting neuritic growth, cells were transferred directly into adhesive 35 mm culture dishes (No. 3001 Falcon) containing 2 ml of conditioned medium (CM). The dishes were made adhesive by pretreatment with 0.1% poly-l-lysine (PLL) in a 0.15M Tris buffer. CM was bulk-cultured in silicone-treated (Sigmacote) glad petri dishes generated by incubating 2 central ring ganglia per 1 ml of DM for 3 days. CM was subsequently transferred to PLL-coated dishes before plating of cultures. Brain-derived factors in CM are required for neurite outgrowth in these cultures (Wong et al., 1981). Neurons were maintained in different cell culture conditions depending on the experiment. In general, two culture configurations were used: 1) soma-soma neuronal pairs and 2) 3-cell neuronal networks. Each of these protocols are described in the following sections.

2-cell soma-soma pairs

Identified neurons were transferred into non-adhesive, 35 mm culture dishes (No. 1008 Falcon) containing 2 ml of CM. Culture dishes had been pre-treated with a 0.5% solution of bovine serum albumin (BSA) to make their surface non-adhesive. Neurons were incubated for 3 days of initial culture as single, spherical cells before being

transferred into fresh CM dishes and paired into contact. Cell pairs were cultured for an additional 1 or 5 days for synapse formation on BSA-coated dishes. The soma-soma pairs, having not produced neurites on non-adhesive substrates, were then transferred to recording chambers (PLL-treated culture dishes containing 2 ml DM) for electrophysiological study.

3-cell network formation

Identified neurons were again transferred into non-adhesive, 35 mm culture dishes (No. 1008 Falcon) containing 2 ml of CM, as described in the previous section. Neurons were incubated for 3 days of initial culture as single, spherical cells before being transferred into fresh CM dishes and paired into contact. Unlike soma-soma pairs, these neuronal pairs were plated onto adhesive substrate cultures coated with PLL and incubated for 4 days while neurites extended and processes established contacts. Simple 3-cell networks were formed by the additional of a third neuron and the network was cultured in CM for another 24h prior to electrophysiological studies. The end result of this culture protocol resulted in two kinds of synapses existing within the network (Fig. 14A). The first synapse type was 5 days of age and formed at contacts between the two original (central) neurons. The second synapse type was 1 day of age and formed between the newly added neuron and the original pair. Following the 5d of synaptogenesis, the neural network was assessed for connectivity. The electrical synapses initially forming between central neuron pairs, less than 24h of contact, were designated as forming electrical synapses (FES; Fig. 14B). The older, 5d electrical

synapses were designated as either transient (TES) or sustained (SES), depending on the nature of the electrical coupling expressed. The newly formed 1d old synapses were designated as new forming electrical synapses (nTES).

General electrophysiology

Electrophysiological properties of neurons were examined using intracellular recording techniques. Glass microelectrodes (borosilicate; FHC), possessing tip resistances ranging from 10-20 M Ω , were filled with 1.5 M KCl or 1.5 M KAc. Current-clamp recordings of neuronal membrane potentials were amplified using a bridge-balanced electrometer (World Precision Instruments) and records were viewed as digitized outputs using a PowerLab 4/35 data acquisition system (ADInstruments) using an iMac computer running Chart software (ADInstruments). In most experiments unless otherwise stated, neuronal membrane potential was maintained with base current injection at approximately -70 mV. Electrical coupling was measured by injecting constant amplitude, hyperpolarizing current pulses (3 s in duration) into one neuron (0.2 - 3nA), while simultaneously recording membrane voltage changes in the presynaptic (injected) neuron (approximately 30-50 mV) and its synaptic partner. Electrical coupling coefficients (ECC) were determined as the ratio of postsynaptic to presynaptic voltage changes (Bennett 1977). Data analyses for coupling ratios were taken at the peak of membrane hyperpolarization. To assess evoked chemical neurotransmission presynaptic cells were generally given a series of 10 depolarizing stimuli of 40-60 mV at a rate of 1 stimulus every 3 seconds. Evidence of evoked chemical release was

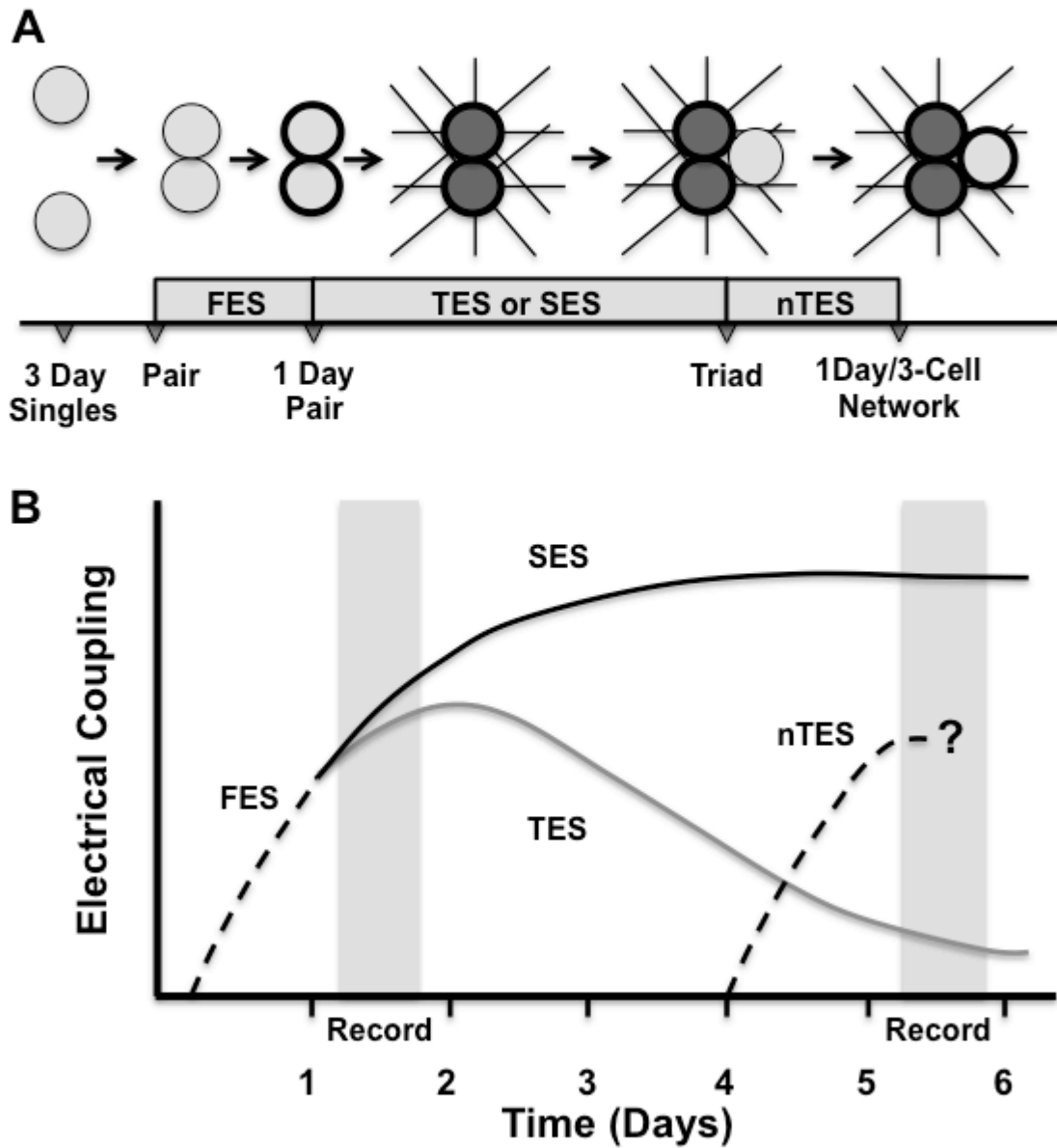


Figure 14. Cell culture protocol for analysis of electrical synapse formation in pair and triad neural networks. (A) Illustration of the cell culture protocol used to produce electrical synaptic connections at soma-soma synapses and subsequent 3-cell, triad, networks. Neurons were isolated into cell culture and incubated for 3 day as single cells. At time point zero, 2 neurons were paired as giant somatic synapses. These cells were plated onto adhesive culture dishes for neurite growth and formation of electrical synapses, either transient (TES) or sustained (SES). For indicated studies certain pairs are recorded from during the first day of synapse formation. On day 4 of culture a new neuron was plated into contact with the existing cell pair to create a triad of neurons. One day later, the 3-cell network was studied. (B) Timecourse of electrical synapse formation, either SES or TES. Forming electrical synapses (FES) develop during the first 24h in contact. SES connections persist, whereas TES connections decline in coupling strength over time. Newly forming TES connections (nTES) were initiated on day 4 and were assessed 1 day later. Shaded areas indicate the times of electrophysiological assessments (Record).

measured by the presence of a graded-postsynaptic potential at 95msec post stimulus of the presynaptic neuron.

Drug treatment procedures

For electrical synapse manipulation, either 18 α -glycyrrhetic acid (AGA) at 10 μ M in 0.01% dimethyl sulfoxide (DMSO; Sigma) in DM or carbenoxolone (CBX; Sigma) at 100 μ M in DM, both gap junction blockers, were added to neuronal cultures for durations as indicated. Cholinergic signaling was disrupted using two nicotinic acetylcholine receptor antagonists, hexamethonium bromide (HEX; Sigma) at 10 μ M in DM or curare (CUR; Sigma) at 10 μ M in DM for the specified durations. For blocking vesicular endocytosis an inhibitor of dynamin, known as dynasore (Dyna), at 10 μ M in DMSO was used for 24 hours prior to cell-cell contact. Upon initial pairing of the central neurons, pharmacological agents (e.g., CBX) or given vehicle were added to the culture medium. Following day 4 of contact, the neuron pairs were washed with fresh medium before the third neuron was plated into contact.

Data analysis

Electrical coupling, compared among groups, was analyzed using the Student's *t*-test, while chi-square tests were used to determine if observed numbers of preparations with chemical neurotransmission were different from expected results (Microsoft Excel). Data presented here represents the mean plus or minus the standard error of the mean

(SEM) unless otherwise indicated. Significance of statistical measures was defined as $p < 0.05$.

Results

Disruption of coupling at forming electrical synapses

Deprivation of trophic factors from identified *Helisoma* neurons suppresses the formation of electrical synapses, leading to subsequent increased rates of chemical synapse formation (Szabo et al., 2004). To test the specific involvement of electrical synapses in modulation of chemical synapse formation, electrical coupling was knocked down using gap junction inhibitors. Electrophysiological recordings were conducted to determine if reduction in coupling altered subsequent synapse formation. Soma-soma synapses comprised of homotypic B19 pairs were strongly coupled after 24h of contact and therefore had formed early SES (Fig. 15). However, identical synapses treated for that 24h of initial synaptogenesis with the glycyrrhetic acid derivative, AGA, had reduced electrical coupling ($n=13$; $p < 0.001$) as compared to vehicle-treated controls ($n=16$; Fig. 15C). No B19-B19 synapses exhibited evoked chemical neurotransmitter release, either treated or untreated, consistent with the idea that B19 gains secretory capabilities only following contact with appropriate muscle targets (Zoran et al., 1996). Thus, AGA effectively reduced the formation of SES by 72% without affecting a change in chemical synaptogenesis at mixed giant synapses.

Heterotypic TES connections, consisting of B110-B19 pairs, had electrical coupling coefficients lower than those seen at B19-B19 synapses (Fig. 15). Although treatment for 24h with AGA reduced the average coupling coefficient by 47% (n=19), when compared with DMSO-treated synapses (n=20) no significant differences in coupling were found (p=0.067; Fig. 15D). Unlike B19s, B110s possess promiscuous chemical synaptogenesis and form inhibitory chemical synapses readily with cholinergic target neurons. Nonetheless, AGA treatment had no impact on chemical synapse formation of B110 (treated vs. control; p=0.43). Therefore, AGA is a potent inhibitor of both SES and TES formation, but does not affect the competence of chemical synapse formation at these synaptic pairs.

Although AGA is a potent inhibitor of *Helisoma* electrical synaptogenesis at soma-soma pairs, I opted against using this pharmacological agent for neurite-bearing neuronal networks to avoid the use of DMSO, a solubilizing agent known to disrupt neurite outgrowth of cultured *Helisoma* neurons (Zoran, unpublished observation). Carbenoxolone (CBX), a water-soluble derivative of glycyrrhetic acid used in many studies of electrical coupling (Traub et al., 2001; Margineanu and Klitgaard, 2001), was tested for its efficacy in blocking electrical coupling between *Helisoma* neurons at strongly coupled B19-B19 SES connections. CBX caused a 51% reduction in electrical coupling at treated synapses (n=10; p<0.001), compared to coupling coefficients at untreated synapses (n=3; Fig. 16). Therefore, CBX provided potent inhibition of electrical synapse formation, while its aqueous solubility made it a useful tool for examining chronic reductions in coupling in growing neural networks.

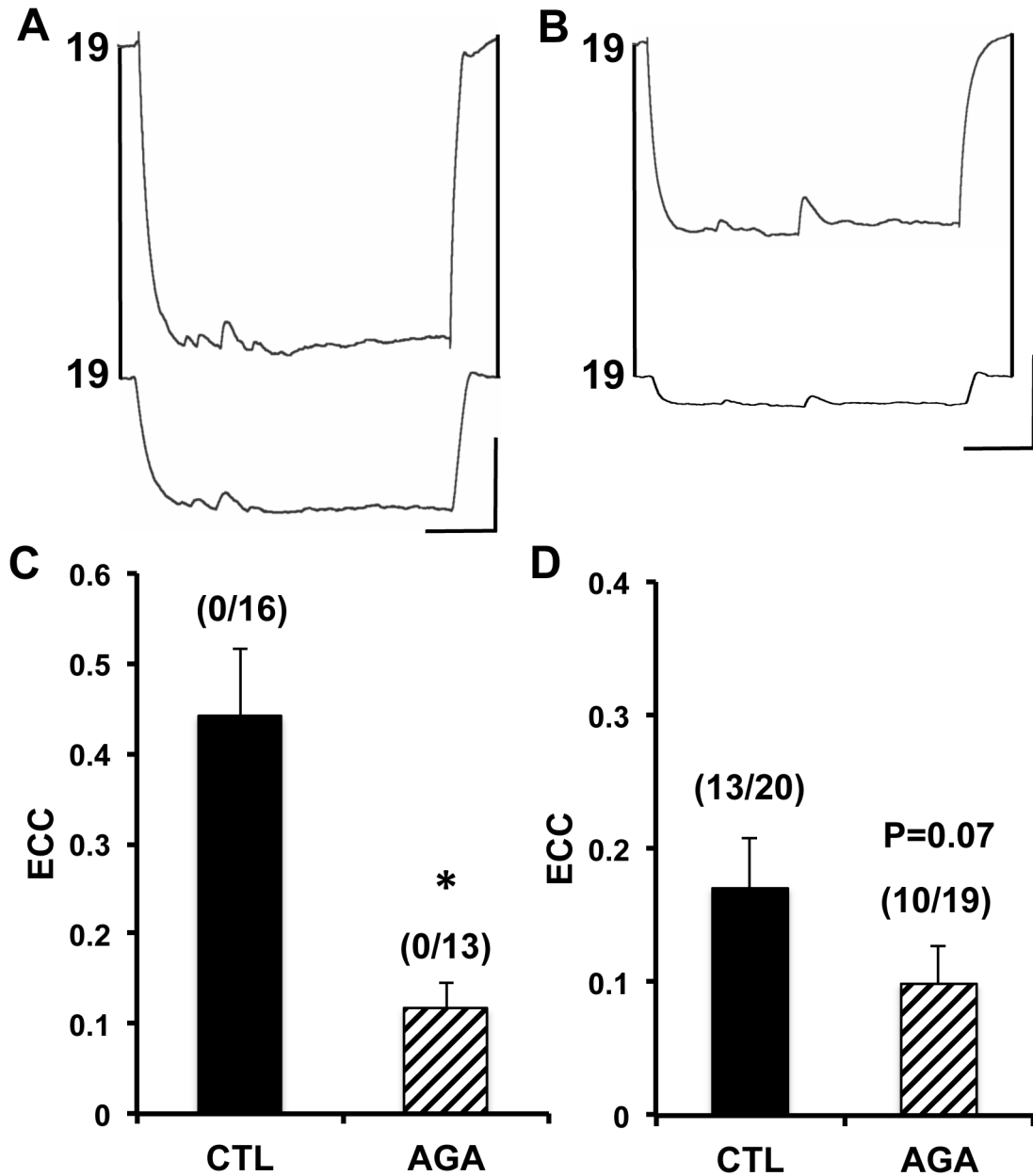


Figure 15. AGA reduces the formation of SES, but not TES, at B19-B19 connections, without altering chemical synapse formation. (A) Membrane potential recordings from a pair of B19 neurons expressing SES. (B) Membrane potential recordings from a pair of B19 neurons expressing reduced electrical coupling following AGA (10 μ M) treatment. Note the spontaneous mPSPs in A and B. Vertical scale bars equal 20 mV. Horizontal bars equal 0.5 s. (C) Histograms represent electrical coupling coefficients (ECC) from control (CTL; n=16) and AGA-treated (AGA; n=13) soma-soma SES connections. ECCs were significantly different (*; $p < 0.05$, Student's *t*-test). Numbers in parentheses represent the ratio of preparations with action potential-evoked chemical synaptic transmission. (D) Histograms represent electrical coupling coefficients (ECC) from control (CTL; n=20) and AGA-treated (AGA; n=19) soma-soma TES connections. ECCs were not significantly different ($p = 0.07$). Numbers in parentheses represent the ratio of preparations with action potential-evoked chemical synaptic transmission. TES preparations in D possess higher percentages of preparations with chemical synapses compared to SES preparation in C, whether control or treated.

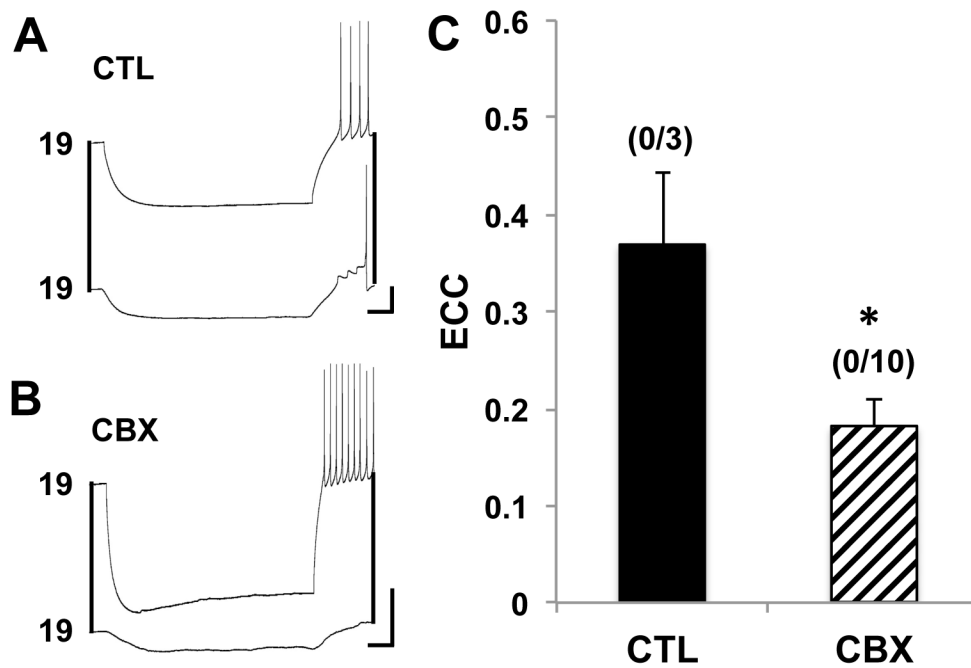


Figure 16. CBX reduces SES formation at B19-B19 connections following 24h of synapse formation. (A) Membrane potential recordings from a pair of B19-B19 control neurons (CTL) expressing strong electrical coupling. Vertical scale bars equal 10 mV. Horizontal scale bar equal 0.5 s. (B) Membrane potential recordings from a pair of B19-B19 neurons treated with CBX (100 μ M) and expressing weaker electrical coupling. Vertical scale bars equal 20 mV. Horizontal scale bar equal 0.5 s. (C) Histograms represent electrical coupling coefficients (ECC) from control (CTL; n=3) and CBX-treated soma-soma SES connections. ECCs were significantly different (*; $p < 0.05$, Student's *t*-test). Numbers in parentheses represent the ratio preparations with action potential-evoked chemical synaptic transmission.

Synaptic outcomes at three-cell networks

Using a simple, three-cell neural network model (Zoran and Szabo et al., 2007), I determined whether or not the nature of electrical coupling at existing neuronal connections altered new synapse formation within that network. In this model, two neurons in contact were either treated with vehicle or drug for 4 days. Following washout, a third neuron was plated into contact with the existing pair and cultured for an additional day. In control SES-centered 3-cell networks, the central B19-B19 connection had significantly stronger electrical coupling (SES; $n=6$; $p<0.05$) than the newly formed B110-B19 synapse (nTES; $n=9$; Fig. 17A). Interestingly, when the central synapse of an SES-centered 3-cell network was treated for 4 days with CBX, this difference in electrical coupling was abolished. In this case, the central B19-B19 connection had virtually identical electrical coupling (SES; $n=4$; $p=0.48$) to that of the newly formed B110-B19 synapse (nTES; $n=6$; Fig. 17A). Similar to the previous demonstration (Fig. 17), CBX reduced SES by 61%. However, due to the smaller sample size used in these 3-cell networks, the difference between control and CBX SES was not significant ($p=0.098$).

These SES-centered 3-cell networks were also assessed for chemical synaptogenesis. Consistent with the knowledge that B19s are constrained in their ability to exhibit evoked neurotransmitter release, no central B19-B19 synapses possessed evoked release in control or CBX-treated networks. However, at the new synaptic connections exhibiting nTES, 30% of control connections ($n=10$) exhibited new cholinergic chemical synapse (nCCS) formation from B110 onto the B19, while no

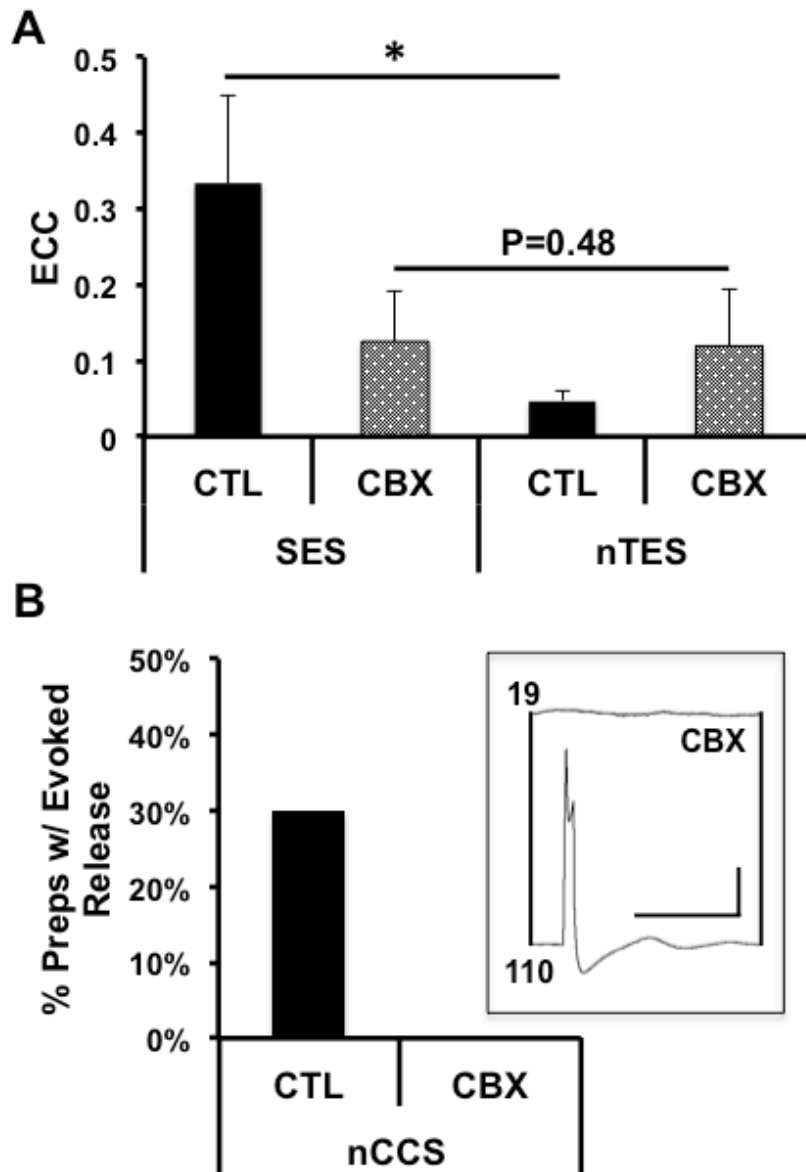


Figure 17. CBX disrupts SES-dependent suppression of new electrical synapse (nTES) and promotion of new chemical synapse (nCCS) formation. (A) Histograms represent electrical coupling coefficients (ECC) from control (CTL) and CBX-treated SES-centered network connections. ECCs were significantly different (*; $p < 0.05$, Student's t -test) between central SES and nTES connections in control (CTL), but not between the CBX-treated SES and nTES connections. (B) Histograms represent the percentage of preparations with new cholinergic chemical synapses (nCCS) in control (CTL) and CBX-treated SES-centered network connections. Inset represents recordings of membrane potential of B110 and B19 neurons at newly forming network connections in CBX-treated SES-centered networks. Vertical scale bars equal 30 mV. Horizontal scale bars equal 0.5 s.

nCCS was detected at new the connections made with CBX-treated pairs (n=4; Fig. 17B). It is unclear if this dissimilar chemical synaptogenesis, which was not significantly different between groups (p=0.22), was in any way connected to differences in electrical coupling imposed by CBX on those networks. It is interesting to note, however, that at new mixed synapses where nCCS formed, nTES coupling was significantly less than SES at the central synapse, suggesting that an inverse regulatory mechanism is at play between chemical and electrical transmission at newly forming synapses.

I next examined the impact of CBX-mediated diminution of electrical coupling in TES-centered 3-cell networks and essentially a reversal of the result seen in SES networks was obtained. In CBX-treated networks, the central B110-B19 connection had significantly lower electrical coupling (TES; n=3; p<0.05) than the newly forming B110-B19 synapse (nTES; n= 5; Fig. 18A). Here, the central synapse of control TES-centered networks had no difference in coupling from the newly forming connection. That is, the central B110-B19 connection had similar electrical coupling (TES; n=4; p=0.42) to that of the newly forming B110-B19 synapse (nTES; n= 4; Fig. 18A). Again, although the CBX-induced reduction of TES was 75%, the difference was not statistically significant (p=0.085). These data suggest that there may be competition between sites of electrical synapse formation in these neural networks, such that existing coupling dictates resource allocation to nTES.

In both control and CBX-treated networks the number of preparations that displayed evoked cholinergic chemical neurotransmission was not significantly different (p=0.74; Fig. 19B), with 50-60% of both groups possessing evoked release. In contrast

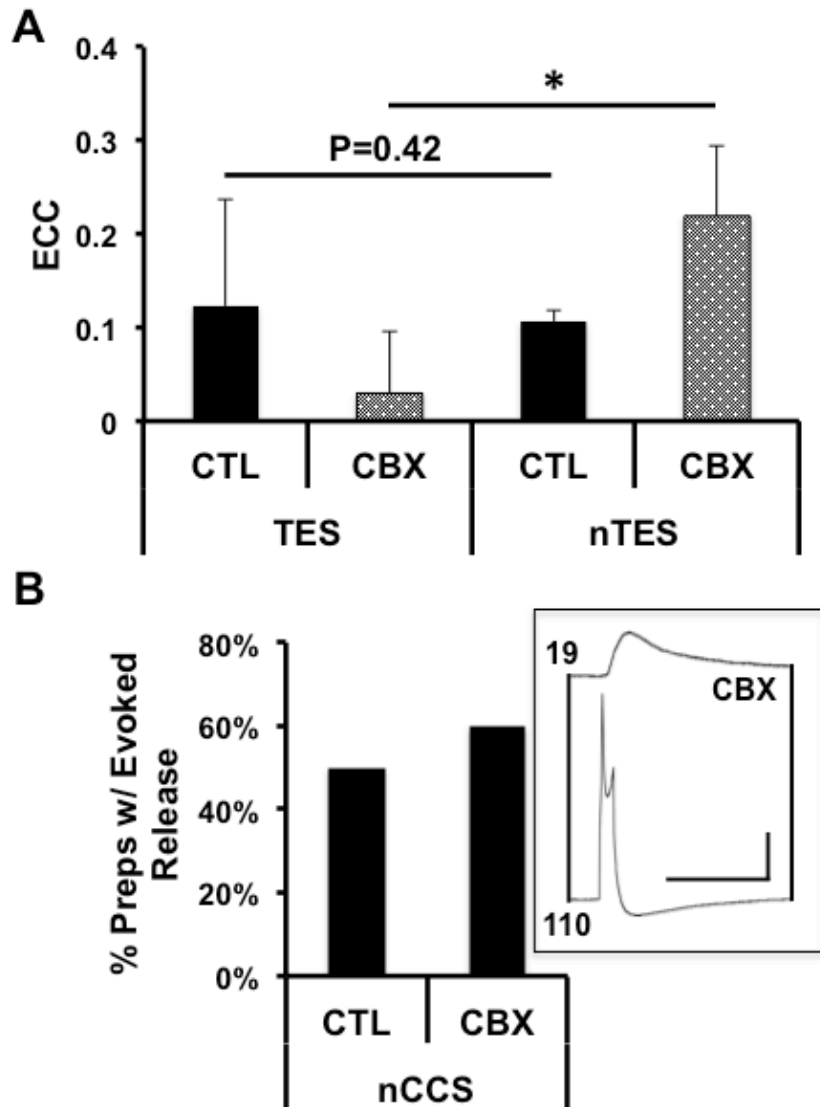


Figure 18. CBX disrupts TES-dependent modulation of new electrical synapse (nTES) formation, but does not alter new chemical synapse (nCCS) formation. (A) Histograms represent electrical coupling coefficients (ECC) from control (CTL) and CBX-treated TES-centered network connections. ECCs were significantly different (*; $p < 0.05$, Student's t -test) between drug-treated (CBX) SES and nTES connections, but not between the control (CTL) SES and nTES connections. (B) Histograms represent the percentage of preparations in TES-centered networks with new cholinergic chemical synapses (nCCS) in control (CTL) and CBX-treated networks. Inset traces represent recordings of membrane potential from B110 and B19 neurons at newly forming network connections following CBX treatment. Vertical scale bar equals 30 mV. Horizontal scale bar equals 0.5 s.

to SES-centered networks, the strength of nTES coupling in TES-centered networks did not appear to alter nCCS formation, thereby raising questions regarding the role of nTES in directly influencing chemical synapse formation at these mixed synapses. In both types of 3-cell networks, reduction of electrical coupling resulted in an overall difference in the nature of new electrical synaptogenesis in those networks.

Changes in electrical coupling through manipulation of chemical synapses

Block of chemical neurotransmission during electrical synapse formation has been suggested to increase electrical coupling (Szabo et al., 2004). To see if ongoing chemical transmission effected formation of TES or SES, I used two known antagonists of *Helisoma* nicotinic acetylcholine receptors, curare (CUR; 10 μ M) and hexamethonium (HEX; 10 μ M) to manipulate the cholinergic signaling at somatic synapses. Following 24 hours in the presence of HEX, B19-B19 SES synapses had significantly different electrical coupling ($p < 0.001$), where untreated control synapses ($n=6$) had lower coupling than the HEX-treated pairs ($n=6$). No significant change in coupling was seen in CUR-treated pairs ($n=5$; Fig. 19A). Following 5 days of synapse formation and drug treatment, long-term cholinergic blockade reduced SES (Fig. 19B). CUR-treated networks ($n=15$) at day 5 had significantly weaker SES than control pairs ($n=13$; $p < 0.05$). In a similar fashion, HEX-treated pairs ($n=17$) also displayed a significant decrease in coupling coefficient from controls ($p < 0.001$).

Similar to SES connections at day 1 of contact, HEX-treated B110-B19 TES pairs ($n=8$) showed a significant increase in electrical coupling compared to control

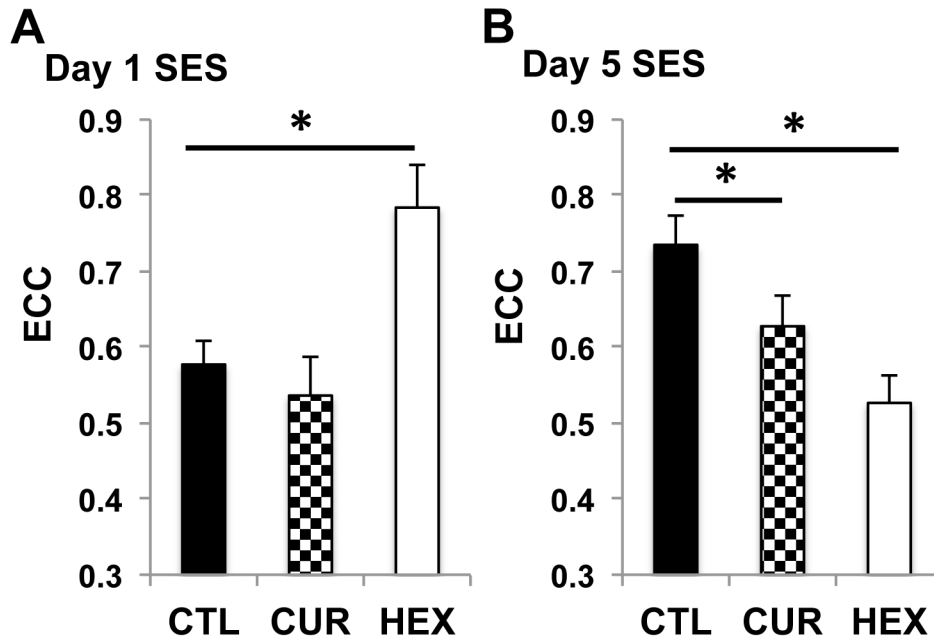


Figure 19. Hexamethonium and curare have potentiating and suppressing effects on SES. (A) Histograms represent electrical coupling coefficients (ECC) from control (CTL), curare (CUR)-treated and hexamethonium (HEX)-treated B19-B19 pairs following 1 day of treatment in cell culture. ECCs were significantly different (*; $p < 0.05$, Student's *t*-test) between HEX-treated and control (CTL) soma-soma SES connections. (B) Histograms represent electrical coupling coefficients (ECC) from control (CTL), curare (CUR)-treated and hexamethonium (HEX)-treated B19-B19 pairs following 5 days of treatment in cell culture. ECCs were significantly different (*; $p < 0.05$) between HEX-treated and control soma-soma SES connections. ECCs were also different (*; $p < 0.05$) between CUR-treated and control soma-soma synapses with SES.

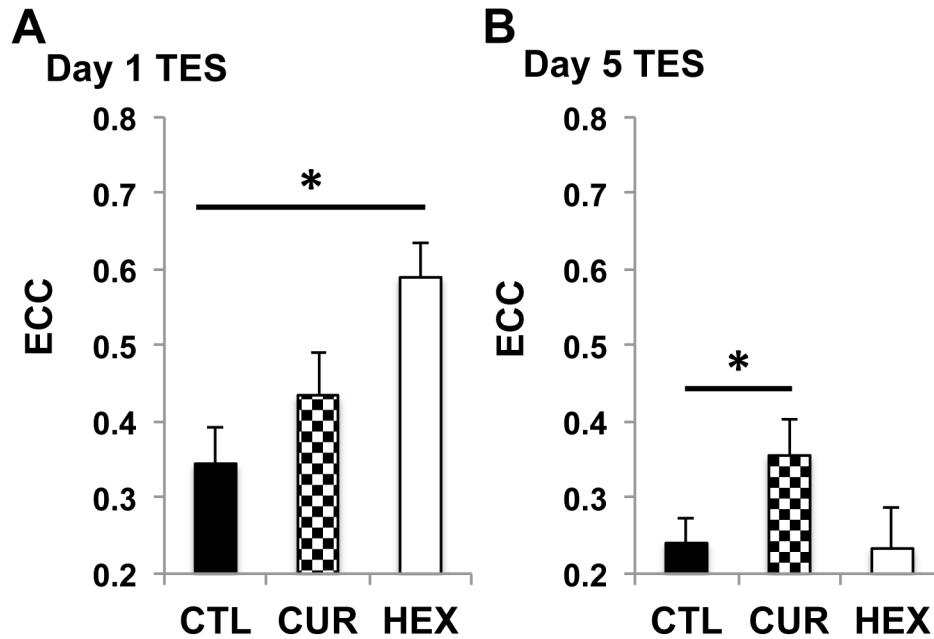


Figure 20. Hexamethonium and curare potentiate TES. (A) Histograms represent electrical coupling coefficients (ECC) at TES connections from control (CTL), curare (CUR)-treated and hexamethonium (HEX)-treated B19-B110 pairs following 1 day of treatment in cell culture. ECCs were significantly different (*; $p < 0.05$, Student's t -test) between HEX-treated SES and control soma-soma TES connections. (B) Histograms represent electrical coupling coefficients (ECC) from control (CTL), curare (CUR)-treated and hexamethonium (HEX)-treated B19-B110, TES, pairs following 5 days of treatment in cell culture. ECCs were significantly different (*; $p < 0.05$, Student's t -test) between CUR-treated and control soma-soma synapses.

synapses (n=28; $p < 0.001$; Fig. 20). TES connections treated with CUR (n=24) were again not significantly different ($p = 0.12$) from control synapses. At 5 days of TES formation, CUR-treated pairs showed a significant increase over controls (n=26; $p < 0.05$), while HEX-treated pairs (n=14) showed no significant differences ($p = 0.45$). Taken together, these results with cholinergic antagonists demonstrate a clear interaction between ongoing chemical synaptic transmission and electrical synapse formation at mixed connections. However, this interaction is not a simple inverse relationship. Rather, the length of antagonist treatment and the identity of the neurons involved in synaptogenesis caused differential potentiating and suppressing effects.

Cholinergic blockade early in FES, particularly with HEX, caused strong potentiation of electrical coupling, but later during TES and SES communication reduction or no change in coupling was induced. This raised the question, what is different regarding the relationship between cholinergic signaling and electrical synapses early in formation versus later in the process of synapse maturation? To address this question, I turned my focus to spontaneous release as a potentially vital regulator of early electrical synaptogenesis. This was in part due to the fact that these *Helisoma* motor neurons have high rates of spontaneous ACh release early during regeneration of neuromuscular junctions (NMJ) both *in vivo* and *in vitro*, when very little evoked release is detected and important target recognition decisions are made (Turner et al., 2011). To determine if similarly high rates of mPSPs were present during FES, I used B110-B19 soma-soma synapses to access miniature potential rates at both 1 day and 5 days of electrical synapse formation (Fig. 21). On day 1, electrical coupling was strong with an

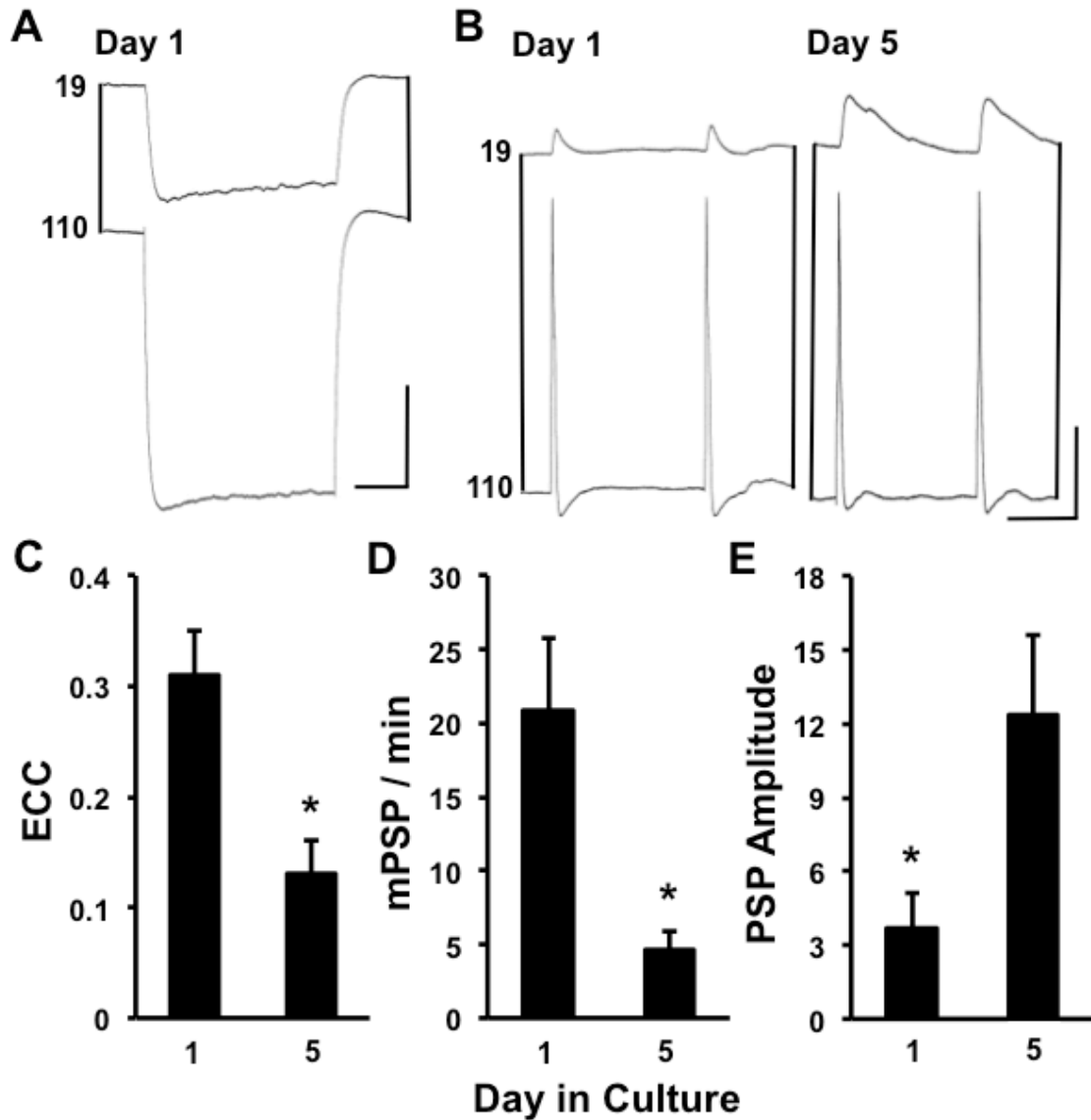


Figure 21. Evoked, but not spontaneous, release is inversely correlated with electrical coupling at TES connections. (A) Membrane potential recordings from a pair of B110-B19 neurons expressing TES at soma-soma synapses. Vertical scale bars equal 20 mV. Horizontal scale bars equal 0.5 s. Note: current was injected into neuron B110. (B) Membrane potential recordings from a pair of B110-B19 neurons expressing primarily electrical coupling following 1 day of synaptogenesis. This pair expressed mixed electrical and chemical synapses following 5 days of formation. Note the extended duration of the PSP at 5 days. Vertical scale bars equal 20 mV. Horizontal scale bars equal 1 s. (C) Histograms represent electrical coupling coefficients (ECC) from soma-soma TES connections at 1 and 5 days. ECCs were reduced and significantly different at day 5 of connectivity (*; $p < 0.05$, Student's *t*-test). (D) Histograms represent spontaneous release rates at soma-soma TES connections at 1 and 5 days. Rates were significantly different and reduced at 5 days of connectivity (*; $p < 0.05$). (E) Histograms represent the strength (amplitude) of evoked postsynaptic potentials (PSP) at soma-soma TES connections at 1 and 5 days. PSP amplitudes were significantly different and strengthened at 5 days of connectivity (*; $p < 0.05$).

average ECC above 0.3 (n=29) that was significant greater than coupling on day 5 (n=19; $p<0.005$; Fig. 21C). At day 1, just as in early NMJ formation, mPSP frequency was high (Fig. 21D) when the strength of evoked ACh release was low (Fig. 21E), as determined by analysis of PSP amplitude. Both mPSP rate and PSP amplitude were significantly different between day 1 and day 5 (Fig. 21D and E; mPSP rate, $p<0.005$; PSP amplitude, $p<0.05$).

It is important to note that cholinergic signaling at central *Helisoma* synapses within the buccal ganglia is inhibitory (Szabo et al., 2004), unlike the excitatory effects of ACh at NMJs (Turner et al., 2011). To determine to what extent spontaneous, inhibitory mPSPs were present very early during cell-cell contact, soma-soma synapses were examined between 1 and 6h following cell pairing in culture. As seen in Figure 22A-C, ACh-mediated mPSPs and evoked PSPs are mediated by a chloride conductance, since use of potassium acetate (KAc) electrodes reverses the sign of these events recorded at B110-B19 synapses or following pressure application of ACh onto the postsynaptic B19. With KAc-filled electrodes, virtually no mIPSPs were detected in the early hours of synaptogenesis at FES. Spontaneous release rates in KAc electrode preparations (n=4) were significantly lower than rates in potassium chloride (KCl) electrode penetrated cell pairs (n=3; $p<0.05$; Fig. 22E). Thus, it is unlikely that such low levels of relatively ineffective IPSPs would have much impact on early FES. To be certain, we used the dynamin inhibitor, dynasore (Dyna) to block the endocytosis pathway and disrupt all vesicle-mediated signaling early in synaptogenesis. Neurons B110 and B19 were treated with 80 μ M Dyna as single cells for 24h and then paired.

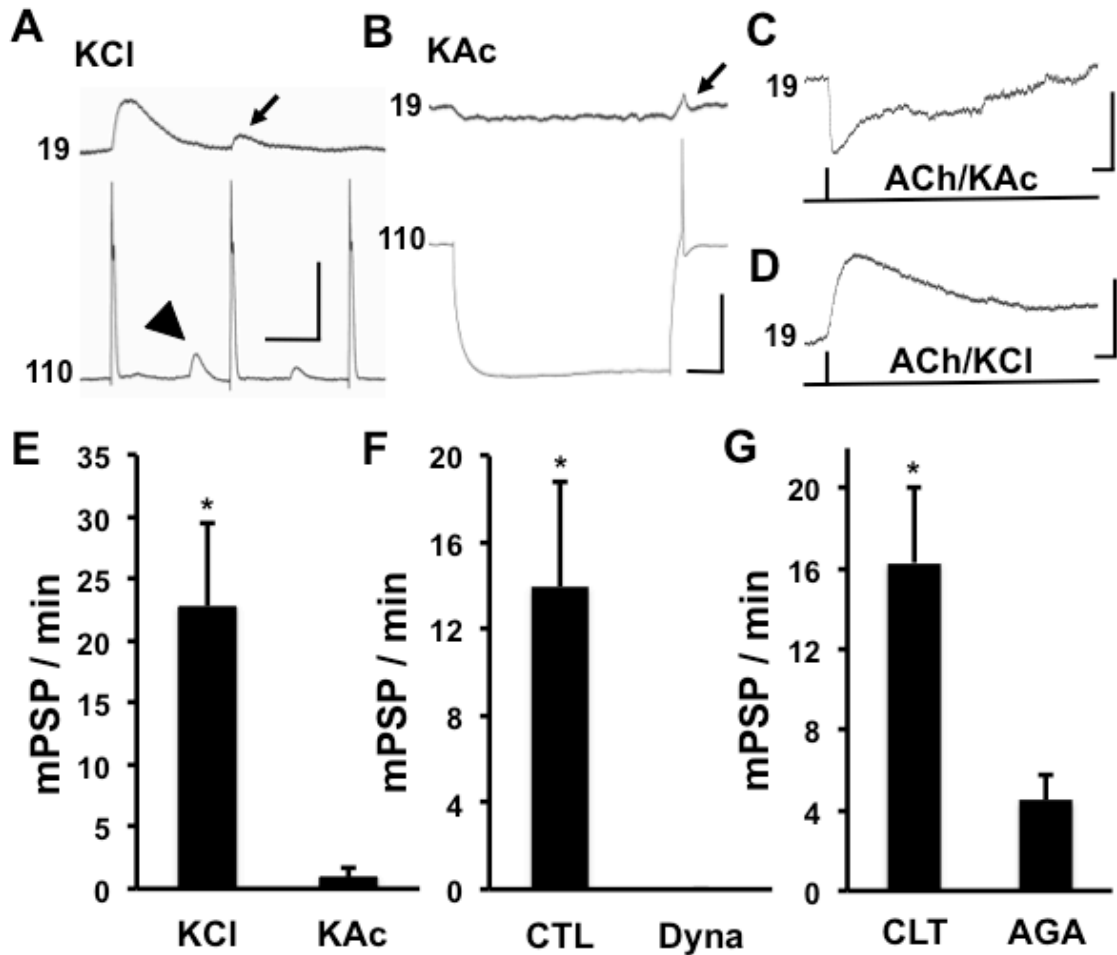


Figure 22. Miniature PSPs are inhibitory during FES and are blocked by dynamin and gap junction inhibitors. (A) Membrane potential recordings from a pair of B110-B19 neurons at forming electrical synapses (FES) between soma-soma contacts, using 1.5 M KCl electrodes. Note the spontaneous mPSPs (arrow head) and evoked PSPs are depolarizing (arrow). Vertical scale bars equal 20 mV. Horizontal scale bars equal 1 s. Note: current was injected into neuron B110. (B) Membrane potential recordings from a pair of B110-B19 neurons at forming electrical synapses (FES) between soma-soma contacts using 1.5 M KAc electrodes. Note the evoked PSP is hyperpolarizing (arrow), when electrical coupling is low. Vertical scale bars equal 20 mV. Horizontal scale bars equal 1 s. Note: current was injected into neuron B110. (C) Membrane potential recordings, using a 1.5 M KAc electrode, from a B19 neuron alone in cell culture. Note the vertical bar in lower traces designates the time of ACh pressure injection onto the neuron. Vertical scale bars equal 20 mV. Horizontal scale bars equal 0.5 s. (D) Membrane potential recordings, using a 1.5 M KCl electrode, from a B19 neuron alone in cell culture. Note the vertical bar in lower traces designates the time of ACh pressure injection onto the neuron. Vertical scale bars equal 20 mV. Horizontal scale bars equal 0.5 s. (E) Histograms represent spontaneous release rates at soma-soma TES connections at 1-6h of contact recorded with KCl or KAc electrodes. Rates were significantly different (*; $p < 0.05$, Student's t -test). (F) Histograms represent spontaneous release rates at soma-soma TES connections at 1-6h of contact recorded with KCl electrodes. TES connections were recorded at control (CTL) and dynasore (Dyna; 80 μ M)-treated synapses. Rates were significantly different and reduced when treated with Dyna (*; $p < 0.05$). (G) Histograms represent spontaneous release rates at soma-soma TES connections at 4h of contact recorded with KCl electrodes. TES connections were recorded at control (CTL) and AGA-treated synapses. Spontaneous release rates were significantly different and reduced in AGA-treated TES pairs (*; $p < 0.05$).

Synaptic physiology was then assayed during the first hours of contact. Treatment of B110-B19 soma-soma synapses with Dyna (n=3) completely abolished spontaneous release compared to control, DMSO-treated synapses (n=4; $p<0.05$; Fig 22F). Electrical coupling was not detected at either control or Dyna-treated synapses (data not shown). Interestingly, although electrical coupling was typically weak or absent during the first hours of cell-cell contact, treatment of these soma-soma synapses with AGA, a gap junction inhibitor, caused a reduction in spontaneous release rate. At B110-B19 synapses treated with AGA (n=14), the number of mPSPs detected per minute was significantly lower than that recorded from untreated control synapses (n=15; $p<0.01$; Fig. 22G).

It is clear that processes of electrical and chemical synaptogenesis in identified neurons influence each other and that that these interactions vary depending on the neural network context (i.e., previous synaptic history; Szabo et al., 2007). Therefore, simple 3-cell TES-centered neural network were again used to determine if cholinergic antagonism during ongoing TES would alter nTES and nCCS at the newly forming synapses. In fact, treatment with 10 μ M curare (CUR) of the central B110-B19 synapse had no effect on emerging nTES ($p=0.24$; Fig. 23A). On the contrary, however, nCCS emerging at CUR-treated TES networks was abolished. Evoked release, present in over 60% of synapses emerging at untreated networks (n=8), was completely absent at new B110 contacts with a CUR-treated, TES-centered network (n=5; $p<0.02$; Fig. 23B).

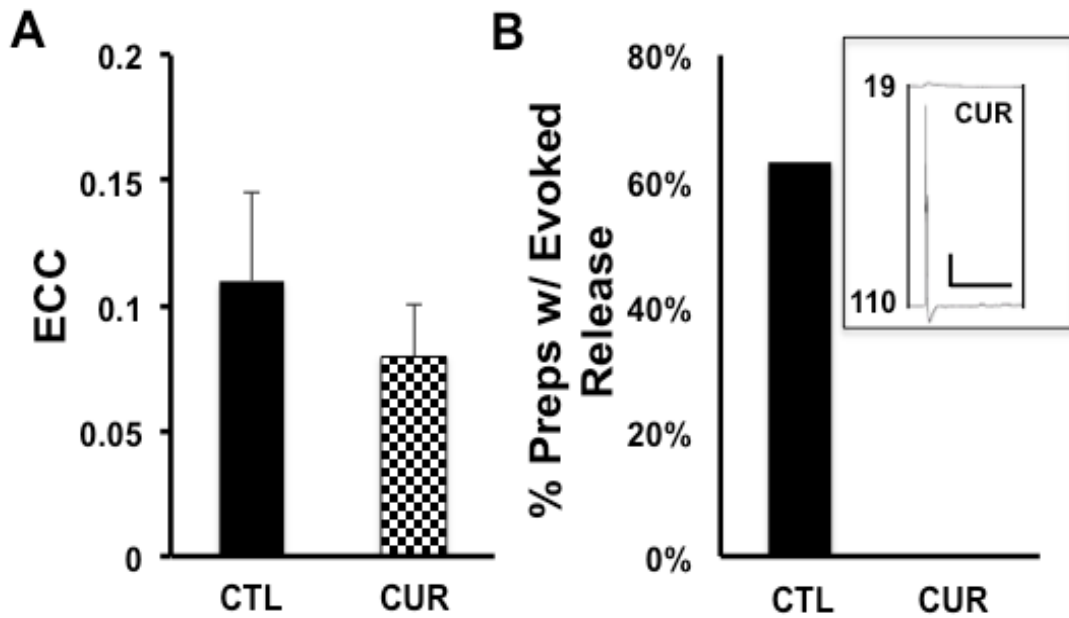


Figure 23. Curare treatment during TES formation has no effect on new electrical synaptogenesis (nTES), but blocks new chemical synaptogenesis (nCCS). (A) Histograms represent electrical coupling coefficients (ECC) at TES-centered networks from either controls (CTL) and curare (CUR)-treated networks. ECCs were not significantly different. (B) Histograms represent the percentage of preparations with new cholinergic chemical synapses (nCCS) at TES-centered networks either in control (CTL) or CUR-treated. Inset traces represent recordings of membrane potential of B110 and B19 neurons at CUR-treated newly forming network connections. Vertical scale bars equal 10 mV. Horizontal scale bars equal 2 s.

Discussion

In *Helisoma* cultures, manipulation of electrical synapse formation alters subsequent formation of synapses. For instance, deprivation of trophic factors from the culture suppresses the formation of electrical synapses and subsequent chemical synapse formation occurs more rapidly in its absence (Szabo et al., 2004). However, trophic factor deprivation also leads to other dramatic cellular consequences, including alterations in ion channel expression (Haydon, 1988) and loss of neurite outgrowth (Wong et al., 1981). Furthermore, trophic factors induce changes in synaptic connectivity between regenerating *Lymnaea* pedal ganglion neurons (Woodin et al., 2002). In Chapter II, I demonstrated that exogenous neuromodulators, specifically DA, enhanced or suppressed electrical synapse formation, depending on the network context, and that modulation impacted future synaptic outcomes of those networks. In the present studies, I determined, not with trophic factors or neuromodulators, but with antagonists directed at electrical synapses, the specific roles of gap junction coupling and inhibitory cholinergic signaling on electrical synaptogenesis at identified mixed synapses in cell culture.

AGA and CBX, both derivatives of glycyrrhetic acid, caused significant reduction in electrical coupling at FES. However, the impact of AGA on SES connections was greater than that induced at TES connections. The specific actions of glycyrrhetic acid and its derivatives are still poorly understood. However, it is speculated that both AGA and CBX either modify phosphorylation states of gap-junctional proteins

(Guan et al., 1996; Liang et al., 2008) or bind the junctional proteins in the membrane (Goldberg et al., 1996), which lead to dysfunctional assembly/disassembly of gap junction plaques, and thereby, reductions in electrical synapse communication. Given the unknown composition of the gap junctions mediating electrical coupling at *Helisoma* synapses, it is reasonable to speculate that hemichannel composition at B19-B19 SES connections is different from that expressed at B110-B19 TES connections. The innexin proteins comprising the B110 hemichannel may therefore not be as sensitive to changes in AGA-induced alterations in junctional coupling. Its assembly may not be as drastically perturbed as a B19, leading to lesser impacts on TES (heterotypic connections) than on SES (homotypic connections). The identity of the innexins specifically expressed dramatically changes the outcome of electrical synapse formation, where ectopic expression and mutation of innexin proteins lead to altered functioning of electrical synapses formed between neurons (Firme et al., 2012). Contrary to previous reports using trophic factor deprivation protocols to alter electrical coupling (Szabo et al., 2004), AGA and CBX did not lead to an enhanced probability of chemical synapse formation. At SES connections, action potentials in B19 do not elicit neurotransmitter release unless the neurons receive contact-mediated cues from their anatomically appropriate muscle targets (Zoran et al., 1990; Zoran et al., 1991). In the case of nTES connections newly forming with an established network, the lack of change in chemical synapse formation is likely due to the decrease in electrical coupling together with the moderate levels of chemical neurotransmission that exist at these connections at 1-day of soma-soma contact.

An intriguing outcome of these studies was the finding that at SES and TES networks the overall profile of electrical coupling was significantly different in control versus CBX-treated networks. Consistent with previous data on *Helisoma* 3-cell networks (Szabo and Zoran, 2007), when extant SES or TES connections were coupled with ECC values higher than 0.2, formation of new electrical synapses had significant reductions in electrical coupling. Here, neuronal networks were created with manipulated central synapses where TES or SES connections were lowered with CBX treatment and the impact of that treatment on new synaptic connections was assessed. When coupling of SES-centered networks was reduced by CBX treatment to an ECC of less than 0.2, then nTES coupling is not significantly different from untreated controls. In contrast, when SES coupling was high, as in controls, nTES coupling was significantly reduced. Also, when TES coupling was lowest following CBX treatment, then emerging nTES coupling was again high. Broadly, this supports the hypothesis that extreme high or low ECC values at central network synapses, whether SES or TES centered, are inversely correlated with the strength of electrical coupling at new synapses connecting with that network. Why does a threshold ECC of 0.2 impose influences on coupling at other synapses within a network and why do extremes in synaptic coupling accentuate these interactions? I suggest that cellular competition for electrical synaptic resources, such as the gap junction proteins, machinery for trafficking innexins, or the regulatory molecules that orchestrate their mobilization, modulation and turnover, exists between multiple developing synaptic sites. A resource threshold for electrical synaptogenesis would constitute a homeostatic mechanism similar to that regulating

neuronal excitability or cytosolic calcium set points; that is, when overall activity goes beyond high or low threshold values compensatory mechanisms are activated to maintain the physiological parameter within appropriate ranges (Turrigiano et al., 1994; Stellwagen and Malenka, 2006). In the case of SES, the construction and persistent maintenance of a strong central electrical synapse, may require a critical amount of finite resources, perhaps those necessary to signal with an ECC of 0.2 or greater. By contrast, when SES was significantly diminished with CBX or during normal TES progression, the ability to assemble gap junctions is equivalent and the competition results in equally weak coupling. The mechanisms underlying this hypothesized electrical synapse competition, however, remain to be determined.

Much of the justification for proposing this synaptogenesis research drew upon previous studies implicating inverse relationships between chemical and electrical synapse formation (Szabo et al., 2004; Kandler and Thiels, 2005; Turner et al., 2011). In SES-centered networks, a low level of nTES coupling was accompanied by evoked chemical neurotransmission in 30% of synapses within 24h, consistent with this inverse relationship hypothesis. However, in TES networks treated with CBX, nTES at developing connections was strong (i.e., ECC greater than 0.2). Still, nCCS formation was robust with 60% of these electrically coupled connections possessing evoked neurotransmission. This result clearly demonstrates that the strength of electrical coupling at *Helisoma* motoneuronal mixed synapses is not necessarily a direct regulator of chemical synapse formation.

Although strong or weak electrical coupling at forming synapses is not an unavoidable determinant of chemical synaptic signaling, cholinergic transmission might still alter electrical synapse strength. Therefore, we disrupted cholinergic signaling with multiple nAChR antagonists to test this idea. Curare acts as a direct antagonist for nAChRs by binding competitively to the ACh binding site on the amino terminus (Hurst et al., 2013). HEX is also a nAChR antagonist typically known for its ubiquitous receptor antagonism in mammalian autonomic ganglia, although it can also affect cholinergic transmission at NMJs (Bibevski et al., 2000). HEX had pronounced influences on the formation of mixed synapses. Interestingly, HEX treatment was associated with significant enhancement of electrical coupling during the first 24 hours of synaptogenesis. On the contrary, prolonged treatment with HEX for 5 days led to a significant depression of electrical coupling, specifically at SES connections. Thus, I found specific disruption of electrical coupling altered chemical synapse formation and specific inhibition of cholinergic signaling altered electrical synapse formation. However, the timing of treatment during the progression of synaptogenesis and identity of the network synapses being treated profoundly impacted the resulting synapse formation. These interactions, therefore, are influenced by multiple factors and complicated by the fact that inhibitory cholinergic transmission is largely absent from B19, and highly variable from B110, with regard to excitation-secretion coupling strength, spontaneous release rates, and percent preparations with evoked release. What are the cellular mechanisms then that link these synaptogenic processes?

Central cholinergic neurotransmission at *Helisoma* motoneuron-to-motoneuron synapses is inhibitory at normal resting potentials. I have demonstrated here membrane potential changes in response to ACh application consistent with a chloride-dependent conductance. Therefore, as observed for other mollusks, nAChRs at these synapses likely mediate inhibitory voltage changes via ACh-activated chloride currents. This finding, taken together with synapse formation results, suggest that electrical and chemical synapses may indirectly interact to potentiate or depress each other through their modulation of synaptic membrane potential. Chemical synaptic regulation of electrical coupling occurs at glutamatergic synapses via membrane potential-dependent signaling. Glutamate and its associated depolarization of membrane potential lead to NMDA receptor activation at goldfish Mauthner cell synapses. NMDA associated calcium influx activates calcium-calmodulin dependent kinase II (CaMKII), which in turn, leads to potentiation of electrical coupling at these synapses (Pereda et al., 1998). In rat hypothalamus, NMDA receptor activation leads to downregulation of gap-junctional coupling, whereas antagonism of NMDA receptors causes persistence of coupling (Arumugam et al., 2005). It is, therefore, difficult to predict the action of ACh-activated chloride channels in *Helisoma* neurons, since the reversal potential of these channels is likely close or slightly more negative than the resting potential. In other words, their activation could be excitatory or inhibitory at different times depending on the cell's membrane potential. Regardless of the sign of the potential, ACh-activated membrane voltage changes would certainly alter voltage-dependent calcium currents at the synapse, thereby providing a mechanism for calcium-dependent modulation of gap

junctions. Currently, though, this is speculation at best and the true interaction between chemical neurotransmission and electrical coupling here and in the field of synaptic physiology remains unknown (Belousov and Fontes, 2012).

The impact of gap junction coupling and cholinergic signaling inhibitors was greatest during early phases of synapse formation near the TES versus SES bifurcation point (Fig. 1) and when the inversion between electrical and chemical communication was at its peak. Also at this time, *Helisoma* neurons had high levels of spontaneous release and low levels of action potential-evoked chemical transmission (Fig. 8), suggesting a special relationship between spontaneous release and electrical coupling. Complete blockade of vesicle-mediated release mechanisms with a dynamin inhibitor, dynasore, had no potentiating effect on electrical coupling. Dynamin inhibitors have been shown to strengthen electrical synaptic connectivity (Flores et al., 2012). Reduction of electrical coupling with AGA caused a significant reduction of mPSPs at TES mixed synapses. Similarly, striatal neurons of CX36 knockout mice have reduced levels of spontaneous inhibitory and excitatory synaptic transmission (Cummings et al., 2008). Still, the mechanism of such interactions remains a mystery

Synaptogenesis is a complex interaction between neurons and targets, even in the simplest of configurations involving factors from targets to neurons and neurons to targets. At mixed electrical and chemical synapses, where multiple types of information must be received and regulated, the process is even more complex. Identified neuronal cultures of *Helisoma* motor neurons provide a simple model for assessing synaptic interactions between these two forms of synaptic communication. It is clear that neuron-

specific synaptogenic strategies have a major influence on how synapses are formed (Zoran and Poyer, 1996) and this is likely a widely transferrable principle, as gap junction-mediated synapses are likely regulated at the level of individual plaques within a neuron via large regulatory complexes that link cellular signaling pathways to electrotonic intercellular communication (Belousov and Fontes, 2012). Neuron-specific strategies for synapse formation do not, however, entirely account for the plasticity in synapse formation reported here. At simple 3-cell neuronal networks, in addition to neuron-specific strategies, there appear to be network-specific factors that guide synaptic outcomes and these are dependent on the emergent nature of synaptic interactions existing within those networks. Further investigation of the mechanisms regulating such complex and emergent synaptic properties is needed, if we are to determine how these synaptic interactions regulate synaptogenesis in more complex neural networks, such as those of the developing or regenerating central nervous system of mammals.

CHAPTER V

GENERAL DISCUSSION AND CONCLUSION

The basis for this dissertation research was the hypothesis that modulation of developing electrical synapses leads to predictable changes in the formation of synaptic connections within known neural networks. The persistence or transience of electrical connections, SES or TES, respectively, produced different modulatory effects and generated varying synaptic outcomes on new connections developing within the neural networks that harbor them. The utility of the *Helisoma* cell culture system as the basis for testing these and other key hypotheses revolve squarely around its ease of manipulation and access, down to the identifiable nature of its specific individual neurons. This allows for the creation of highly specific cell-cell contacts that permit knowledge of neuronal history and synaptogenic characteristics prior to the establishment and modulation of network interactions. The study of individual synaptic interactions and the rules that govern those interactions are used to form hypotheses about synaptic interactions between larger, less tractable neural networks. There is promising evidence that analysis of activity in a large network can be predicted through more focal interactions around any given cell (Nirenberg and Victor, 2007). Additionally the practicality of population monitoring of activity in neural ensembles continues to get more efficient (Grewe and Helchem, 2009; Peterka et al., 2011), however, even at relatively simple and reduced systems the interactions governing network physiology are not strictly stereotyped or even definitively characterized in most instances. Therefore, in

this dissertation I set out to characterize those interactions, such as the regulatory interaction between chemical and electrical components at mixed synapses and modulation of forming electrical synapses (FES) and their influence not only with new emerging electrical synapses (nTES), but with new cholinergic chemical synapses (nCCS) at sites of mixed neural communication.

A question examined throughout this dissertation was the regulatory interaction between chemical neurotransmission and electrical coupling. At certain mixed synapses glutamatergic signaling and associated activity-dependent postsynaptic changes result in dual potentiation of electrical and chemical synaptic strength (Pereda et al., 1998), while at other synaptic connections chemical interactions result in an inverse potentiation/depression at synaptic sites (Arumugam et al., 2005; Szabo et al., 2004). I present findings that at temporally and phenotypically controlled synaptic pairs chemical synapses may be potentiated or weakened in response to changes in electrical coupling. For instance, short-term reduction in electrical coupling (within the first 24h of contact) at FES of B110-B19 connections is correlated with a decrease in spontaneous release of neurotransmitter. Thus, electrical coupling may be important for presynaptic release of neurotransmitter, assembly of presynaptic machinery or recruitment of vesicles, a notion earlier advocated by Neunuebel and Zoran (2005). They showed that in trophic-factor deprived neuronal cultures that the absence of FES caused poor recruitment of vesicles to the synaptic membrane. Chemical neurotransmission develops more readily at mixed synapses when electrical coupling is low, but it does not form as efficaciously.

The exact regulatory contribution of gap-junctional intercellular communication to the regulation of chemical synaptic transmission is unknown. However, several hypotheses have been put forward. Electrical synapses, through coordination of biochemical signaling, hold nearby chemical synapses in a ready, but silent state by conductance of regulatory second messengers like Ca^{2+} or IP3 (Kandler and Katz, 1995, 1998). Additionally, a less studied function of gap junctions is its role in cell-adhesion, where connections to cytoskeletal proteins and signaling complexes link cell-to-cell cytoplasm events. Elias et al. (2007) describe a procedure whereby migration of neurons into the cerebral cortex of rodents is mediated by adhesive properties of gap junctions. Lastly, passage of ionic currents across gap junctions, the definitive properties of electrical synapses, might underlie regulation of chemical synapse formation at these mixed connections, as activity-dependent mechanisms are well-known for their role in refinement of neural circuits (Zhang and Poo, 2001).

It is also clear that chemical synaptic transmission, through some mechanism, regulates junctional coupling, as demonstrated by work using curare and hexamethonium. If transmission of chemical signals is an important regulatory agent for electrical synapses, it seems most reasonable it works through either changes in calcium-dependent signaling or subthreshold voltage-dependent changes in electrical coupling. Given that chemical synapses formed between *Helisoma* motor neurons in culture are entirely inhibitory through the ACh-mediated activation of chloride channels, it is unlikely that chemical neurotransmission, at least at resting membrane potentials, causes voltage-dependent calcium fluxes. However chemically-mediated inhibitory currents

produce shunting effects on junctional conductance. In the inferior olive, chemical synapses between olivary cells reduce electrical synchrony by introducing hyperpolarizing currents near the site of electrical synaptic transmission (De Zeeuw, 1998). Similar shunting effects could be occurring in chemical/electrical synaptic interactions in *Helisoma*, especially given that a similar phenomenon is seen in the gastropod *Navanax* (Spira et al., 1976), although the functional implications depend on the timing of current activation and its proximity to the junctional site. Subthreshold voltage changes could also be effective at directly altering electrical coupling through changes in voltage-dependent conductance state. In Bukauskas et al. (2002) changes in transjunctional voltage induced charge selectivity through the junctional pore. Therefore, small amplitude hyperpolarization induced by cholinergic neurotransmission between *Helisoma* neurons might alter the conductance state and introduce selectivity in the conductance of the intercellular channel.

The first 24 hours of synaptic contact were intriguing with regard to regulation of emerging electrical synapses, where treatment with hexamethonium yielded large increases in electrical coupling. This time corresponds to the presence of pronounced spontaneous synaptic potentials, but with relatively little ability to evoke release of neurotransmitter. This would suggest that hexamethonium-mediated increases in first-day coupling are not likely a product of evoked ACh release. Therefore, to determine if a direct interaction was present between spontaneous release and electrical synapses, I manipulated the vesicle recycling pathway in the first few hours contact, but saw no evidence of upregulation of electrical coupling. It is entirely possible that neurons are

incapable of building electrical synapses in the first few hours of contact. However, two ideas make it reasonable to hypothesize their presence in the absence of endocytosis and hence any chemical signaling: 1) Electrical synapses can be constructed and deconstructed in hours; and 2) B110-B19 synapses do form strong coupling as early as 12h post contact (Szabo et al., 2004).

An unexpected but intriguing finding was that electrical coupling either at a synapse or in a network appeared to display forms of self-regulation to maintain coupling within a certain range. This was particularly evident at SES- and TES-centered networks using CBX-treated manipulation of electrical coupling. At these neuronal networks moderate coupling at a central synapse (around 0.1 ECC) resulted in similar coupling at all electrical synapses. However, central synapses with much higher electrical coupling, in excess of 0.3 ECC, maintained an unbalanced electrical coupling between synapses. It is interesting to note that this balancing interaction may also occur when central synapses have excessively low coupling (~ 0.05 ECC), but this seems to be variable. While forming, both TES and SES show higher coupling during pharmacological inhibition of chemical synapses using HEX. Subsequently by day 5 of treatment those same synapses have the lowest coupling values in HEX-treated pairs. Interestingly, *in vivo* the transient nature of B4-B5 synapses was dependent on the presence of coupling between the two B5s (Bulloch and Kater, 1981). In the absence of that coupling, B4-B5 electrical connections were maintained roughly twice as long, suggesting that balancing of coupling inputs may even be important to the regulation of the expression of electrical synapses. It is possible, particularly at the network level, that

balancing of electrical coupling could be the outcome of a form of resource competition. In this instance the timing and strength of the synapse might be important in dictating which connection preferentially receives the finite resources. The building and trafficking of hemichannels is a particularly attractive suggestion. It is known that production of hemichannels is largely an ER-golgi process and that the construction of an electrical synapse is dependent on directional trafficking to the synapse. Even if the construction of hemichannels doesn't constitute a finite resource they may still compete for those hemichannels once they are constructed and directed to needed synaptic sites.

Exogenous application of DA is capable of initiating the full three phase feeding behavior (Quinlan and Murphy, 1996). Feeding stimulants that induced the tri-phasic feeding behavior activate neuron N1a, a phase 1 dopaminergic interneuron, which subsequently initiates feeding. Exogenous DA application at neuronal networks, might therefore, represent a similar mechanism by which the snail nervous system modulates its own existing or developing synapses. Neuromodulation via DA was shown to act either directly (TES) or indirectly (SES) to manipulate certain biophysical aspects of electrical coupling. DA was previously shown to suppress neuronal activity of some buccal neurons via activation of D2-like receptors that gate K⁺ channels to open (Zhong et al., 2012). This provides a potential non-junctional means of modulating B19-B19 synapses. This would act to alter functional signaling compartments within the network and produce a variety of complex input-output patterns depending on the modulated state of the junction. This is particularly useful because dopamine is suggested to be an intrinsically used modulator of buccal central pattern generation and therefore may

define the chemical/electrical interaction *in vivo*. Although in a simple invertebrate feeding network this might not be critical, however, in systems such as the mammalian CNS this could be vital in fine-tuning of sensory inputs and decision-making associated with complex motor outputs.

Synchronization of synaptic signaling is a critical and universally accepted role of gap-junctional intercellular communication. However, electrical synapses are not the only means of achieving synchronization. Combined with the challenging nature of pharmacological agents against junctional coupling, this has made attributing synchrony to gap junctions, at least in a clinical sense, trying. The especially useful thing about this model network system is that it is unlikely that synchrony can be attributed to anything, but coupling through gap junctions. Thus, there is utility to examine effects of synaptic coupling on broader network coupling, without the need to penetrate neurons using intracellular electrophysiology. These data could also indicate the nature of coupling in feeding circuits of *Helisoma* and how those circuits are used to produce feeding behaviors. B19 *in vivo* is strongly dye-coupled to other S3 motor neurons, B17 and B18 (Szabo, unpublished), suggesting that the entire output of phase 3 motor activity is synchronized via gap junctions to ensure that behavioral outputs occur with high fidelity.

Synaptic interactions even in a simple 3-cell neuronal network vastly increase the complexity of synaptic outcomes of the network that are not strictly related neuron-specific strategies. In fact, even pairwise interactions are not solely competent of explaining modulation of chemical/electrical synapses at mixed connections. Take for instance three scenarios: 1) In a network where nTES is significantly lower than its

central SES counterpart, chemical synapses appear with a higher probability; 2) In a network where nTES is significantly higher than its central counterpart, such as a CBX-treated TES-centered network, no difference in chemical formation is seen; 3) In networks where all coupling is relatively low, when the central SES is treated with DA, there is a larger presence of nCCS, without DA-induced modifications to postsynaptic sensitivity for neurotransmitter. Taken together, these data demonstrate that multiple sets of rules govern neuronal network formation and are likely to include, neuronal strategies in the formation of chemical synapses, either constrained or promiscuous, interactions between chemical/electrical components at a synapse and network-specific historical influences. Therefore, there are at least 3 layers of regulation that interact in a complex fashion to govern synapse formation and these principles might sufficiently describe broader network activity within much larger neural networks.

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